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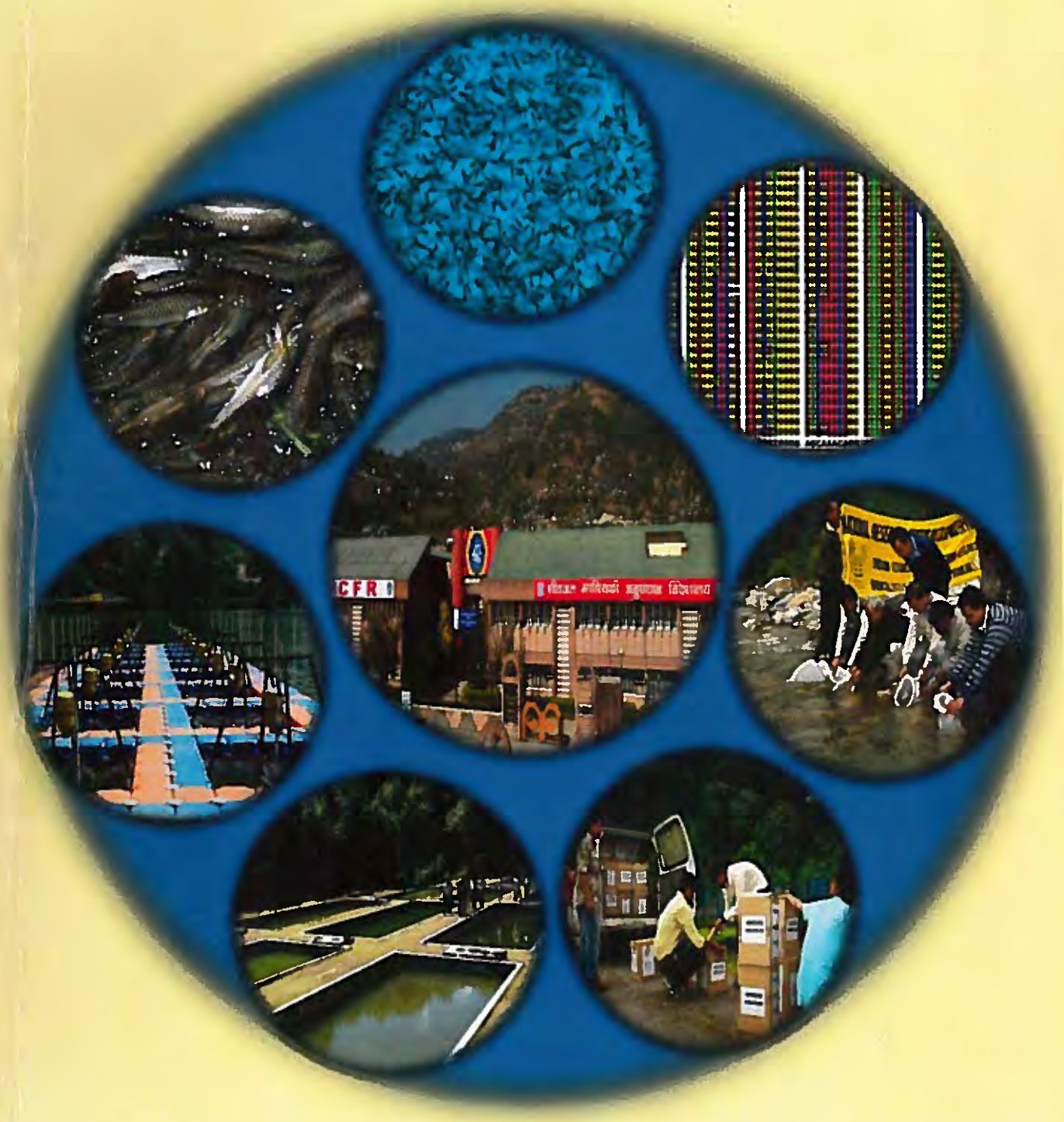

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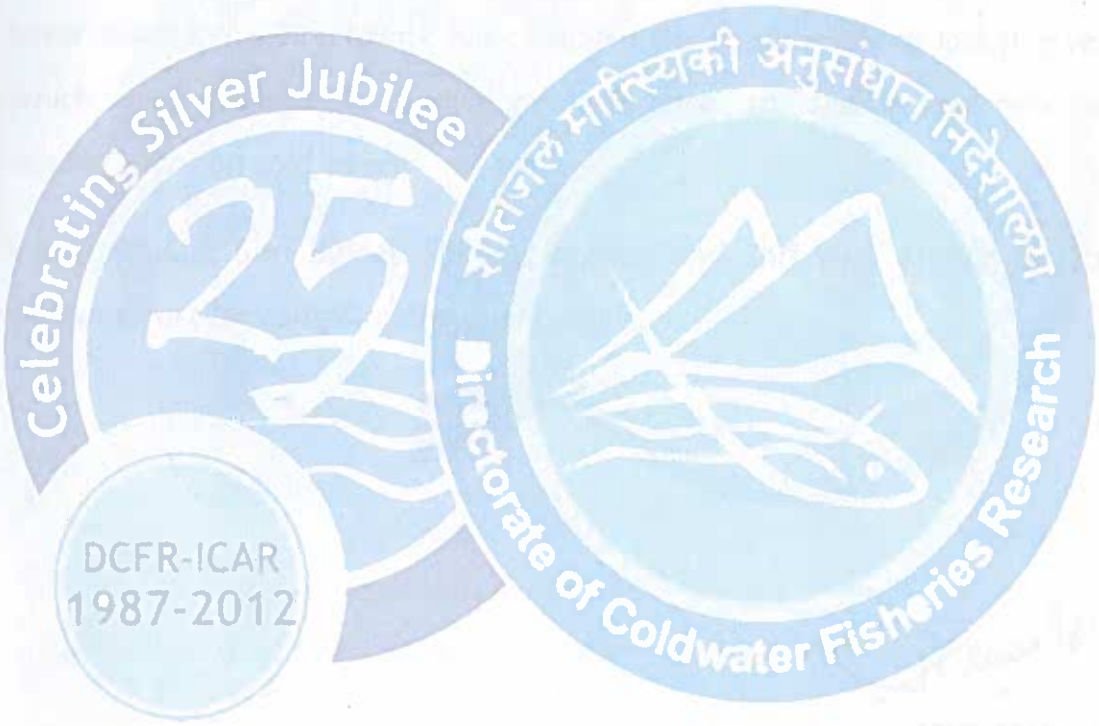

शीतजल मात्स्यिकी अनुसंधान निदेशालय

(भारतीय कृषि अनुसंधान संस्थान)

भीमताल – 263136, नैनीताल, उत्तराखण्ड (भारत)

Preface

It is a matter of great pleasure that Directorate of Coldwater Fisheries Research has completed 25 years of its establishment. These twenty five years of journey witnessed the success of scientists and researchers in several field like aquaculture suitable to hill condition, species diversification to increase the production in coldwater sector, breeding of coldwater fish species, molecular genetics, fish nutrition, fish health management, extension and conservation. The success of research is reflected through publications in reputed journals which brought glory to Directorate. To commemorate the Silver Jubilee the Directorate has compiled the publications of last five years which has generated readymade reference to the researchers and academicians on cold water.

I congratulate Shri Baldev Singh, Librarian and Shri Vijay Kumar for their sincere effort for compiling the publications.

DCFR-ICAR
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(P.C. Mahanta)
Director

October 23, 2012
Bhimtal

Introduction

The Directorate of Coldwater Fisheries Research (DCFR) came into existence as National Research Center on Coldwater Fisheries as an independent Research Center on 24 September, 1987 during the VII Five Year Plan. This is the only national facility in the country to take up the research investigation on capture and culture aspects with a focus on exotic and indigenous coldwater fish species. Since its inception, the DCFR in spite of constraints in terms of manpower and infrastructure has made significant contribution for proper appraisal of coldwater fishery resources and developed suitable technologies to propagate important coldwater fish species in hills. Keeping in view the ever expanding activities of NRCCWF and the greater potential of coldwater fisheries in different Himalayan states, in a significant decision during the XI plan, it has been made Directorate of Coldwater Fisheries Research (DCFR), to develop location, situation and system specific technologies by utilizing and augmenting resources in all the Himalayan states from Jammu & Kashmir to Arunachal Pradesh. The Directorate has completed 25 years of establishment and now has entered into its "Silver Jubilee Year". During this period the Directorate has done sincere efforts to harness the available resource in a sustainable manner and equipped itself to face new challenges in coldwater fisheries research and development. The progress made in terms of infrastructure and research facilities are commendable. The DCFR is on its glorious path of virtually actualizing its vision by imparting boon of quality research in sustainable coldwater fisheries production, management and conservation. During these 25 years, DCFR has published some peer reviewed articles/bulletins/abstracts etc. In this edition, it was tried to compile all those publications of five years for ready reference to the academicians, researchers and students.

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Aquaculture Management, Pages 105–113
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CULTURE OF CARPS IN HIGH ALTITUDE: SOME IDEAS ON ITS SUCCESSFUL MANAGEMENT

P.C. Mahanta, B.C. Tyagi and Debajit Sarma

Abstract

The biological productivity in the foothills of high altitudes is quite high which can suitably be harnessed into fish biomass. Considering the available natural resources, the production from coldwater fisheries sector can make a dent and contribute to the economy of hilly regions especially by adopting carp culture with a certain series of successful management practices. The experiment was performed to develop a composite carp farming system for the mid-Himalayan region with a special case study carried out in the Himalayan states of Uttarakhand, Arunachal Pradesh and Manipur (800-1740 m asl). More biomass was obtained by stocking fish seed @ 6/m^2 but growth reduced to 15-20 gm/ month against the average growth of 33.4-77.1 gm /month when stocked @ 2-4 fish /m^2. Higher production and growth was recorded at 1740 m asl as compared to 800-1200 m asl irrespective of the altitudinal location of the ponds and temperature ranges. The average fish production was ranging from 3289-4972 kg/ha/yr during the experimental period in mid-high altitudinal region. The fish feed in a culture system is a single input and share 45% of total expenditure. The present work will certainly indicate that the culture of Chinese carps in hills is easy to operate, economical and eco friendly since it is able to convert/recycle the cattle yard/ agricultural/kitchen wastes into value added fish food.

Introduction

India has a vast upland aquatic resources in terms of upland rivers/streams, high and low altitude natural lakes, man made reservoirs, both in the Himalayan region and Western Ghats, which is the native of a large population of indigenous and exotic, cultivable and non cultivable fish species. The important coldwater aquatic resources of the country comprises 10,000 km of streams/ rivers, 20,500 ha natural lakes, 50,000 ha reservoirs and 2,500 ha brackish water lakes which harbors 258 coldwater fish species (Mahanta, *et al.*, 1998). The biological productivity in high altitudes is limited by a number of factors; however, in the foothills, the biological productivity is quite high which can suitably be harnessed into fish biomass (Sarma *et al.*, 2003; Tyagi *et al.*, 2005). The per unit production of cold water fisheries to the total inland production basket is not very significant but these precious natural resources has the potential to contribute to the economy of our

hilly regions and uplands specially by adopting carp culture with a certain series of successful management practices.

Mountain and hill regions can contribute substantially to the economic development of India. Fish being cold blooded animal need water for all activities but hardly consume 10-15% volume of it for survival and growing. The water, a scared commodity for survival of all living beings, in hills can be harvested from rains or by diverting from stream/rivulet into ponds and that can be used for growing crop/vegetables, cattle needs, fish rearing at a time. Carps efficiently feed on insects, worms etc. available in the ponds and can convert wastes from cattle yard, agricultural, kitchen into fish flesh of high nutritive value ready for consumption. Harvesting and conservation of water has multiple benefits, in one way it will improve the sub-soil water availability and in another way it will also augment the farm productivity and up gradation of environment in carp farming in high altitudinal regions of India.

Common carp phenotypes viz. scale carp, *Cyprinus carpio communis* and mirror carp, *Cyprinus carpio specularis* constitute the bulk of the commercial fishery of certain upland lakes and reservoirs of J&K, Himachal Pradesh, Uttarakhand, North Bengal, Arunachal Pradesh, Nagaland, Meghalaya, Tamilnadu and Kerela. Monoculture of *Cyprinus carpio* was introduced initially in these regions to create awareness about aquaculture. By exploiting the natural productivity of rural ponds and regulating the kitchen refuse and other run-off from the village into pond an estimated production in the range of 2.0-2.5 tonnes /ha was achieved in a period of 12 months (Vass, 2005). However, monoculture without integrating with other farming system is uneconomical and considered as waste of natural as well as human resources.

The work carried out by NRC on Coldwater Fisheries evaluated status and potentials of cultivable carps of indigenous and alien origin in Hills (800-1740 m asl) and developed an integrated carp farming system for the mid-Himalayan region. The article embodied to develop a composite carp farming system for the mid-Himalayan region with a special case study carried out in the Himalayan state of Uttarakhand, Arunachal Pradesh and Manipur.

Materials and Methods

A total numbers of 60 ponds located at altitudinal range of 800-1740 m asl in the districts of Nainital, Almora, Bageswar and Champawat of Uttaranchal state, Zero, Along and West Kameng district of Arunachal Pradesh and Ukhrul and Chandell district of Manipur were selected to demonstrate composite carp farming system developed for high altitudinal region during 1998-2004 based on a survey and data on biological status of ponds in addition to socio economic levels of clients. Three species namely grass carp (feeding on all type of aquatic and terrestrial weeds), silver carp (feeding on natural microscopic insects-plankton) and common carp (feeding on faecal matter of grass carp, worms on bottom and unutilized feed) are stocked @ 2.8-4 fishes/m^2 in April when water temperature

was above 17^0C with the provisions of supplementary feed and fertilizers. The selected ponds were renovated to hold harvest water. Pre stocking management i.e. filling ponds, liming, seed transport and stocking @ 2.8-4 fish /m^2 having grass carp 35-50%, silver carp 25-30% and common carp 30-35%0f 20-40 mm size were carried out. The fishes were daily fed @ 3-4% of their body weight on supplementary feed prepared from kitchen wastes/ local cheap food items and terrestrial weeds. The performance of each species of fish in terms of growth, survival, contribution to local biomass, FCR, pond conversion ratio and economic viability based on expenditure and income were analysed by adopting standard methodology. The share of fish culture in total farm productivity and income were also calculated.

Results

The experiment conducted on carp farming in high altitude showed that yield depends on the growth rate and retrieval of the stocked species at the time of harvest. The coldwater located in the hills at different altitudes are no exception. The temperature tolerance studies of important cultivable carps under farm conditions were carried out and recorded for optimum water temperature, average water temperature and adverse water temperature. During the study the optimum water temperature, the average water temperature and the adverse water temperature for the cultivable carps were found to be ranging between 22-28°C, 20-29°C and 6.3-17°C respectively (Table-1). The growth of cold water fishes like Himalayan mahseer and snow trout was recorded 6.6-12.9 and 3.3.-4.9 g/month at 800-1200 m asl (Table-2).

Table-1. Temperature tolerance of cultivable fishes under farm conditions in hills

Species	Optimum water temperature (°C)	Average water temperature (°C)	Adverse water temperature (°C)
Common carp	28.0	20.0-26.0	6.3-9.1
Silver carp	28.0	20.0-27.0	6.3-10.0
Grass carp	27.0	20.0-29.0	6.3-17.0
Mahseer	22.0	20.0-27.0	9.2-10.0
Mrigal	–	22.0-28.9	9.1-17.0

The experimental ponds were treated with different stocking densities and growth of individual ponds was recorded in relation to the stocking densities. It was observed that more biomass could be obtained by stocking fish seed @ 6/m^2 but growth reduced to 15-20 gm/ month against the average growth of 33.4-77.1 g /month @ when stocked with 2-4 fish /m^2. The growth of grass carp was recorded in relation to the quality and quantity of the grass fed. The fishes were also fed with *dub grass* that grows in hills. In a span of 7-8 months @ 40-50% of stocking density it has attained the maximum weight of

1060 g. The importance of supplementary feed in carp poly culture or other fish culture system is well documented. In hills, irrespective of altitude, the quantity and quality of supplementary feed played very important role. Higher doses of feed @ 3-4% of body weight of fishes produced an average production of 4235-5610kg/ha/yr during 1998-2005. On reducing the feed to @2% of the body weight, the production recorded low to 2371-3246 kg/ha/yr in the corresponding period. The ponds where feeding were given occasionally (2-3days /week) got an average production of 1183-2736 kg/ha/yr (Table-3). The trials (148) showed that 900kg feed/ha for a rearing period of 8 months (temperature17.6-26.5^{0}C) is sufficient to achieve fish production @ 5610 kg/ha. The quantity can be reduced if the temperature is low and also can be safely applied @ 10000-12000 kg/ ha/yr at higher temperature of 27-28^{0}C. Apart from this, slightly

Table 2. Performance of different fish species under farm conditions in hills

Sl No	Species	Density (nos/m^2)	Growth (g/month)	Production (%)	Rank
1	Common carp***	1.0-2.0	12.0-27.1	23.3-46.3	2
2	Silver carp***	0.6-2.0	14.7-26.8	11.1-32.4	3
3	Grass carp**	0.4-2.9	30.2-62.8	24.2-66.8	1
4	Mahseer***	0.1-1.2	6.6-12.9	4.0-18.0	5
5	Snow trout***	0.1-0.3	3.3-4.9	0.1-0.6	6
6	Rohu*	0.3-0.5	7.1-8.2	5.0-6.6	4
7	Mrigal**	0.3	5.0	NIL	7

* at 1400 m asl ** at 1620 m asl *** at 800-1740 m asl

Table 3. Fish production in relation to altitude, density and husbandry practices adopted in hill ponds

Particulars	1998-99	1999-2000	2000-01	2001-02	2002-03	2003-04
Total no of ponds	8	15	24	40	60	50
No of ponds harvested	6	14	14	30	50	40
Location (m asl)	800-1740	800-1740	800-1740	800-1740	800-1740	800-1740
No. of fish species	3-4	3-4	3-4	3-4	3	3
Density (no./m^2)	1.6-5	2.3-5	3-4	3-4	3-4	3
Average production (kg/ha/yr)	3289	3404	3590	3698	3508	4972
Highest production (kg/ha/yr)	4345	5041	5987	5981	6942	9860
Lowest production (kg/ha/yr)	2028	1183	1330	1335	1200	2736
Production under intensive culture (kg/ha/yr)	4956	5041	4235	4902	4271	5610
Production under semi intensive culture (kg/ha/yr)	2630	2700	2371	2504	3100	3246
Production by recycling kitchen wastes (kg/ha/yr)	3379	3824	3071	3470	3421	4106

higher protein (21-30%) and fat (15-25%) required in cold water fish feeds. Raw Soya floor is a growth inhibitory feed but roasted one is good and cheap source of protein. Admixture of vitamin A+ C+ E is found to be beneficial in cold-water fish feed.

The fish feed in a culture system is a single input and constitute 45% of total expenditure (Fig.1). The use of local feed items or recycling of cattle yard /kitchen wastes was found to be one way to reduce the cost of fish production. Admixture of backery /kitchen wastes to the extent of 50% @ 3% of body weight of fish helped to achieve a production of 3379 kg/ha/yr. Further trials on recycling cattle/kitchen wastes in different ponds revealed impressive growth of fishes and showed production of 3421-4106 kg/ha/yr indicating utility of integrated farming based on fish culture (Table-3). The experiment of carp farming under different management practices were carried out and recorded at high altitudinal region. Under extensive culture system the production was recorded 1183-2736 kg/ha/yr during 1998-2004 (n=108); semi intensive practices showed higher production of 2371-3246 kg/ha/yr and intensive program led to the highest fish production of 4232-5610kg/ha/yr during the corresponding period (Table-3). The share of fish culture in the total income from agriculture and allied activities were analysed and recorded under the integrated fish farming system adopted in the three hilly states. The fish culture contributed upto 30% to total income from agriculture and allied activities (Fig.2). The average annual production during the experimental period were analyzed and recorded in Fig. 3.The economic evaluation of fish culture was carried out and average expenditure on fish unit has been enlisted in Table-4 and Fig.2. The net profit is reported to be Rs. 47/kg against the cost of production of Rs. 23/kg. Based on the present experiment the operational calendar for composite carp farming system was developed as a model according to the scientific requirements and needs of the people of hilly regions (Table-4). The variants of the technology for different altitudinal regions have been evolved and found to be economical and operative (Table-5).

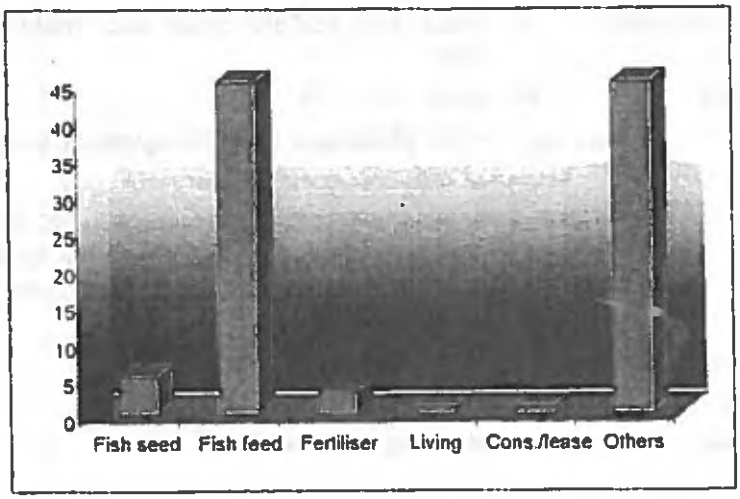

Fig.1. Expenditure on different inputs in fish culture

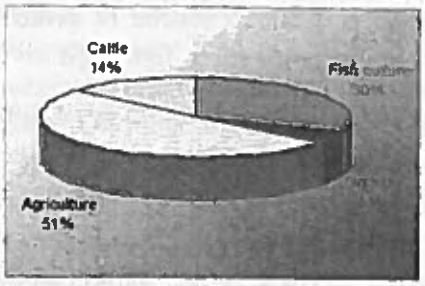

Fig. 2. Share of fish culture income in total income from agriculture and allied activities

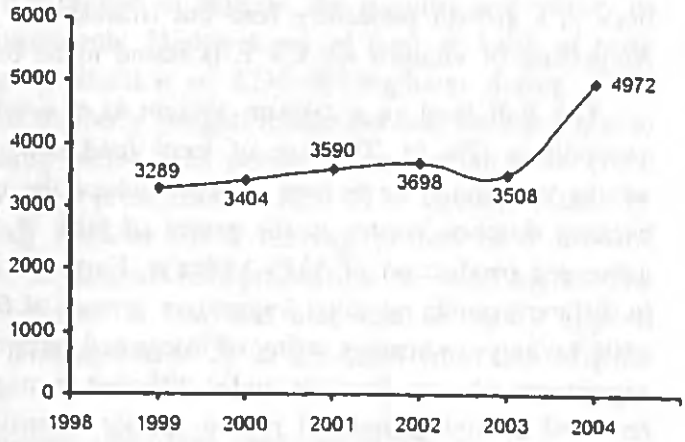

Fig. 3. Average fish production (kg/ha/yr) during 1999-2004

Table 4. Fish farming technology and its operational calendar

Area of application	**Sub- Himalayan area (800-1740 m asl)**
Pond size	Minimum100-150 m^2
Depth of the ponds	Minimum 1.8-2.0 m
Type of the ponds	Earthen/RCC having soil at bottom
Water	Harvested rain water or from the stream
Period of operation	April-October/early November
Fish species	Grass carp, Silver carp, Common carp (Mahseer and Rohu below 1200m asl)
Fish density	2.8-4 fish/m^2
Fish species combination	Grass crap 35-50%, Silver carp 20-30%, Common carp 30-35%
Size of fish stock	30-40 mm
Fertilization	RCD 15000kg + Urea 50 kg/ ha at every 10^{th} day
Liming	@ 200-350 kg/ha/yr every 10^{th} day
Feeding	Soaked in dough form made from OC 30% + WB 40% + RP 20% + Soya beans 10% @ 9-11000 kg/ha/yr. Two times daily in trays. Vegetable waste/terrestrial grass for grass carp daily on platform
Monitoring growth/water quality	At least once in a month
Harvesting time	Early November
Expected harvest	90.00 kg or 0.596 kg/m^2

Table 5. Composite carp farming and its variants suitable for West and East Himalayan region

Particulars	CFCV-1	CFCV-2	CFCV-3	T-1
Area of application	260-800	800-1300	1300-1800	>1800
Operational period	March-November	March-October	Apr to Oct	Round the year
Temp.range (^{0}C)	15.0-31.0	9.1-29.5	4.5-27.6	3.0-18.0
Nature of pond	Earthen	Earthen/RCC	Earthen/RCC	RCC/GRP
Size (m^2) & shape	1000 rectangular	400-1000 rectangular	150-400 any shape	150-200 rectangular
Pond depth (m)	1.8-2.5	1.8-2.0	1.5-2.0	1.0-1.5
Source of water	Rain, canal, tube, well	Rain/stream	Rain/stream	Stream/springs
Fish species	Catla, Rohu, Mrigal, Grass carp, Silver carp, Common carp	Rohu, Grass carp, Silver carp, Common carp, Mahseer/ Katli	Grass carp, Silver carp, Common carp,	Rainbow trout
No/m^2	0.6-1.0	2.0-3.0	3.0-4.0	10-15
% of species	10,10,10,25,25,20	10,35,20,25,10	50,20,30	100
Size of fish seed (mm)	25-30	25-30	30-40	40-50
Feed				
i. % of body weight	1.5-3.0	2-3	2-3	5-8
ii. @ kg in 1000/ha	14-19	12-15	9-11	As required
iii. Frequency	Daily	Daily	Daily	Daily
iv. Items***	OC+RB+WB+FM	OC+RB+WB+FM	OC+RB+WB+sbM	Formulated
v. Grass	Daily 15% of wt.	Daily 10% of wt.	Daily 10% of wt.	Nil
Fertilizer: Every 10th day (kg/ha/yr)				
i. Raw cattle dung	15000	8000	5000	Nil
ii. Urea	250	50	–	–
iii. S. phosphate	300	100	–	–
iv. Oil cake	100	50	50	
Liming every 10th day (kg/ha/yr)	250	300	300	Nil
Monitoring growth, health, water quality	Every month	Every month	Every month	Every 10th day
Harvest	Early December	Mid November	Early Nov.	Marketable size
Harvest (kg/m^2)	1.0-1.2	0.6-0.8	0.4-0.6	–
Range of wt. of fish	0.8-2.1	0.5-1.0	0.4-0.9	0.6-0.8
Net profit Rs./kg	23.00	30.00	47.00	–

Discussion

From the result of the temperature tolerance studies it could be inferred that the Indian major carps can not survive and grow if the water temperature is below 7.0^0C where as grass carp, common carp and silver carp were found growing above this temperature and surviving at 6.3^0C. The growth and survival of fishes found directly correlated with water temperature, quality and quantity of supplementary feed provided, natural food available in ponds and *modus operandi* of the operator in both tropical and temperate waters. The coldwater located in the hills at different altitudes are no exception. The water temperature is inversely correlated to altitude and is single vital factor to control the growth, survival and production of fish. Each species has its own thermal regime but adverse impact can be neutralized in lower and upper range of it by adopting scientific management practice. It is to be noted in this context that temperature plays a vital role in the physiology of fishes (Takashi, 1982).

Higher production and growth was recorded at 1740 m asl as compare to 800-1200 m asl irrespective of the altitudinal location of the ponds and temperature ranges. The stocking density was found regulating individual fish growth irrespective of other treatments. The management practices adopted by the farmers were found directly correlated to production level. It is to be mentioned here that Tyagi and Behl, 1998 and Bhuyan *et al.*,, 2003 also found similar result when studies were conducted in different altitudinal region. The growth of grass carp was found related to the quality and quantity of the grass fed. It grew equally well in the hills on *dub grass*. The fertilizers of organic manure like raw cattle dung, oilcake together in prescribed doses at 8 0 days interval are important to produce natural food in ponds. Such fertilization procedure play vital role in getting higher survival and growth of fish. The use of inorganic fertilizers in hill ponds is not required at all as in tropical aquaculture. There are much more scope to replace the fertilizers by recycling the cattle yard washing or wastes.

The result of the production figures under different management practices certainly indicated that the culture of Chinese carps in hills is easy to operate, economical, eco friendly since it is able to convert/recycle the cattle yard/ agricultural/ kitchen wastes into value added fish food. It is to noted that the integrated farming system based on fish culture as one of the component is highly paying as fish culture contribute upto 30% to total in come from agriculture and allied activities. The economical evaluation of fish culture under Transfer of Technology programme indicated higher profitability from integrated farming system based on fish culture.

The knowledge and aptitude of the prospective clients, financial constraints in constructing fish ponds, non availability of quality fish seed of candidate species at right time and non persuasive approach of the concerned agencies are some factors which restricts the adoption oh composite carp farming system in hill areas. Imparting training to the clients through demonstration program on fish culture, providing financial and technical assistance initially can pave the way to adopt the integrated farming based on

fish culture in Himalayan uplands which in turn can help the people to improve their socio-economic status and availability of high protein food especially for the people of North Eastern region who relish fish most.

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SPECIES DIVERSITY OF POLYCHAETE FAUNA OF DIGHA-TALSARI REGION OF WEST BENGAL, INDIA

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Polychaetes are one of the important natural foods of many economically important fishes, containing high calorific value and rich in protein. In the present investigation, taxonomic account, distribution pattern and habitat ecology of 24 species of polychaetes belonging to 14 genera in 12 families are studied. The analysis of the data shows that the majority of these species are restricted to areas located at the lower reaches, attributed to increased flow of sewage and wastes pouring into the sea. Many polychaetes are being used in aquaculture and agricultural sectors as live feed and biopesticide, respectively. The study has identified prospect of capturing and further culturing this group.

Key words : Polychaetes, taxonomic account, live feed, biopesticide.

Introduction

Polychaetes constitute the most abundant and diverse faunal group in all marine sediments from intertidal to deep sea (Annandale, 1922). Around 10,000 species have been described worldwide. Most of them are small and short-lived, with a high secondary production. Generally they form an important link in marine food webs. Because of the high calorific value and rich protein content, both the adult and larvae of polychaetes are considered to be one of the main food supplies to many economically important fishes (Meunpol, *et al.*, 2005). In China, large quantity of Nereidae are exported to Japan as bait for recreational fishing besides being a delicacy in South China and Southeast Asia.

Since decades, yeast, bacteria, micro algae, ciliates, rotifers, copepods, cladocerans, brine shrimps and tubifex have been used as live feed in aquaculture practices. But global increase in establishment of number of shrimp hatchery units, the aquaculture sectors including larval and grow out feed producers and the aquarium trade, the demand for organisms like polychaetes as a live feed has also increased (Craig and Rutherford, 2005). In view of large demand for polychaetes as live feed, some companies have specialized the culture and supply of high quality polychaete worms to aquaculture hatcheries. This increased demand for these invertebrates is directly linked to their high palatability, attractability, digestibility and rich nutritional profile.

Polychaetes are also excellent bio-indicators of benthic environment, being the dominant macrofauna within the fine sediment (Palomo, 2002). A considerable work on the taxonomy of polychaetes has been done around the world. In India, the Zoological Survey of India has recorded 300 species of polychaetes, including 40 species from the brackishwater environment (Fauvel, 1932; Mishra, 1995, 1998). In addition, the taxonomy of polychaetes has also been done by Southern (1921); Fauvel (1953) and Mishra *et al.*, (1984). The present investigation was carried out to identify the presence of diverse groups of polychaete fauna in Digha-Talsari region of West Bengal, India for possible exploration in fisheries development on a regional basis.

Materials and methods

The Digha-Talsari site located close to the Gangetic mouth on the East coast of India, facing Bay of Bengal at a latitude 21 °36' N and longitude 87 °30' E was selected for the present study, especially in the backdrop of prevailing shrimp farming in this region, over the period May, 2004 to April, 2005. The polycheate fauna was sampled once in a week. Pelagic annelids were easily sampled by towing plankton net of 100 µm mesh size. Night fishing with artificial light was also employed to collect a lot of syllids, epitokous, nereids and many rare small species of polychaete larvae. The other necessary implements used for sampling polychaetes were stout spade, crowbar, chisel and canvas bucket, fisherman's basket with several glass jars and a number of glass tubes. Large specimens of polychaetes were easily collected once the dredge or trawls were on board and the contents scattered on deck. The contents were brought to the laboratory and allowed to dip inside the wash basin or glass vessels containing seawater which enabled the small specimens to come out from their hiding. The collected specimens were washed in brackishwater and allowed to slacken off up in 7% magnesium chloride ($MgCl_2$) prepared in brackishwater to avoid twisting or contravening of the specimens. Polychaetes of the family Phyllodocidae, Nereididae and Glyceridae were treated with sudden addition of absolute alcohol for overting their pharynx. They were preserved in 70-75% alcohol. The large species were kept for narcotization for a short time in 5% commercial solution of formalin before preserving in spirit. Delicate and brittle species were also narcotized prior to fixing in spirit. The narcotization was done gradually with increasing concentration of alcohol (5 to 10 %) to the seawater. Other anesthetics like cocaine, chloral were also used for narcotization occasionally. The collected polycheates were identified using identifying key stated by Fauvel (1953).

Results and discussion

In the present investigation, taxonomic account, distribution pattern and habitat ecology of 24 species of polychaetes, belonging to 14 genera under 12 families were studied. They were observed to inhabit in freshwater, brackishwater and sea. The errantiates and sedentarian polychaete fauna dominated the species composition at Digha (W.B.), Talsari (Orissa) and other adjacent areas. Certain species of polychaetes collected under the present study are presented in Fig. 1. A checklist of all polychaetes collected is also enlisted below.

Checklist

i. Family - POLYNOIDAE Malmgren, 1867.
Sub family - Lepidonotinae Horst, 1917
Genus - ***Lepidonotus*** Leach, 1816
Species - (1) *Lepidonotus tenuise* (Gravier)
Sub family - Harmothoinae Horst, 1917
Genus - ***Gattyana*** McIntosh, 1900.
Species - (2) *Gattyana fauveli* (New species)

ii. Family - AMPHINOMIDAE Savigny, 1818
Genus - ***Chloeia*** Savigny, 1818
Species - (3) *Chloeia parva.*

iii. Family - PHYLLODOCIDAE Williams, 1851
Genus - ***Eteone*** Savigny, 1818
Species - (4) *Eteone barantollae* Fauvel, 1932
(5) *Eteone ornata* Grube, 1878

iv. Family - NEREIDIDAE Jonston, 1865
Genus - ***Dendronereides*** Southern 1921
Species - (6) *Dendronereides heteropoda* (Southern)
Genus - ***Neanthes*** Kinberg, 1866
Species - (7) *Neanthes chingrighattensis* (Fauvel)
(8) *Neanthes chilkaensis* (Southern)
Genus - ***Perinereis*** kinberg, 1866
Species - (9) *Perinereis nigropunctata* (Horst)
(10) *Perinereis cultrifera* (Grube)
(11) *Perinereis nuntia* (Savigny)
(12) *Perinereis nuntia* Var. typica

(Savigny)

v. Family - NAPHTYDAE Grube, 1850
Genus - *Nephtys* Cuvier in Audouin & Milne Edwards, 1833
Species - (13) *Nephtys dibranchis* (Grube)
(14) *Nephtys oligobranchia* (Southern)

vi. Family - GLYCERIDAE Grube, 1850
Genus - *Glycera* Savigny, 1818
Species - (15) *Glycera convoluta* (Keferstein, 1862)
(16) *Glycera rouxii* (Audouin and Milne Edwards, 1878)

vii. Family - ONUPHIDAE Kinberg, 1865
Genus - *Diopatra* Audouin and Milne Edwards, 1833
Species - (17) *Diopatra cuprea* (Bosc, 1802)

viii. Family - EUNICIDAE Grube
Genus - *Marphysa* (Quatrefages)
Species - (18) *Marphysa gravelyi* (Southern)

ix. Family - LUMBRINERIDAE Malmgren, 1867
Genus - *Lumbrineris* Blainville, 1828
Species - (19) *Lumbrineris heteropoda* (Marenzeller, 1879)
(20) *Lumbrineris polydesma* (Southern, 1921)
(21) *Lumbrineris notocirrata* (Fauvel, 1932)

x. Family - SPIONIDAE Sars
Genus - *Polydora* Bosc
Species - (22) *Polydora ciliata* (Johnston)

xi. Family - CAPITELLIDAE Grube, 1862
Genus - *Parheteromastus* Monro, 1937
Species - (23) *Parheteromastus tenuis* (Monro)

xii. Family - TEREBELLIDAE Malmgren
Genus - *Loimia* Malmgren, 1966
Species - (24) *Loimia medusa* (Savigny, 1818).

The density and diversity of polychaete fauna at Digha beach and Talsari area were sufficiently rich, which may be due to relatively stable sand, flat substratum and abundance of organic detritus (Mishra, 1998). The flat substratum was also rich in meiofauna population. Availability of rich meiofauna is highly essential for completing the food chain of beach fauna (Varshney and Govindon, 1995). But, it is difficult to explain the differences in density and specific occurrence of polychaetes at different horizontal levels in these areas. It may be due to their environmental preference and tolerance of component species. *Lumbrineris polydesma* was highly populated species recorded towards the canal area (muddy substratum) of the upper littoral zone of Talsari. The analysis of the data showed that the majority of these species were concentrated in the lower reaches, which decreased gradually towards the upper reaches. It was observed that the fluctuation of salinity in brackishwater zone highly affected the population of colony forming polychaetes with a declining trend with the increase in distance from the sea. Similar observation was also made by Mishra (1995). High abundance of *Lumbrineris polydesma* was recorded during June- July, 2004 (70 nos./sq.ft.), but it decreased in number (20-25 nos./sq.ft) during mid November to December, 2004. Some species of Nereididae family were also found in mud spattered area. The density of *Dendronereides heteropoda* was recorded as 20-30 nos/sq.ft while the population rate of *Glycera sp.* in mid littoral and sub littoral zone of Talsari was only 4-5 nos/sq.ft and in 2-3 nos/sq.ft, respectively. The population density of *Glycera alba* in upper littoral zone was found to be 2-3 nos /sq.ft. Some species of Nereididae, Onuphidae and Glyceridae were also recorded between 5 and 7 nos/sq.ft in the sub-littoral zone. Anthropogenic activities and indiscriminate waste disposal in Digha-Talsari region was observed during the present investigation. The *Parheteromastus* sp. was typically found in areas affected with sewage pollution. The dominant polychaetes of this region mentioned earlier found scattered in different places of the study area.

The main limitation using polychaetes as a biomonitor for pollution study is the paucity of its taxonomic and biological information (Dirk *et al.*, 1998). There is also dearth of information on the life history and seasonal variation of endemic

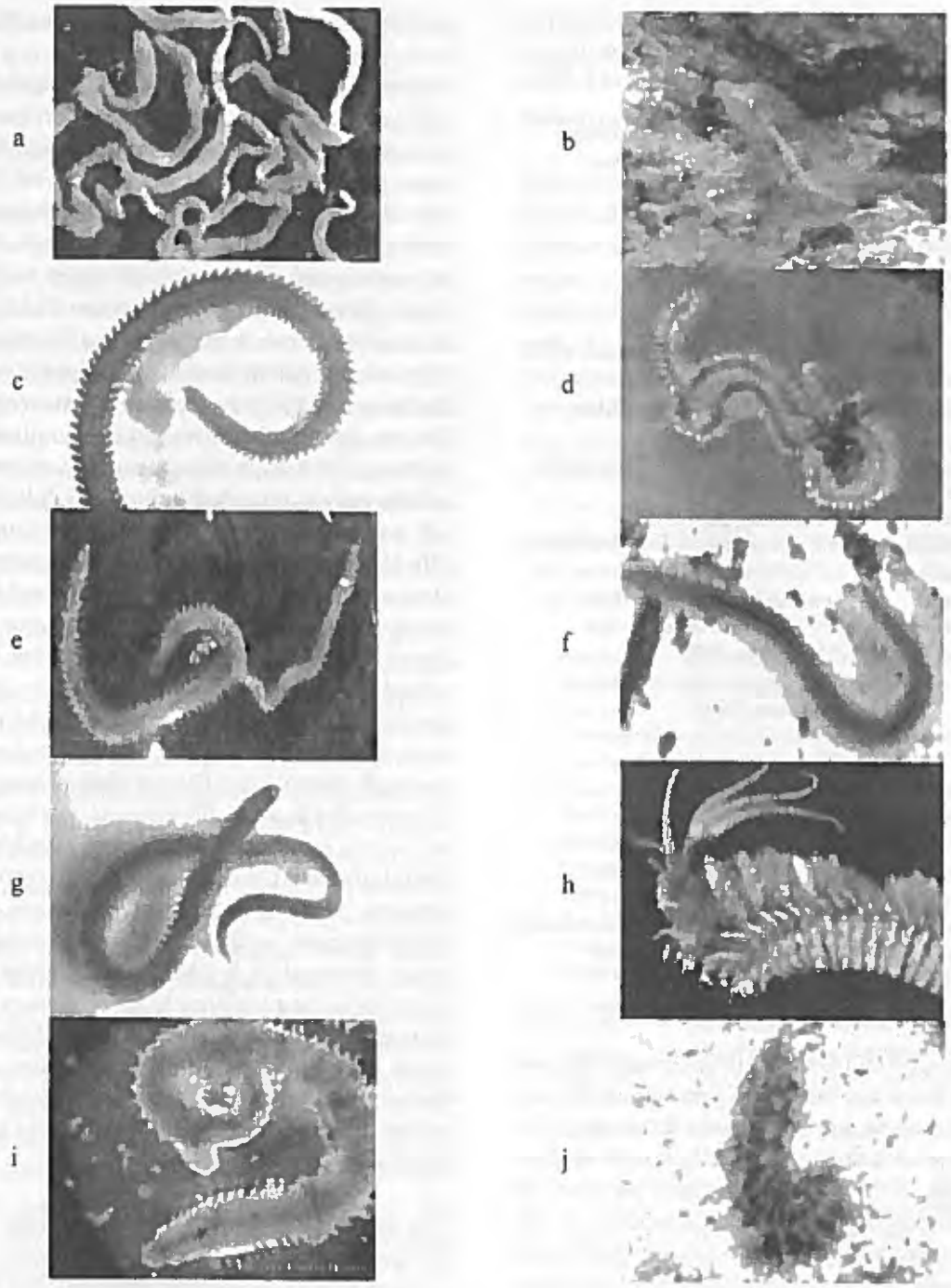

Fig 1. *a* - *Gattyana* sp.; *b* - *Chloeia parva*; c - *Eteone* sp ; d - *Neanthes* sp., e - *Perinereis cultrifera*. f - *Nephtys* sp.; g - *Glycera convoluta*; h - *Diopatra cuprea*; i - *Lumbrineris* sp.; j - *Loimia medusa*.

polychaete population. The knowledge on their natural responses to the various environmental parameters is limited and investigation on the ecotoxicology is still at an infant stage, complicating interpretation of field data. Application of organic pesticide obtained from *Lumbrineris heteropoda* is reported to be safe to human and domestic animals (Nitta, 1934). As this species is also available in the present collection, it indicates the possibility of judicious exploitation of it for the production of bio-pesticide.

Though the present study has only identified diverse group of polychaetes, there is a scope for establishing some kind of a co-management practice, roping in market oriented expertise, culture experts and Government agencies. It offers immense potential for economic and social benefits to the local community, and so, demands further investigation.

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Molecular Phylogeny of Cyprinid Fishes of India Using 12S rRNA Gene Sequences

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Taxonomic position of the four important coldwater fish species of India—namely, the snow trout, *Schizothorax richardsonii* (Gray); golden mahseer, *Tor putitora* (Hamilton-Buchanan); Indian trout, *Raiamus bola* (Hamilton-Buchanan) and garra, *Garra gotyla* (Gray)—varies according to different sources. In order to confirm the subfamily level taxonomic positions, a pair of universal primers amplified 12S rRNA genes of these species. The universal primers uniformly amplified 456 bp lengths of 12S rRNA genes of all the species. The amplified genes were sequenced further, and the base composition and the alignment of sequences were compared. The presence of a common conserved core region of all the four fish 12S rRNA genes indicates that all these species belong to the same family (Cyprinidae). The phylogenetic tree constructed based on these 12S rRNA gene sequences suggests the possible occurrence of three subfamilies, namely, Schizothoracinae, Cyprininae and Rasborinae, within the family Cyprinidae.

Keywords: Cyprinid fishes, Mitochondrial DNA, Phylogeny, Genetic distance, 12S rRNA

Introduction

Coldwater fishery is one of the emerging contributors to inland fish production of India through aquaculture and capture fisheries. The trouts, mahseers, schizothoracids, minor carps and exotic carps widely distributed in the Himalayan and peninsular regions of India are important both for food and recreation (Tripathi, 2005). *Tor putitora* and *Schizothorax richardsonii* are the most commonly cultivated species due to their wide range of distribution in the hilly region (Sehgal, 1987). *Raiamus bola* and *Garra gotyla* are the emerging potential candidate species for coldwater aquaculture in the Kumaon region of India. But the systematic classification of these fish species is still unclear. Even though they are classified under the family Cyprinidae, the subfamily level classification is ambiguous. Different authors have classified them under different

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subfamilies, leading to improper taxonomy of the species (Berg, 1940; Munro, 1982; Kapoor *et al.*, 2002; and The Catalogue Fishes, Online).

Recent developments in the field of molecular biology have made it quite feasible to determine the sequence of a genome directly from a large number of individuals in a short duration (Kocher *et al.*, 1989). Mitochondrial DNA markers have been successfully employed in the systematic classification of fishes too. It helps in the characterization of systematic positions of fishes even at species level (Kocher *et al.*, 1989; Thomas and Beckenbach, 1989; Barlett and Davidson, 1991; Carr and Marshall, 1991; Orti *et al.*, 1994; Lydeard *et al.*, 1995; Zardoya *et al.*, 1995; Murphy and Collier, 1996; Brito *et al.*, 1997; Halanych and Robinson, 1997; Dergam *et al.*, 2002; Moran, 2002; and Shikano and Taniguchi, 2003) because of the availability of 'universal amplification primer sequences' (Kocher *et al.*, 1989). The mtDNA 12S rRNA gene has been one of the most widely targeted genes for the phylogenetic analysis of different taxa such as families (Alves-Gomes *et al.*, 1995; Douzery and Catzeflis, 1995; and Ledje and Arnadon, 1996) and genera (Gatesy *et al.*, 1997; and Murphy and Collier, 1996).

Due to the ambiguity in the taxonomic position of coldwater fish species of India, this study seeks to characterize 12S rRNA gene of four cyprinid species—snow trout, *Schizothorax richardsonii* (Gray); golden mahseer, *Tor putitora* (Hamilton-Buchanan); Indian trout, *Raiamus bola* (Hamilton-Buchanan); and garra, *Garra gotyla* (Gray)—to understand their subfamily level taxonomic positions.

Materials and Methods

Fish Samples

Samples of *S. richardsonii*, *T. putitora*, *R. bola* and *G. gotyla* were colleted ($n = 5$) using gill nets and traps from the lakes and streams of Kumaon region of Uttarakhand, India. Samples of *H. molitrix* were obtained from our Experimental Farm.

Extraction of DNA

The extraction of DNA from the muscle tissues was undertaken by using Wizard® Genome DNA purification kit (Promega), according to the manufacturer's instructions. The concentration of the DNA was estimated by measuring the absorbance at 260 and 280 nm in an UV-visible spectrophotometer (Lambda 35 UV/VIS spectrometer, Perkin-Elmer Ltd., USA) according to Sambrook *et al.* (1987), and the good quality DNA having the OD ratio at 1.7 to 1.9 was subjected to Polymerase Chain Reaction (PCR) amplifications.

PCR Amplification

Universal primer pairs based on the published sequences of highly conserved regions of 12S rRNA of the mitochondrial genome from the GenBank for mammal (Anderson *et al.*, 1981) were used to amplify the partial 12S rRNA genes from the samples. The PCR was set up in 50 µL reaction volume. Based on initial trial, the reaction mixture was optimized

as follows: 5 μL of 10X Assay buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM $MgCl_2$) from Bioron GmbH, 1 μL (200 μM each) of dNTP mix (sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water, pH 7.5 from Promega, Madison, WI USA), 1 μL or 20 Pico moles each of forward (5' – CAA ACT GGG ATT AGA TAC CCC ACT AT-3' 26 mer) and reverse (5' – GAG GGT GAC GGG CGG TGT GT-3' 20 mer) primers (Bangalore Genei, India), 1.66 U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron GmbH, Germany), 50 ng of purified DNA and autoclaved HPLC grade water (Merck, Germany) to make up the volume. The PCR tube containing the reaction mixture was flash spun on a micro centrifuge to get the reactants at the bottom. The reactions were performed in a Thermal Cycler (Applied Biosystems, USA) having a gold plate and heating lid.

The cycling conditions involved an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 45 sec denaturation at 94 °C, 45 sec annealing at 60 °C, and 1 min elongation at 72 °C. After the reaction, tubes with PCR products were held at 4 °C until further analysis/confirmation by agarose gel electrophoresis or stored at –20 °C for further use. The amplification patterns were analyzed on 1.4% agarose gel using a horizontal electrophoresis unit (Atto, Japan) in TBE buffer (1X, pH 8.0). An aliquot of 5 mL of PCR product was added with 1 μL of loading dye (Promega) for electrophoresis at a constant voltage 3 V/cm. The amplicons on the gel were visualized in the Gel Doc system (Alpha Imager, USA) after staining with ethidium bromide. The amplified products of 456 bp were purified by the QIA quick gel extraction kit (USA) and were used for direct sequencing without cloning.

Sequencing of Amplicons

PCR products were directly sequenced without cloning using ABI Prism 377 DNA sequencer at DNA sequencing facility (Bangalore Genie, Bangalore). The comparison of sequence was done by Clustal X method (Thompson *et al.*, 1997) using MegAlign™ software package (DNA STAR, Inc.). The mt 12S rRNA gene sequences of related fish species were retrieved from GenBank nucleotide sequence database (www.ncbi.nlm.nih.gov/entrez) and compared.

Sequence Alignment

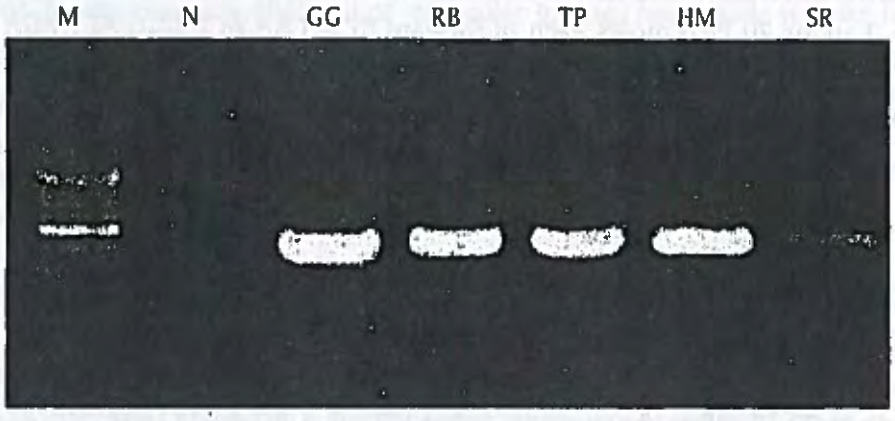
The sequence alignment, phylogenetic tree construction, percentage identity/similarity, sequence base pair distances and base substitutions were calculated using Clustal X method with weighted residue weight table.

Results

Amplification of the 12S rRNA Gene by PCR

The designed set of universal primers consistently amplified a DNA fragment of about 456 bp length of mtDNA in all the cyprinidae fish species analyzed (Figure 1). The amplified PCR product from these fish species shows the amplicon length variations

Figure 1: PCR Product of Mitochondrial 12s rRNA Gene Amplified from 5 Coldwater Fish Species



Note: M: 100 bp molecular marker; N: Negative control; GG: *G. gotyla*; RB: *R. bola*; TP: *T. putitora*; HM: *H. molithix*; and SR: *S. richardsonii*.

between the species, even though the fragments were partially overlapping each other (> 76%). The nucleotide sequences of 12S rRNA gene of different fish species submitted to EMBL nucleotide sequence database and their GenBank accession numbers are given in Table 1.

Table 1: Mitochondrial 12S rRNA Gene Sequences of Coldwater Fishes Submitted in NCBI with Accession Numbers

	Species	Accession Number
1	*R. bola*	AM778104
2	*G. gotyla*	AM778106
3	*H. molitrix*	AM778105
4	*S. richardsonii*	AM778103
5	*T. putitora*	AM778102

Size and Base Composition of 12S rRNA Genes

The sequence of the 12S rRNA gene (from 1-456 bp) was determined in these five fish species. The amplified products were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and were compared with other fish species 12S rRNA gene sequences availed from the GenBank. The positions of the structural gene region were inferred on the basis of homology with previously reported sequences from other fish species. Base frequencies of 12S rRNA genes of fishes are given in Table 2. The overall base composition of 12S rRNA gene was characterized by an overrepresentation of adenines (27.29 to 32.13), whereas the other nucleotides were almost equally distributed. The total number of nucleotide sites used in this study for phylogenetic analyses was 2223 bp. The nucleotide compositions of the 12S rRNA among these species were quite similar in all the species, with average percentages of 29.60 (A), 26.18 (C), 23.30 (G) and 20.92 (T). The comparison of gene sequences among the five species is given in Figure 2. The conserved core regions of the 12S rRNA molecule were particularly rich in purine, which was conserved in all the five species studied. The ratios of transitions and

transversions based on pairwise comparison of base sequences among these species ranged from 0.58 to 1.08. The genetic distance data among the five species are given in Table 3. The similarity among the sequences of different species ranged from 76.3 to 89.3%. The ratio of transitions and transversions between any two species from the nucleotide composition data was from 1.18 to 2.35. Pairwise comparisons of observed substitutions were similar, though differ slightly among the fish species. Dissimilarity in the range of 8.1 to 15.3% was found among the fish species studied. The highest percentage of genetic similarity was observed between *S. richardsonii* and *H. molitrix* followed by *T. putitora*. The maximum divergences were observed between *R. bola* and *G. gotyla* followed by *T. putitora*. With respect to the percent divergence data *G. gotyla* and *R. bola* had the highest divergence (15.3%), but on the other hand, least divergence was found between *S. richardsonii* and *T. putitora*.

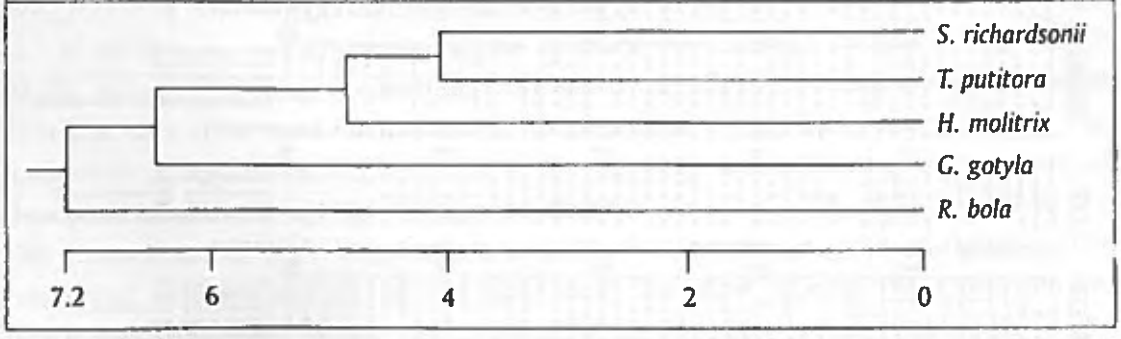
Table 3: Paired Genetic Distances Among the Coldwater Fishes Using Clustal X Method

		Percent Similarity				
Percent Divergence		*G. gotyla*	*R. bola*	*S. richardsonii*	*T. putitora*	*H. molitrix*
	G. gotyla	–	76.3	82.5	83.9	81.7
	R. bola	15.3	–	77.1	80.2	79.5
	S. richardsonii	11.4	13.2	–	86.8	89.3
	T. putitora	13.4	14.3	8.1	–	84.0
	H. molitrix	13.7	13.0	8.9	10.5	–

Phylogenetic Tree

The nucleotide sequences of 12 S rRNA genes were aligned in order to determine the phylogenetic relationships among the fish species. The phylogenetic tree constructed based on 12 s rRNA gene sequences of the five species is given in Figure 3. Unambiguous alignment was obtained for most of the 12 S rRNA genes in the conserved region. The Maximum Likelihood (ML) analyses of 2,223 nucleotide site from the mitochondrial genomic data yielded an ML topology with resolution of the branching pattern in Cyprinidae family.

Figure 3: Construction of Phylogenetic Tree Using Clustal X method



Discussion

The commercially important fish species, *S. richardsonii*, *T. putitora*, *R. bola* and *G. gotyla*, were selected for the study because of their ambiguity in taxonomic position (Berg, 1940; Munro, 1982; Kapoor *et al.*, 2002; and The Catalogue Fishes: http://www.calacademy.org/research/ichthyology/catalog/fishcatmain.asp), and the species. *H. molitrix* was used as a standard out-group since it is an established representative from the Cyprininae subfamily. According to the conventional classification, all these species are grouped under the family Cyprinidae of the Order cypriniformes (Berg, 1940; Kapoor *et al.*, 2002; and The Catalogue Fishes, Online). According to Jhingran (1991), based on the classification by Berg (1940), *S. richardsonii* and *T. putitora* belong to Schizothoracinae, *R. bola* (Previous name *Barilius bola*) to Rasborinae, and *G. gotyla* to Cyprininae subfamilies. In the subsequent classification (Talwar and Jhingran, 1991; and The Catalogue Fishes, Online), *S. richardsonii* was classified in the Schizothoracinae and T. putitora in the Cyprininae subfamily. In all the classifications, *G. gotyla* was put under Cyprininae and *R. bola* under Rasborinae. According to Munro (1982), the family Cyprinidae is having only two subfamilies, viz., Cyprininae and Rasborinae.

The universal primers uniformly amplified 456 bp lengths of 12S rRNA gene of all the species, which indicate that all the species are having a common conserved region. Moreover, the amplified gene sequences were matching with that of other fish species available from the GenBank. This implies that all the fishes analyzed were belonging to the same family, that is Cyprinidae. Wang *et al.* (2002) also amplified the 12S rRNA genes of different vertebrates by similar universal primers and was compared with other available gene sequences.

As per the base frequencies analysis, the purine and pyrimidine bases were distributed almost equally in the conserved core region of all the species analyzed. But the overrepresentation of the purine base observed in all the sequences may be a unique characteristic of the family Cyprinidae. The range of similarity among the sequences of different species (76.3 to 89.3%) was found to be sufficient enough for construction of phylogenic relationship among the species. Hillis and Dixon (1991) suggested that an optimal similarity of DNA sequences between 70% and 100% was sufficient for phylogenetic studies among vertebrates. Moreover, the range of transition and transversion ratios (0.58 to 1.08) indicate several million years of evolution among the Cyprinidae species (Springer and Douzery, 1996; and Wang *et al.*, 2002) required for the genetic evolution of the species.

The sequence pair distance data and the phylogenetic tree indicate the possible occurrence of three subfamilies among the fish species studied, viz., Schizothoracinae, Cyprininae and Rasborinae. The species *S. richardsonii* and *T. putitora* showed the least genetic divergence (8.1%) and showed as parallel branches of the phylogenetic tree, indicating that they can be classified under the same subfamily, Schizothoracinae. *G. gotyla* emerged as a different branch closer to *H. molitrix* in the phylogenetic tree, and

thus can be kept under the subfamily, Cyprininae. The conventional classification by Berg (1940) and Kapoor *et al.* (2002) also suggested that these species can be placed under the same subfamily, Cyprininae. The species, *R. bola* being the species showing maximum genetic divergence among the species analyzed (15.3%) can be placed in a separate subfamily, Rasborinae. The results are absolutely matching with the most widely accepted classification given by Berg (1940).

The present study confirms that 12S rRNA sequences can be used successfully for the phylogenetic studies of fishes even at the subfamily level. This might be due to the fact that 12S rRNA gene sequences contain several regions having variable evolutionary rates, for example, the stems of the 12S rRNA gene secondary structure sequence evolved slower than that of the loops (Wang *et al.*, 2002). The 12S rRNA sequence can be used for deducing phylogenic relationships across a broad spectrum, since transitional substitutions in the loops region have accumulated as fast as 10-20 million years after the divergence event occurred. On the other hand, transversional substitutions in stems and loops remain unsaturated for more than 100 million years (Springer and Douzery, 1996), which enables the 12S rRNA gene to be more comprehensive for phylogenetic reconstruction (Wang *et al.*, 2002). The 12S rRNA gene has been widely used in phylogenetic studies in fishes for the genomic level classification by several researchers (Alves-Gomes *et al.*, 1995; Douzery and Catzeflis, 1995; Ledje and Arnadon, 1996; Gatesy *et al.*, 1997; Murphy and Collier, 1996; and Wang and Lee, 2002). The study further suggests that the universal primers have proved to be successful for the amplification of 12S rRNA gene sequence for the molecular systematic of fish species.

Conclusion

It is concluded that the 12S rRNA gene sequence variation was sufficient for revealing the subfamily level phylogenetic relationship among the fish species. The universal primers proved to be successful for the amplification of 12S rRNA gene sequence for the molecular systematic classification of different taxas of fishes. The 12S rRNA genomic sequence-based phylogenetic tree suggests the possibility of three subfamilies within the family, Cyprinidae, viz., Schizothoracinae, Cyprininae and Rasborinae. ❋

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Effect of Spirulina Fortified Diets on Growth and Survival of Chocolate Mahseer *(Neolissochilus hexagonolepis)*

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ABSTRACT

Chocolate mahseer, considered as a new candidate species for hill aquaculture, was selected in the present study to assess the efficacy of *Spirulina (S. platensis)* fortified diets on its growth and survival. Five isoproteic diets (with 35% protein) with different percentages of *Spirulina* (0, 3, 5, 7 and 10%) were formulated. Fish were fed @ 5% of their body weight in two split doses for 90 days. Highest weight gain (302 and 313%) and lowest food conversion ratio (FCR) were recorded in case of 5% and 10% *Spirulina* fortified diets (1.48 and 1.43) while, lowest weight gain (182%) and highest FCR (2.46) were recorded for the control diet. The specific growth rate (SGR) was recorded highest (8.46%) for 10% *Spirulina* fortified diet and lowest (5.77%) for the control diet. Comparing the cost of the formulated diets, it is revealed that 5% *Spirulina* fortified diet resulted in better growth performance, effective utilization of feed and maximum survival percentages in chocolate mahseer.

Key words: Chocolate Mahseer, *Spirulina*, Isoproteic, Growth, Survival.

INTRODUCTION

Chocolate mahseer *(Neolissochilus hexagonolepis)* is a very important endangered fish of North Eastern Himalayan region in terms of its sports and food value (Menon, 1999). This fish is considered as a new candidate species for hill aquaculture particularly in Western Himalayan region (Sarma, 2009). It is widely cultured in Jhora (spring water fed) ponds fisheries of Darjeeling, West Bengal along with other carps (Barat et al. 2005). Chocolate mahseer is a voracious feeder with bottom feeding habit subsisting on gastropod shell, filamentous and planktonic algae and vegetable debris (Dasgupta, 1988; Sarma, 2006 and Sarma et al. 2007).

In India, fisheries have always been playing a provital role in the food and nutritional security of people, especially in rural areas (Sugunan, 2002) and many fish species had already been transplanted from one place to other since long on the availability of suitable eco-biological conditions to increase the fish production and rural income. *N. hexagonolepis* was introduced from wild environment of Arunachal Pradesh to Kumaon, Uttarakhand in 2008 as new candidate fish in hill aquaculture keeping in mind their feeding habit, nutritional and eco-biological needs and the similarities in both the geoclimatic conditions (Sarma, 2009).

In an aquaculture system, natural food is not sufficient to sustain optimum productions. Hence it is

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vital to provide a nutritionally balanced diet for optimum aquaculture productivity. Nutritionally balanced diet contains carbohydrate, fat, protein, minerals, vitamins and carotenoids in sufficient quantity for proper growth and pigentation (Jha et al. 2009). Many feed additives like vitamin-C, α - Tocopherol, marigold flower, red pepper, rose petals, *China rose, Chlorella, Spirulina* meal, etc. have also been utilized to improve growth, survival, fecundity, nutrient profile and pigentation in fin fish and shell fish (Rema and Gouveia, 2005; Buyukcapar et al. 2007 and Ezhil, 2008).

As feed additive, dried algae improve growth, feed efficiency, carcass quality, and physiological response to stress and disease in several species of fish (Mustafa and Nakagawa, 1995). Amongst various algae, *Spirulina* is considered a rich source of protein having 65-70% protein (Lornz, 1999), vitamins, minerals, essential amino acids, fatty acids (gamma - linolenic acid (GLA), antioxidant and pigments, such as carotenoids (Belay et al. 1996). In addition, it is also effective as an immunomodulator (Takeuchi et al. 2002). *Spirulina* is the only microalgae additive, which demonstrates benefits to growers that offset the initial cost and provide a significant cost/performance ratio. *Spirulina* has been studied over the globe by the scientists as a feed supplement for various fishes and found to significantly improve growth, survival, and feed utilization (Belay et al. 1996; Takeuchi, 2002). Several studies have been conducted using dried *Spirulina platensis* as a feed supplement (Chow and Woo, 1990; Watanabe et al. 1990).

The present study evaluates the effect of *Spirulina platensis* meal, as feed additive on growth and survival of Chocolate mahseer *(N. hexagonolepis)* in its new culture environment.

MATERIAL AND METHODS

Five isoproteic diets (with 35% protein) fortified with *Spirulina* meal @ 0 (D-1), 3 (D-2), 5 (D-3), 7 (D-4) and 10% (D-5) were tested in a totally randomized design during 90 days of experimental period, using *Neolissochilus hexagonolepis* fingerlings obtained from the hatchery rearing pond of Directorate of Coldwater Fisheries Research, Bhimtal. Fingerlings were stocked in 100 liter capacity fiber glass tanks (provided with inlet

Table 1. Proximate composition of ingredients (on % dry matter basis) in percentage

Ingredients	Moisture	Dry matter	Crude Protein	Crude Lipid	Ash
Wheat Bran	12.8	87.2	13	2.64	5.51
Rice Bran	11	89	13	4.2	11.5
SOC*	10.1	89.9	40	5.51	9.46
Fish Meal	6.8	93.2	60	8.3	14.8
Spirulina	4.5	95.5	70	4.32	9.5

*Soybean oil cake

Table 2. Proximate composition (on % dry matter basis) of different diets used in the experiment

	Diet				
	D-1	D-2	D-3	D-4	D-5
Moisture	13.28	13.40	13.15	13.34	13.30
Crude protein	35.21	35.32	35.10	35.20	35.09
Crude lipid	7.65	7.80	7.25	7.97	7.45
Ash	10.85	11.01	10.90	11.00	11.08

and outlet) @ 10 fingerlings per tank in triplicates for each diet. Constant water flow (2-3 Liter/ minute) was maintained. To eliminate fecal residues and food remains, siphoning was done daily. Water quality parameters were registered weekly following the APHA (1995) methodology before the siphoning in the morning. Total water exchange and washing of tanks with $KMnO_4$ were carried out every week to protect the fish from fungal infection. Diets were formulated using four basic ingredients (fish meal, soybean oil cake, rice bran and wheat bran) and Spirulina meal as an additive. Vitamin-Mineral mixture and sodium alginate (as binder) were also added in every diet @ 2% each. Quality of different ingredients for formulation of diets was determined by Pearson-square formula. Feeding was carried out twice a day (morning and evening) @ 5% (2.5% at a time) of their body weight. Proximate composition of diets was determined according to the procedures of AOAC (1995). Fish growth in terms of length and weight gain were recorded fortnightly and survival, specific growth rate and food conversion ratio were calculated at the end of the study using the formula described by Ezhil et al. 2008. All analysis was done in triplicate and data were subjected to statistical analyses using the statistical package- SPSS, version 12.01 for Windows ($p<0.05$).

RESULTS AND DISCUSSION

The proximate composition of ingredients is given in the table-1. Crude protein was estimated as 70% while crude lipid was estimated as 4.32% on dry weight basis for *Spirulina*. It is clear that *Spirulina* is a rich source of protein (Belay et al. 1996). In the present study, different percentages of *Spirulina* were used keeping protein value constant (35 %) for all the diets (table 2). Since *Spirulina* is a costly ingredient, the cost of diet also increases with increase in *Spirulina* content (Figure 1). But, due to lower value of food conversion ratio (FCR), the cost of fish produced from D-1 and D-5 diet was almost same (Figure 2.). The water parameters such as temperature, oxygen and pH were registered as 10-15 °C, 6-8.5mg L- 1 and 7.3-8.2 respectively during the complete study period.

The proximate composition of 0 (control), 3, 5, 7 and 10% *Spirulina* fortified diets did not vary significantly ($P<0.05$) in terms of moisture, crude protein, crude lipid and ash as shown in the Table-2. Survival was 100% in all the tanks due to proper management and quality diets provided (table 4). Mean value of initial and final length and weight for the fishes fed with all five diets are given in the table-3 and it is clear that final weight differ significantly in case of D-2 and D-5. It was highest (10.05g) for D-5 and lowest (8.05g) for D-1. Weight gain percentage (Table-4) was observed highest for D-5 and D-3 (313.240% and 302.407%, respectively). It was lowest for D-1 (182.456%). The specific growth rate (SGR) is given in the table-4 and was observed highest (8.46%) for D-5, slightly low (7.81%) for D-3 and lowest (5.77%) for D-1. In the present study, only 35% protein with almost 7.5% lipid showed better SGR

Table 3. Growth of fish fed on different diets

	Initial Length and Weight		Final Length and Weight	
	Length (cm)	Weight (g)	Length (cm)	Weight (g)
D-1	6.89 ±1.45	2.85 ± 0.08	9.65 ± 1.33	8.05 ± 0.06
D-2	6.48 ± 1.05	2.61 ± 0.15	10.55 ± 1.28	9.75[a] ± 0.04
D-3	6.15 ± 0.96	2.32 ± 0.09	10.60 ± 1.19	9.36 ± 0.34
D-4	6.56 ± 1.22	2.52 ± 0.10	10.70 ± 1.43	9.50 ± 0.08
D-5	6.25 ± 1.14	2.43 ± 0.07	11.08 ± 1.55	10.05[a] ± 0.25

Values are mean ± SD of three replicates (n=10 fishes).

and FCR in Chocolate mahseer when *Spirulina* was a source of protein in contrast to the result shown by Kuruger et al. (2001) who found 45% protein and 6% lipid level is needed for the best specific growth rate and feed conversion ratio of fish.

FCR was observed highest 2.46 for D-1, 1.64 for D-2 and 1.62 for D-4 while, D-5 and D-3 showed the lowest value (1.43 and 1.48, respectively). It is proved that lower the FCR higher is the quality of the feed as observed in many fresh water fishes and FCR is known to decrease with increasing dietary protein content (Jauncey, 1982; Jha et al. 2009) and the effects also vary with species (Dabrowski, 1997). However, in the present investigation, it was clear that even with constant protein level, the FCR decreased from 2.46 (D-1) to 1.64 (D-2), 1.48 (D-3), 1.62 (D-4) and 1.43 (D-5). A decreasing trend of FCR was observed with increase in *Spirulina* content in the feed. Lower FCR resulted in less feed intake, higher weight gain and also lower cost for per kg of fish produced (Figure 2.).

The cost of prepared diets (Figure 1) varied from Rs. 19.21/kg for D-1 to Rs. 46.91/kg for D-5 due to the high price of *Spirulina*. But, the cost of fish produced did not vary significantly for D-1, D-2 and D-3 (47.03, 45.01 and 48.85 Rs. / kg fish, respectively) diets, whereas, cost of feed does (Figure 2.).

Fig 1. Ingredients used (%) and cost of feed (in Rs.)

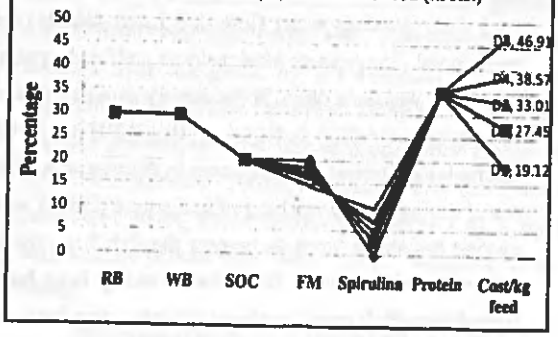
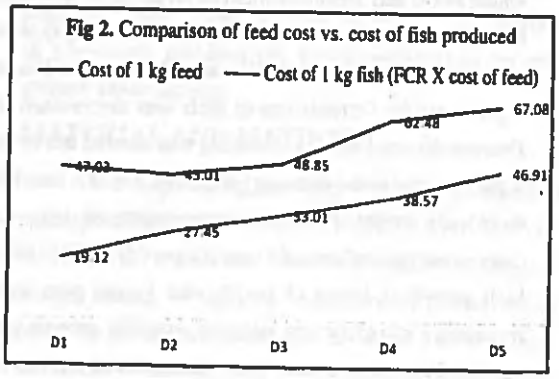


Fig 2. Comparison of feed cost vs. cost of fish produced



Symbol used in the figure 1. is RB for rice bran, WB for wheat bran, SOC for soybean oil cake and FM for fish meal. Cost of produced feed is calculated taking RB and WB @10 Rs/kg, SOC @25 Rs / kg, FM @40 Rs / kg and SM @300 Rs/kg (Procured from Aquaculture Department, Barkatullah University, Bhopal (M.P.).

Table 4. Growth parameters of Chocolate Mahseer fed on different diets

Diet	Survival (%)	Weight gain (g)	*SGR (%)	#Weight gain (%)	**FCR
D-1	100	5.20 ± 0.4	5.77 ± 0.8	182.45 ± 4.535	2.46 ± 0.12
D-2	100	7.13 ± 0.9	7.93[a] ± 0.9	273.27[c] ± 2.708	1.64[a] ± 0.08
D-3	100	7.03 ± 0.5	7.81[a] ± 0.6	302.40[b] ± 2.245	1.48[a] ± 0.06
D-4	100	6.97 ± 0.7	7.75[a] ± 0.6	276.23 ± 2.924	1.62[a] ± 0.09
D-5	100	7.61 ± 0.5	8.46[a] ± 0.5	313.24[a] ± 2.547	1.43[a] ± 0.16

Values are mean ± SD of three replicates (n=10 fishes).

Values in the same column sharing a common superscript are not significant (P<0.05).

*SGR (%) = [(ln final weight - ln initial weight)/ Rearing period (days)] x 100

#Weight gain (%) = (Final weight (g) - Initial weight / Initial weight) x 100

**FCR = Feed consumed (dry weight) / Body weight gain (wet weight)

The present study revealed that Spirulina fortified diets hold immense potential in inducing higher growth in Chocolate mahseer. The 5% *Spirulina* fortified diet was found to be cost effective when correlated with FCR. Thereby, the diets fortified with *Spirulina* may be considered as an effective feed for Chocolate mahseer during its nursery rearing period in its new culture environment. In addition, it may be also concluded that the nutrient quality and pigmentation of the fish is likely to be improved, keeping in mind the growth promoting, immunomodulating, antioxidant and pigment enhancing properties of *Spirulina platensis*.

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Occurrence of fish louse (*Argulus* sp.) on Indian snow trout (*Schizothorax richardsonii*) and golden mahseer (*Tor putitora*) in subtropical Himalayan Lake of Bhimtal, Uttarakhand, India

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ABSTRACT

Indian snow trout (*Schizothorax richardsonii*) and golden mahseer (*Tor putitora*) from cage culture unit of Bhimtal Lake were examined for fish louse (*Argulus*) infestation from August 2008 to January 2009. The percentages of *S. richardsonii* infected were 51.21, whereas abundance and mean intensity of infestations were 1.05 and 2.06 respectively. Maximum prevalence (70.1%) was observed in September with least in December. Percentages of host infected in *T. putitora* were 25 with abundance and mean intensity of 0.34 and 1.36 respectively. One to two percentage of fish stock was fungal infected. Measurement of crustacean parasite illustrated carapace length (CL) comprised 75% of average total body length (5.4 mm). Prevalence was positively correlated (*S. richardsonii*: r = 0.88; and *T. putitora*: r = 0.91) with decrease in water temperature. The results of mean intensity of *Argulus* infestation indicated an initial stage of infection in both the species. So the present study draws immediate attentions of fish health management towards enhancement of fish production in coldwater aquatic resources of India.

Key words: Argulus, Coldwater, Fish health

The crustacean ectoparasite fish louse (*Argulus*), is one of the major threats for fish health management and aquatic crop production in fisheries in tropical and temperate regions (Costello 1993). This crustacean parasite punctures the skin of the hosts with modified mouthparts and feeds on blood by releasing anticongulants into fish (Hakalahti *et al.* 2004). Thus in *Argulus* infestation (Aurgulosis), the chance of fish death due to secondary infection increases. Aurgulosis (Crustacea: Branchiura) was observed in many European freshwater lakes and fish farm (Buchmann and Uldal 1995, Grignard *et al.* 1996, and Valtonen *et al.* 1997). Trout and carp stocks were severely damaged by epizootics of this parasite (Menezes *et al.* 1990, Rahman 1996). *Argulus* infestations have been reported round the globe (Shimura 1983, Gusev 1987, Menezes *et al.* 1990, Gault *et al.* 2002, Hakalahti and Valtonen 2003 and Bhuiyan *et al.* 2008).

In India, fish louse mainly infested inland capture and culture fisheries of cyprinids, as temperatures of these water bodies provide optimum environment for life-cycle of *Argulus*. There was report of *Argulus* infestation in carps from West Bengal (Clers *et al.* 1992). Present study is the first report on incidence of *Argulus* in cage reared Indian snow trout (*Schizothorax richardsonii*) and golden mahseer (*Tor putitora*) from Bhimtal Lake of Uttarakhand in India.

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MATERIALS AND METHODS

Description of study site

Bhimtal lake (29°20′5″N 79°33′3″E) is a sub-tropical lake, situated 4494.7 feet (1370 m) above the mean sea level, with an area of 44 ha (Kumar *et al.* 2007). It is situated in Nainital district of Uttarakhand in India. This lake is perennial in nature and a habitat to a large variety of aquatic flora and fauna. Bottom is muddy with high organic load.

Sample collection

Live specimens of *Schizothorax richardsonii* and *Tor putitora* were collected from cage culture unit of Bhimtal lake, from August 2008 to January 2009. Sampling was done once in a month. Collected specimens were examined externally for *Argulus* infestation.

Data collection

Total of 2187 numbers of *S. richardsonii* and 186 numbers of *T. putitora* were examined during the study period. The external surface of host (skin, fins, base of the fins and opercula region) was examined thoroughly for presence of *Argulus*. Parasites were easily seen with naked eyes and were

picked from the infested host with help of a blunt forceps and needle, and fixed in 70% ethanol for identification and measurements. Then they were examined by light microscope at 40X and 100X for identification. *S. richardsonii* stock was divided into 2 groups (4.5–5.5 cm and > 5.5 cm in length) depending on their size to find out the effect of host size on prevalence, abundance and mean intensity of *Argulus* infestation as per Margolis *et al.* (1982) and Abdus *et al.* (2008). Surface water temperature of the lake at cage culture site was measured by mercury thermometer (Accuracy 0.1°C).

Data analysis

The statistical program SPSS 15.0 for Windows was used for Spearman rho correlation analysis.

RESULTS AND DISCUSSION

Prevalence, abundance and mean Intensity of Argulus sp. *infestation on* Schizothorax richardsonii

A thorough observation of collected sample showed that, these ectoparasites were generally attached to the base of the fins, operculum, dorsal and ventral surface of the fish body and caudal peduncle regions. The incidence of parasitic attachment was maximum in the operculum and base of the pectoral fins of the parasitized fishes. Total numbers of *S. richardsonii* examined for parasite infestation were 2187. Out of 2187 hosts, 1120 fishes were infested with *Argulus* sp. The number of *Argulus* sp. recorded from all the infested hosts was 2312. Prevalence (percentages of fish infested), abundance and mean intensity (number of parasite per infested host) were 51.21%, 1.05 and 2.06 respectively. The mean intensity is low, which indicates that fish are of parasite infestation at initial stage. Data on month-wise abundance and mean intensity of *Argulus* infestation are shown in Table 1.

Prevalence, abundance and mean intensity of Argulus *sp. on* Tor putitora

Total number of *T. putitora*, examined during study period was 186. Out of this, 47 number of host were infected with parasite. The month-wise percentages of host infected, abundance and mean intensity are given in Table 2, which showed 25% of stocks were infected with *Argulus* sp., whereas mean intensity revealed initial stage of infection. During sampling period, authors could not sample more numbers of hosts due to sampling difficulty, which explained a relatively small sample size of *T. putitora*.

Monthly patterns of prevalence of Argulus *sp. on* S. richardsonii *and* T. putitora

Maximum prevalences of *Argulus* sp. on *S. richardsonii* (Fig. 1; 70.1%) as well as on *T putitora* (Table 2; 42.2%) were recorded in September 2008. During winter in January, fishes of both the stocks were almost free from *Argulus* (Figs 2 and 3).

Argulus *free hosts*

The hosts (Indian snow trout and Golden Mahseer), which did not show the infestation of *Argulus* sp. at the time of sampling, were observed with presence of small patches of grey-blue colour lesions on the skin, operculum and dorsal surface of their body. It may be due to the irritation and tissue

Table 1. Abundance and mean intensity of *Argulus* infestation on *S. richardsonii* from August to December, 2008

Month of sampling	Total number of host		Total number of parasite recorded	Abundance	Mean intensity
	Examined	Infected			
August	505	343	780	1.54	2.27
September	426	299	627	1.47	2.09
October	411	266	576	1.40	2.16
November	402	179	291	0.72	1.62
December	443	33	38	0.08	1.15
Total	2187	1120	2312		

Table 2. Prevalence, abundance and mean intensity of *Argulus* infestation on *T. putitora* from August to December, 2008

Month of sampling	Total number of host		Total number of parasite recorded	Prevalence (%)	Abundance	Mean intensity
	Examined	Infected				
August	40	13	18	32.5	0.45	1.38
September	45	19	27	42.2	0.60	1.42
October	42	8	11	19.04	0.26	1.37
November	33	5	6	15.15	0.18	1.2
December	26	2	2	7.6	0.07	1
Total	186	47	64			

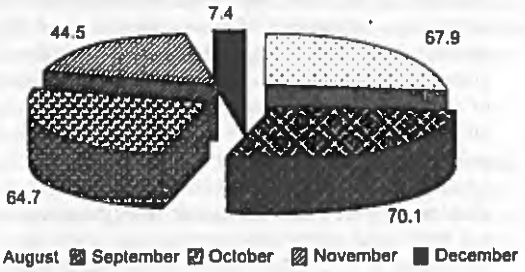

Fig 1. Monthly (August to December 2008) prevalence (%) of *Argulus* infestation on *S. richardsonii* from cage culture unit of Bhimtal lake.

damage by detached parasites, which left the host with small patches of grey-blue and lesions on their body. Alexandre *et al.* (2002) reported that once the mating is over on fish body, the female *Argulus* swims away from hosts and lays eggs on plants, stones, nets and other submerged objects. So, it may form one of the reasons why observed fish were left with small patches of grey-blue and lesions. One to two percentage of total stock was also found infected with cottony wool like appearance on base of the dorsal, pelvic and caudal fins, which most likely a fungal disease. This infestation of *Argulus* sp. may be the possible cause of the secondary infection such as fungal diseases. The attachment of *Argulus* paves the way for entry of opportunistic pathogens (Ravichandran *et al.* 2001 and Alexandre *et al.* 2002). Mortality of the fishes was not observed during sampling. Fish infected with cotton wool like growth, were sampled and brought to laboratory for identification of fungi. With application of hemp-seed method (Pottinger and Day 1999, Sati and Mer 1989) it was found to be *Saprolegnia* sp., which caused this secondary infection (cottony-wool like growth) on fish body. On experimental basis, the entire host (*S. richardsonii*) in one cage was examined thoroughly; *Argulus* sp. were removed from the infected host and *Argulus* free stock was allowed to restock in the same cage. After 10 days the cage was lifted and sampled again and it was found same stock heavily infested with *Argulus* sp. again. It is reported that an adult *Argulus* can survive without a host for up to 15 days, while a newly hatched larva for 1 or 2 days. The life cycle of this ectoparasite depicts a newly hatched larva actively seeks host and continues its development on fish (Alexandre *et al.* 2002). The netted cage wall forms a suitable submerged object for egg laying, hatching and completion of life-cycle of *Argulus* sp. As fishes were restocked in the same cage, as said above, the adult *Argulus* sp. and newly hatched one might have re-infested the stock.

Description of isolated Argulus sp.

In present study the collected *Argulus* sp. was identified based on their external characteristics. The measurement was based on 20 specimens. Morphometry of crustacean parasite illustrated 5.4 mm average total length (TL), 4.1 mm carapace length (CL), which comprised 75% of total body length, 4.0 mm carapace width (CW) and 2.1 mm abdominal length (ABL). Yildiz and Kumantas (2002) reported that total body length of *Argulus foliaceus* ranged from 6 mm to 7 mm, whereas *Argulus japanicus* and *Argulus coregoni* are from 4 mm to 8 mm and 12 mm respectively. Tam *et al.* (2005) reported 5.5 mm average body length, 3.03 mm average length of carapace (53% of overall body length), 2.9 mm of average width of carapace and 2.1 mm of average length of abdomen in male *Argulus personatus* (measurement based on 8 male specimens of *Argulus personatus*). In female *Argulus personatus*, the total body length, carapace length, carapace with and abdomen were 5.6 mm, 3.7 mm, 3.4 mm and 1.5 mm respectively (measurement based on 1 female

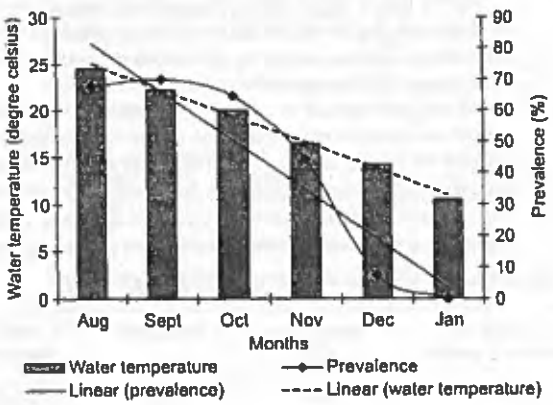

Fig 2. Decreasing trend of prevalence with decrease in water temperature, Stock: *S. richardsonii* (August 2008 to January 2009).

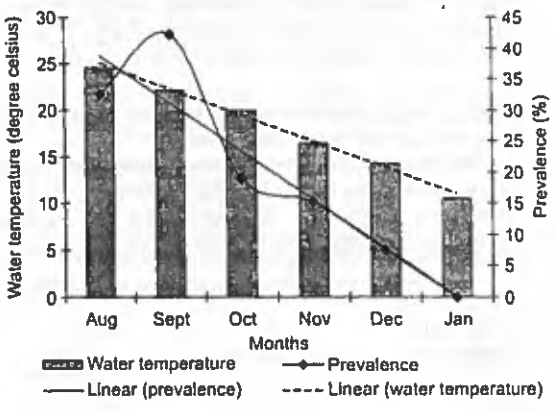

Fig 3. Decreasing trend of prevalence with decrease in water temperature, Stock: *T. putitora* (August, 2008 to January, 2009).

specimen of *Argulus personatus*). Wadeh *et al.* (2007) observed total body length ranged from 4.9 mm to 8.2 mm with an average of 6.5 mm and abdomen length 1.2 mm to 1.7 mm with an average of 1.4 mm in adult female *Argulus japanicus* Thiele 1900 (Crustacea: Branchiura) collected from Shunde of Guangdong, China. They have also reported average total body length 3.6 mm of male *Argulus japanicus* Thiele, with carapace length comprising 75% of total body length.

Effect of host size on prevalence, abundance and mean intensity of Argulus *infestation on Indian snowtrout*

In present study, prevalence, abundance and mean intensity of *Argulus* infestation did not vary significantly between fish size groups in length (Stock: *S richardsonii*, Table: 3). The apparent result indicated infected hosts in size class 45 to 55 mm acquired more *Argulus* (mean intensity 2.14) as compared to size class > 55 mm (mean intensity 1.87). Poulin (1999) demonstrated prevalence and mean intensity correlate positively with host body size for copepod ectoparasite. Grutter (1994) also found a positive correlation between host fish length and gnathiid parasite loads. According to island biogeography theory (MacArthur and Wilson 1967), larger hosts are likely to harbour more parasite species and higher number of individual parasites than smaller host. The fact that parasites attached to body surface of larger host may mean that they provide greater surface area and are attacked more often than smaller host (Cochran 1985). Clers *et al.* (1992) also recorded the similar observation of increase abundance of *Argulus* infestation with increase in host size, but the observation recorded in the study for effect of host size group on prevalence, abundance and mean intensity did not concur with the reports stated above. As fishes were stocked in cage net and reared in a confined environment, which hardly allow them flee to open water, the parasites might have easily accessed the stock for infestation irrespective of size of the hosts. The effect of host size on prevalence, abundance and mean intensity of *Argulus* infestation was not studied in Golden mahseer, *T. putitora* because of a relatively small sample size of the fish.

Effect of water temperature on percentage of host infected (stock: S. richardsonii *and* T. putitora)

The effects of water temperature on prevalence showed positive correlation (Fig.2; *S. richardsonii:* r = 0.88; Fig. 3, *T. putitora*: r = 0.91) in the observation. The maximum prevalence for both fishes (*S. richardsonii* 70.1% and *T. putitora* 42.2%) was monitored in September. Then a decrease in percentage of host infection from October onwards was recorded with fall of water temperature in the present study. As optimum water temperature for completion of *Argulus* life-cycle ranges between 18 and 22°C, lessen in prevalence in November and December may be the due to unfavorable water temperature during the said months. The whole life cycle may take 30–100 days depending on the water temperature of the rearing unit. So eggs can over-winter and hatch later when water temperature starts increasing (Alexandre *et al.* 2002). Clers *et al.* (1992) also observed that high water temperature promotes the increase in *Argulus* population.

There is marked deterioration of water quality of Bhimtal lake with recession of its margin and shallowing in the past few years by anthropogenic activities. There is also a marked decrease in depth of the lake during summer with increase in water temperature, appearance of aquatic weed infestation and muddy fauna. All these may be the basis for infestation of *Argulus* sp. on coldwater fish in cage culture system. The present infestation of *Argulus* sp. may be at initial stage, but it can be categorized in high-risk group, where organism is termed as typically pathogenic and requires immediate treatment and mechanical removal. Because this crustaceans infestation not only retards the growth of the fish, but also acts as a vector for entry of certain fish viruses (Clers *et al.* 1992) and secondary infections such as bacterial and fungal diseases. Lester and Roubal (1995) also reported that growing *Argulus* sp. juveniles may expose fish to fungal and bacterial infections by irritating the skin that can affect the fish's feeding habit, which results in retarding growth and sometimes death of the host. Thus a periodical monitoring program on fish health is considered necessary to keep the stock in healthy state. The present study sensitized us to examine the fish culture tank at higher altitude raceways culture system. The Indian snowtrout and Rainbow trout stock at coldwater fish farm, Champawat, which is situated at 5688.97 feet (1734 m) above the sea level, were examined for Argulosis. But the stocks did not show any sign of *Argulus* infestation. During sampling period, the average water temperature of Champawat fish farm was 11.5 °C.

Virtually no extensive survey of parasites in coldwater aquatic resources of mid-Himalayan region of India has been done so far. As there is no information on distribution and abundance of *Argulus* infestation in coldwater bodies of India, this occurrence of *Argulus* sp. on coldwater fishes, Indian snowtrout and critically endangered Golden mahseer in cage

Table 3. Prevalence, abundance and mean intensity of *Argulus* infection on *S. richardsonii* in different length group

Size group (length in mm)	No. of host examined	No. of host infested	Number of parasite recorded	Prevalence (%)	Abundance	Mean intensity
45–55	459	227	486	49.45	1.05	2.14
>55	312	151	283	48.39	0.90	1. 87

culture unit of Bhimtal Lake draws immediate attentions of fish health management and extensive survey of parasites towards enhancement of fish production in coldwater aquatic resources of hilly states of India.

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DEVELOPMENT OF EIGHT EST-DERIVED MICROSATELLITE MARKERS IN INDIAN SNOW TROUT (*SCHIZOTHORAX RICHARDSONII* GRAY 1832)

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Indian snow trout, *Schizothorax richardsonii* is distributed in fast flowing torrential snow-fed streams, rivers and lakes in various altitudes of the Himalayas. Developed fifteen microsatellite markers from approximately 32923-expressed sequence tags (ESTs) of common carp, *Cyprinus carpio* and evaluated eight of them in 42 unrelated specimens of *S. richardsonii*. The number of alleles per locus ranged from 3 to 6. The observed heterozygosity (H_o) ranged from 0.099-0.937 and the expected heterozygosity (H_e) ranged from 0.675-0.755. Because of high level of polymorphism the microsatellite markers reported here would be useful to study population genetics and linkage mapping of Indian snow trout.

Keywords: Indian snow trout, *Schizothorax richardsonii*, expressed sequence tag (EST), microsatellite markers

Introduction

Schizothorax richardsonii, also known as Indian snow trout, inhabits fast flowing torrential snow-fed streams and lakes in the higher altitudes of the Himalayas and Central Asia (Talwar and Jhingran, 1991). In recent years, a significant decline of snow trout in the wild was observed due to over-exploitation of natural stock and deterioration of environmental conditions (Sehgal, 1999) and the species has been categorized as "threatened" (CAMP, 1998). A comprehensive literature survey revealed that there were few partial *cytochrome b* gene sequences (AF532075-AF532088) and two microsatellite sequences (FN568062 and FN568062) available for *S. richardsonii* in GenBank. Thus the snow trout has a very narrow genetic database and more number of markers needs to be developed. Development of species-specific primers for PCR amplification of microsatellite loci by screening of size fractionated genomic library is expensive and time consuming. In recent years the development of microsatellite markers from expressed sequence tags (ESTs) has become useful tools in several fish species (Westgaard *et al.*, 2007; Kim *et al.*, 2008; Delghandi *et al.*, 2008). It can be rapidly and inexpensively developed from freely available EST database. EST-derived microsatellite markers have the potential to be functional markers and may be used in gene associated polymorphism studies (Vasemagi *et al.*, 2005). In this paper we report the development of 8 EST-derived microsatellite loci from *Cyprinus carpio* cDNA library. These eight microsatellite markers would be useful to study population genetics and linkage mapping of Indian snow trout.

Materials and methods

Forty two live samples of snow trout were collected from wild populations in the Goula, Uttarvahini, Chirapani, Kosi and Alaknanda rivers in Kumaon and Garhwal region of

Uttarakhand during February to August 2009. Individual fin-clips were stored immediately into 95% ethanol. For each specimen, DNA was extracted from 50 mg of tissue using the standard phenol-chloroform extraction protocol (Sambrook and Russell, 2006) and re-suspended in TE (10Mm Tris-HCL, pH.8.0, 1mM EDTA). DNA concentrations were determined using UV-Vis Spectrophotometer (Thermo Scientific, England) and accordingly diluted to 50ng/µl.

FASTA sequences of ESTs of common carp (*C. carpio*) were downloaded from GenBank dbEST (http:// www.ncbi.nlm.nih.gov/dbEST/ index.html). Redundant clones were removed using a local nucleotide BLAST search with Bioedit sequence alignment editor software version 7.0.1 (Hall, 1999). Rest of the sequences was analyzed for the presence of repeat motifs using Tandem Repeats Finder software (Benson, 1999) with the parameters: match 2; mismatch 7; indel 7 and max period size 500. Initially a set of 80 primer pairs were designed from EST sequences containing di-and trinucleotide repeats using Primer Select (DNASTAR) and 15 primers were screened for PCR amplification.

PCR amplifications of microsatellites were carried out on a 9700 Thermal Cycler (ABI, USA) using the following program: 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, locus specific annealing temperatures (Table 1) for 60 s and 72°C for 60 s and a final extension of 72°C for 10 min. The amplification reaction was performed in 10 µl reaction mixture containing 50ng template DNA, 10X PCR-buffer (100 mM Tris, pH 9.0, 500mM KCl, 15mM $MgCl_2$, 0.1% Gelatin), 200 µM of each dNTPs (Genei, India), 5pmol of each primer (Ocimum Biosolutions, India) and 0.5U *Taq* DNA Polymerase (Genei, India). One negative control (absence of DNA template) was included for each set of amplifications. PCR product was separated in 6% non-denaturing polyacrylamide gel and visualized by silver staining. Allele sizes were determined by comparison with a molecular marker ÖX 174 DNA/ Hinf I (Fermentas, USA) on UV-Gel Documentation Unit (Alpha Imager 3400, Alpha Innotech Corporation, USA). The number of alleles per locus (A), observed heterozygosity (*Ho*), expected heterozygosity (*He*) and exact test for the conformance to Hardy-Weinberg Expectations (HWE) were calculated using GDA (Lewis and Zaikin, 2001). Linkage disequilibrium was calculated using Fisher's exact test by permuting all two-locus genotypes within all populations.

Results and discussion

In the process of EST database mining, a total of 32,923 EST sequences were downloaded from NCBI GenBank. From the above databases 548 (1.66%) sequences contained 333 (60.76%) di-nucleotide; 75 (13.68%) tri-nucleotide; 85 (15.51%) tetra-nucleotide; 25 (4.5%) penta-nucleotide and 30 (5.21%) hexa-nucleotide repeats. The most frequent di-nucleotide repeat motifs were CA/GT (75.37%) and GA/CT (23.12%). Out of 80 primers set, 15 primers were selected for amplification on the basis of high frequency of repeat motifs (more than 15 perfectly continuous repeats for di nucleotides and 6 to 14 continuous repeats for tri-nucleotides) and out of 15 primer pairs, only 8 primers were successfully cross amplified with expected size and high polymorphism (Table 1). The other primers were excluded from analysis because they showed less than 2 alleles per locus and also showed some non-specific amplification. The number of alleles in the remaining loci varied from 3 to 6 with an average of 3.96 alleles per locus. The observed heterozygosity (H_o) ranged

Table 1. Characterization of 8 EST-SSRs in Indian snow trout, *Schizothorax richardsonii*

Locus	Acc. No	Repeats	Primer sequence (5'-3')	T_a	S	A	H_o	H_e	*P*
CWF114	EX884609	$(CA)_{16}$	F-AAGGCAGTTTTCGGTTTC R-TATGGCAGTGCACTATTGTTTA	50	187	4.4	0.280	0.755	**0.00**
CWF117	EX883239	$(CA)_{19}$	F-TCTGGGGCTCTACGGGCTTATTGT R-TTCCTCTCATCGTTTCGGGTGGT	62	197	4.0	0.875	0.731	0.15
CWF126	EX885497	$(AGG)_{14}$	F-CTACGCAAAGTGGCTAAGG R-CACCCGTGACACAGAAGAC	56	170	4.4	0.099	0.705	**0.00**
CWF127	EX885584	$(GAG)_{10}$	F-TTCCTGGATGGCAAAGA R-CAGAAACAAATATCAAGCAGTAGA	59	490	3.8	0.326	0.711	**0.00**
CWF135	EX880053	$(GAT)_9$	F-CAAAGCCTTCAGTCCCATCAGC R-TTAGCGAAACAGCAGCCGTCAT	64	164	3.6	0.200	0.675	**0.00**
CWF142	EX825418	$(CCT)_6$	F-GCCCGGCCCGTGTCTCT R-GTGCGGTGCTTCAGTGCTTTGTC	65	116	4.0	0.477	0.717	0.05
CWF149	EX823621	$(GAT)_8$	F-GCGGAGGAGCTGGAGGACT R-TTAATCATCATCATCATCATCACC	63	150	3.4	0.480	0.732	**0.00**
CWF151	EX821974	$(ATT)_8$	F-GGCCAACCCATTACCTCTCAC R-TACCCCAATAACCGACCCTCTAC	59	216	4.2	0.937	0.697	0.07

P-values are bold at the Hardy-Weinberg equilibrium (HWE) departure locus; T_a: annealing temperature (°C); S: allele size range (bp); A: number of alleles; *Ho*: observed heterozygosity; *He*: expected heterozygosity; *P*-value, exact *P*-value ($p < 0.05$) for the HWE test

Table 2. Annotation of Gene-associated microsatellite based on BLAST searches against the NCBI GenBank non-redundant database

Locus	Gene Identity	E-value	Species	Accession no of closest homology
CWF114	AHNAK nucleoprotein mRNA	9e-154	*D. rerio*	XM682604
CWF117	Vacuolar ATP synthase 16Kda proteolipid subunit and AtP6vOc	4e-117	*D. rerio*	NM173255
CWF126	Unknown	-	-	-
CWF127	Hypothetical protein LOC793800	1e-30	*D. rerio*	XP001333116
CWF135	Peptidase D	7e-16	*D. rerio*	XP702030
CWF142	Rassf1 PROTIEN (Tumour suppressor protein with Ac-terminal Ras-associated domain)	1e-55	*D. rerio*	AAH932667
CWF149	Calsequestrin 2	1e-63	*D. rerio*	NP001002686
CWF151	Glycoprotein M6Aa	2e-34	*D. rerio*	AAI50456

from 0.099-0.937 and expected heterozygosity (H_e) ranged from 0.675-0.755. No significant linkage disequilibrium was found between any pair of the loci. However, five loci (CWF114, CWF126, CWF127, CWF135 and CWF149) significantly deviated from Hardy-Weinberg equilibrium. The departure from HWE may be due to small sample size and presence of null alleles. BLAST analysis showed highly significant similarities for seven, out of eight, loci (Table 2). These represent well-characterized genes thus defining the associated microsatellites loci as type-I markers, all located to the coding region of the genes. In conclusion, the microsatellite markers reported herein provide a valuable molecular tool for detailed genetic analysis of stock structure in wild populations of Indian Snow Trout. Such studies may contribute significantly to the

development of a sound management plan for this species.

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Research Article

Genetic diversity of two riverine populations of *Eutropiichthys vacha* (Hamilton, 1822) using RAPD markers and implications for its conservation

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Abstract

Eutropiichthys vacha were collected from two rivers namely Ganga (Patna, India) and Kosi (Madhepura, India) for population genetic and phylogenetic studies. Five OPA primers were used to generate the fragment patterns from the samples collected. Polymorphisms within and between populations were assayed using 5 random primers, and 45 loci were amplified ranging from 250 to 2,000 bp. The percentage of polymorphic loci was found 51.1% and 55.6% for Ganga and Kosi populations, respectively. Total genetic diversity was 0.2173, and the average coefficient of genetic differentiation was 0.0958. The highest level of genetic diversities within population as well as lower between populations suggested that lower differentiation rate between populations. Gene flow between Ganga and Kosi populations was 4.7. Nei's unbiased measure of genetic identity and genetic distances of two populations were found 0.9606 and 0.0402, respectively. Phylogenetic analysis by RAPD showed one common cluster between two wild populations (Patna and Kosi) though they are quite distant from each other but belongs to same drainage system.

Keywords: *Eutropiichthys vacha*, population structure, RAPD, conservation, phylogenetics

***Eutropiichthys vacha*'nın (Hamilton, 1822) iki riparyen populasyonunda RAPD markörleriyle genetik çeşitliliğin gösterilmesi ve korunması için çıkarımlar**

Özet

Eutropiichthys vacha, populasyon genetiği ve filogenetik çalışmalar için Ganga (Patna, India) ve Kosi (Madhepura, India) isimli iki nehirden toplandı. Toplanan örneklerden parça paterni oluşturmak için beş OPA primeri kullanıldı. Populasyonlar içinde ve arasındaki polimorfizmler rastgele 5 primer kullanılarak test edildi ve 250-2000 bp arasında 45 lokus çoğaltıldı. Polimorfik lokusların oranı Ganga ve Kosi için sırası ile %51.1 ve %55.6 bulundu. Toplam genetik çeşitlilik 0.2173 ve ortalama genetik farklılaşma katsayısı 0.0958'di. Populasyon içi en yüksek, populasyonlar arası daha düşük genetik çeşitlilik derecesi populasyonlar arasında daha az farklılaşma hızı fikrini verdi. Ganga ve Kosi popülasyonları arasında gen akımı 4.7'ydi. İki popülasyonun genetik benzerliği ve genetik uzaklığı Nei'nin tarafsız ölçümü ile sırası ile 0.9606 ve 0.0402 bulundu. RAPD ile filogenetik analiz birbirlerinden oldukça uzak ama aynı su havzasına ait olmalarına rağmen iki yabanıl popülasyon (Patna ve Kosi) arasında ortak bir grup gösterdi.

Anahtar sözcükler: *Eutropiichthys vacha*, populasyon yapısı, RAPD, korunum, filogenetik

Introduction

Eutropiichthys vacha is an economically important fish of the family Schilbidae and has gained popularity among consumers due to its high nutritional value (Hasan et al., 2002) and good taste. The genus *Eutropiichthys* is distributed throughout India, Pakistan, southern Nepal, Bhutan, Bangladesh, Myanmar and Thailand (Talwar and Jhingran, 1991). The culture practices of this species are not available so far and the entire demand for this fish in the domestic market is met through capture from rivers; thus, the effective management of wild stocks is critical. Mijkherjee et al. (2002) found *Eutropiichthys vacha* as vulnerable species and predicted that, the species will disappear from their natural habitat in West Bengal. Basic knowledge of the biology, including information on population structure of the species is important for sound management of fisheries resources. This type of information is useful for the development of management strategies that will conserve the biodiversity associated with different species, sub-species, stocks and races (Turan et al., 2005). Thus, detailed knowledge on the population structure of *E. vacha* is needed for sound management, successful commercial fishing and conservation of this species.

Various molecular markers such as RFLPs, AFLPs, VNTRs, SSRs and RAPDs have been used in fish population genetics. RAPD analysis has been described as a simple and easy method to detect polymorphisms based on the amplification of DNA segments with single primers of arbitrary nucleotide sequence (Williams et al., 1990; Welsh and McClelland, 1990). RAPDs have gained considerable attention particularly in population genetics (Lu and Rank, 1996) for species and subspecies identification (Bardakci and Skibinski, 1994), for gynogenetic fish identification (Chen and Leibenguth, 1995; Corley-Smith et al., 1996) and for gene mapping studies in fish (Postlethwait et al., 1994; Kazianis et al., 1996).

The aim of this study was to obtain a general view of the genetic profile of *Eutropiichthys vacha* in two rivers. The levels of variability of two different riverine populations of *Eutropiichthys vacha* were evaluated; in fact, the present investigation is the first report on the structure of the genetic diversity of the *Eutropiichthys vacha*.

Materials and methods

Animals

Eutropiichthys vacha were collected from two different rivers namely Ganga (Patna, 25°61' N, 85°14'E) and Kosi (Madhepura, 26°07' N, 86°19'E), respectively (Figure 1). The fish were caught with the help of cast nets. Caudal fin of each fish was cut with the help of scissor and washed thoroughly with clean water to remove mucus and other dirt. Clean fins were placed in 2ml vials containing 80% ethanol. These vials were brought to DCFR, Bhimtal and placed in -20 °C refrigerator.

DNA Isolation

Genomic DNA was isolated by the phenol–chloroform procedure (Sambrook et al., 1989). Analysis on agarose gels and spectrophotometric methods were used to determine DNA quality and quantity.

RAPD Primers

The sequences of the primers were taken from the literature (Ambak et al., 2006) and oligonucleotides were got custom synthesized by Genei Pvt. Ltd. Bangalore, India (Table 1). Altogether 20 (10-mer) random primers of OPA series from Operon technology were screened. Of these, 10 primers yielded amplification products and among them 5 primers that produced the strongest amplification with reproducible polymorphic bands were selected for this study.

RAPD-PCR Assay

RAPD-PCR amplifications were performed in a total volume of 25 µL containing: 1X Taq polymerase buffer (Bangalore Genei, India), 2 mM $MgCl_2$ (Bangalore Genei, India), 100 µM each dNTP (Bangalore Genei, India), 5 pmol primer (Bangalore Genei, India), 0.75 U Taq polymerase (Bangalore Genei, India) and 25 ng template DNA.

In order to detect any DNA contamination, negative control reactions was setup without genomic DNA. Amplifications was performed using a Eppendrof Thermocycler and that was programmed for 35 cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 2 min. An initial denaturation step of 4 min at 94°C and a final extension step of 7 min at 72 °C were included in the first and last cycles, respectively. The amplification products were size-fractionated in a 1.4% agarose gel containing ethidium bromide in TAE buffer.

The molecular sizes of the RAPD product were estimated by their comparison with standard molecular size marker (Lambda DNA-Hind III/Eco RI digest and/ or 100 bp DNA ladder, Bangalore Genei, India), which was run parallel to the amplified products in the gel or by the help of a computer program.

Statistical Analysis

The RAPD bands were scored as present (1) or absent (0) in each pattern. All calculations were carried out using the population genetic analysis software, POPGENE 1.31 (Yeh et al., 1999). The UPGMA dendrogram of population was constructed based on Nei's (1972) and genetic distances using TFPGA (Tools for Population Genetics Analysis) software (Miller, 1997). Genetic differentiation (G_{st}) was calculated by using formula: Genetic dif (G_{st}) = 1- H_s/H_t, Where, H_s is sample gene diversity and H_t is total gene diversity. Gene flow was indirectly estimated among the populations by using the formula: $Nm = 0.5(1 - G_{st})/ G_{st}$ (McDermott and McDonald, 1993). Shannon's diversity index was calculated to provide a relative estimate of the degree of genetic variation within each population using POPGENE 1.31(Yeh, 1999).

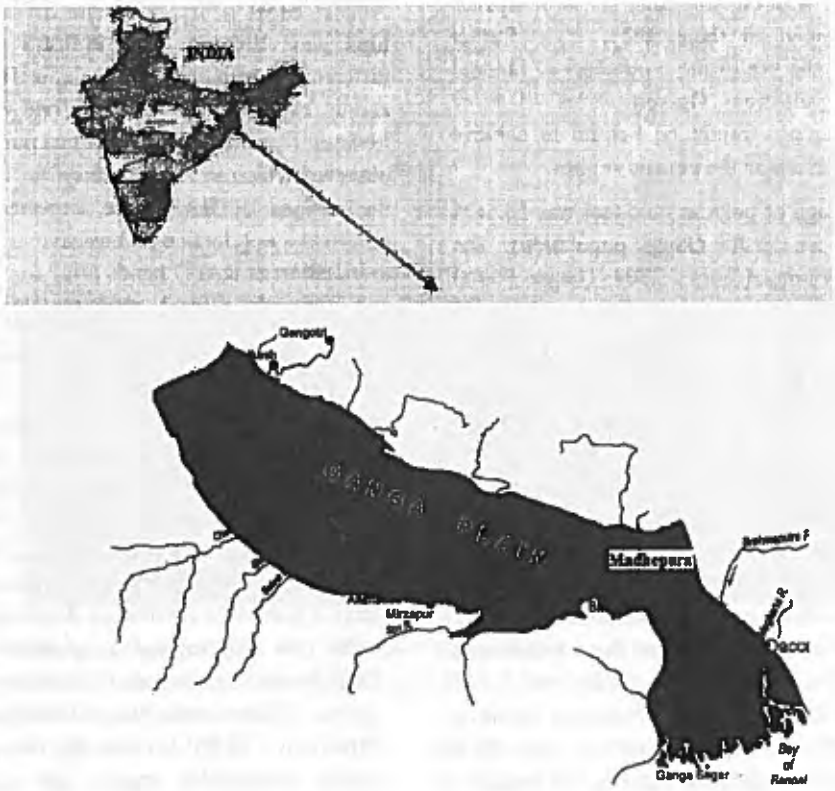

Figure 1. Map showing the two sampling sites in the Ganga river system in India.

Table 1. Primers and primer sequences used for the detection of polymorphism in *Eutropiichthys vacha*.

Sl.No.	Primer	Sequence (5 to 3)	GC content (%)
1	OPA-02	TGCCGAGCTG	70
2	OPA-04	AATCGGGCTG	60
3	OPA-05	AGGGGTCTTG	60
4	OPA-08	GTGACGTAGG	60
5	OPA-09	GGGTAACGCC	70

Results and Discussion

In the present study five primers generated a total number of 45 fragments, with the approximate size ranging from 250 to 2000 bp (Figure 2-5). This wide range of band sizes is comparable to the results of studies carried out by other authors. For example, Yoon and Kim (2001) employed only 5 random primers and observed 1344 numbers of amplicons. However, Liu et al. (1999) observed 462 amplified fragments (200-1500bp) by using 75 primers. The presence of more numbers of fragments might be due to the presence of more priming site at the template DNA with the particular series of Operon primers employed in their study. It is further suggested that the use of more numbers of random primers from different Operon series in more numbers of samples might be helpful to achieve more reliable results in the genetic studies.

The percentage of polymorphic loci was 55.56% for Kosi and 51.1% for Ganga populations. The Shannon index ranged from 0.2804 (Ganga, Patna) to 0.3001 (Kosi, Madhepura). Polymorphic loci indicate that the genetic variation among Madhepura (Kosi) populations was higher than the Patna (Ganga) population. In the present study, higher genetic diversity was found within the Kosi population (0.203), and lower genetic diversity was found for the Ganga (0.189). This means that Kosi population has a higher proportion of heterozygous genotypes than the Ganga population, which was in accordance with the result of Shannon's Information index. Das et al. (2005) observed the varied range of 42.6%, 31.7%, 30%, 19.2%, 16.8% and 14.3% polymorphic loci in different carp species. However, Li and Chu-Wu, (2006) calculated very high (86.00 – 92.11%) polymorphic loci ratio in five species of snappers using the RAPD technique. Hence, in the present study polymorphisms of alleles found to be less in comparison to other reports.

The total gene diversity (H_t) in the population was 0.2173, and the genetic diversity within population (H_s) was 0.1965. The genetic differentiation (G_{st}) of all populations were 0.0958, which can be interpreted to mean that 90.5% of total genetic variation was within populations and 9.5% was among populations. The gene flow (Nm) between populations was 4.72. *Eutropiichthys vacha* is a potamodromous fish i.e. migrate from one river to another for spawning and nursery ground, this may explain the high levels of detected gene flow. Almost equal population gene diversity (H_s) and total gene diversity (H_t) indicate small genetic differentiation among the populations (G_{st} = 0.0958) and is explained by the high rate of gene flow. Lower differentiation rate between populations were observed which is very common for RAPD data as the regions of RAPD are expected to be less responsive to selection and to have higher tolerance to mutation as RAPD bands arise from both coding and non-coding DNA regions (Williams et al., 1990). This also may be due to small sample sizes. Geographical isolation, limited dispersal and phylopatric behaviour of populations should promote genetic differentiation, particularly in freshwater habitats (Carvalho, 1995). Similar to the present study, Omar et al. (2004) also found very little genetic differentiation (0.086) in L. geminis population and suggested that these organisms may have a pattern of continuous distribution associated with the hydrographic patterns of basins. Furthermore, Sands et al. (2003) also observed low genetic differentiation (G_{st} =0.0165) and high gene flow (Nm = 29.83) between the two populations of squid, Moroteuthis ingens and interpreted that extensive adults migration is enough for the low genetic diversity.

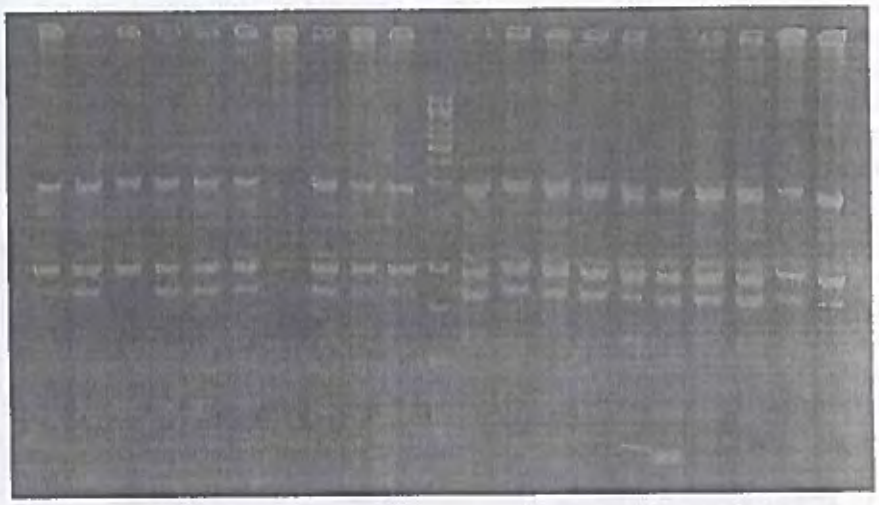

Figure 2. OPA-8 Amplified products on 1.4% agarose gel. Each lane shows different individual amplified DNA Samples from River Ganga (First 10 lane) and Kosi (Last 10 lane). Marker: 1 kb molecular weight marker (Bangalore Genei, India).

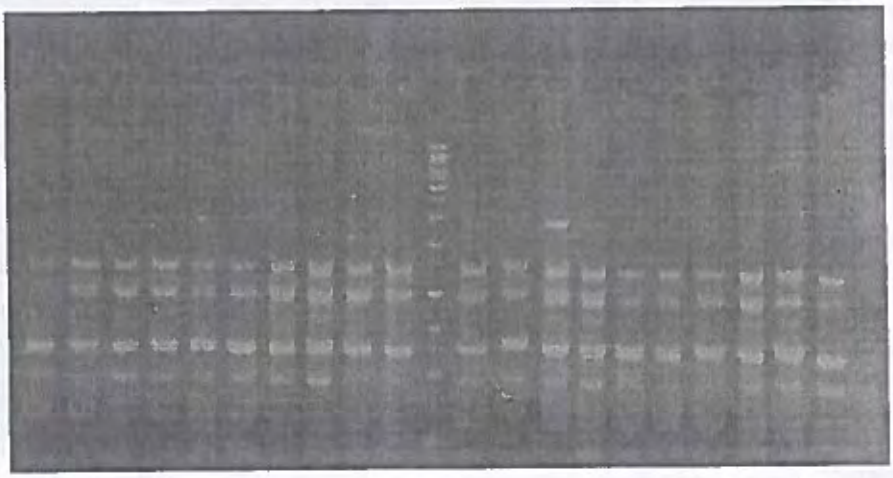

Figure 3. OPA-9 Amplified products on 1.4% agarose gel. Each lane shows different individual amplified DNA Samples from River Ganga (First 10 lane) and Kosi (Last 10 lane). Marker: 1 kb molecular weight marker (Bangalore Genei, India).

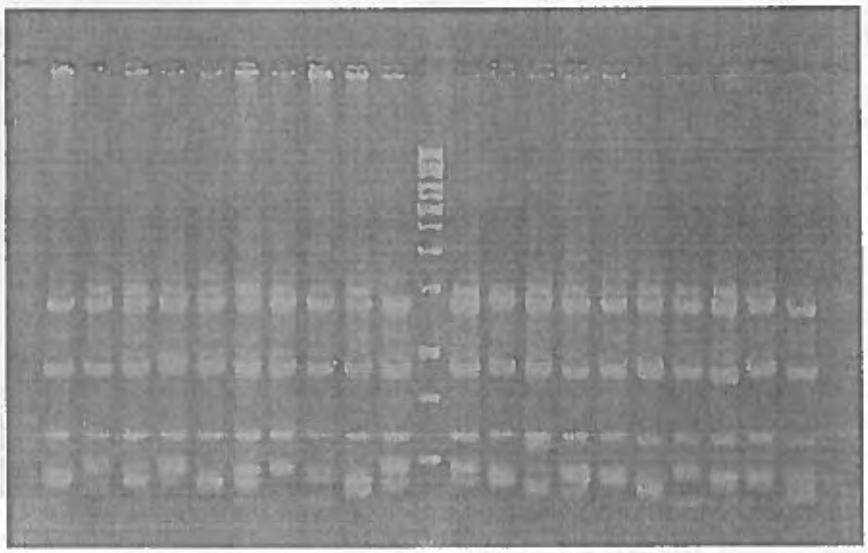

Figure 4. OPA-4 Amplified products on 1.4% agarose gel. Each lane shows different individual amplified DNA Samples from River Ganga (First 10 lane) and Kosi (Last 10 lane). Marker: 1 kb molecular weight marker (Bangalore Genei, India).

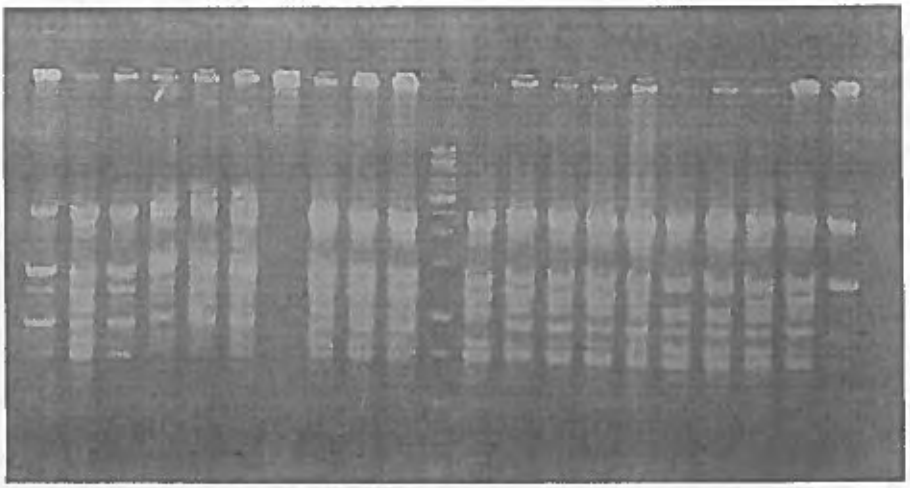

Figure 5. OPA-5 Amplified products on 1.4% agarose gel. Each lane shows different individual amplified DNA Samples from River Ganga (First 10 lane) and Kosi (Last 10 lane). Marker: 1 kb molecular weight marker (Bangalore Genei, India).

Using RAPD data the genetic distance between Patna and Madhepura populations was found to be, 0.0402. Based on these genetic distance measures, the dendrogram was made. The dendrogram showed one cluster, using RAPD markers (Figure 6). The genetic identity between the populations from the amplified patterns of five random primers was 0.9606 (Table 2). Phylogenetic analysis by RAPD showed one common cluster between two wild populations (Patna and Kosi) though they are quite distant geographically from each other but belongs to same drainage system. None of the primers amplified RAPD bands that were entirely absent in one of the populations but present in the others. This suggests that the populations have not been isolated long enough for specific genes to be gained or lost in particular areas during the course of evolution. Further, it is suggested that large scale screening of random primers are necessary to develop a specific markers and should be confirmed either by repeating the PCR reaction or by sequencing.

Table 2. Nei's unbiased measures of genetic identity and genetic distance (1978).

Pop ID	Patna	Madhepura
Patna	****	0.9606
Madhepura	0.0402	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal): (POPGENE ver 1.32).

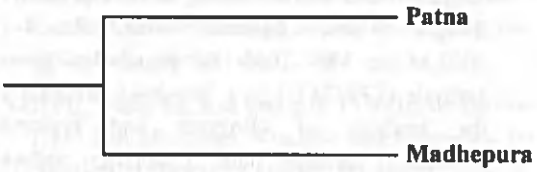

Figure 6. Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA

Implications for conservation

Characterization of genetic diversity is a necessary requirement for the improvement, use and conservation of genetic resources. Maintaining genetic diversity has become a major issue in conservation biology as it is generally thought to be important for the overall species viability and the potential for evolutionary responses to environmental change (Meffe and Carroll, 1997).. Loss of genetic diversity could lead to a decline in a species's ability to cope with changing environment and demographic fluctuations both in the short and long term (Milligan et al., 1994).

The reproductive biology of *E. vacha* is an unexplored area as little is known about the breeding and embryonic development; research is needed to develop and standardize techniques for their induced breeding and artificial propagation. Such technology can then be used to conserve the species through captive breeding programs and also to generate new employment opportunities. Study should be carried out to know the physico-chemical as well as ecological requirements for the successful proliferation of the species. Furthermore, different strategies for fish conservation such as in situ (Protected sites), ex situ (live gene banks, cryopreservation of fish gametes and embryos) can contribute to conservation, optimum utilization and recovery of bioresources. There is an urgent need to aware the fishing communities about the importance of environment and fish resources conservation and integrating stakeholders and particularly local communities in all stages of project planning and implementation (Lakra et al., 2007). This will particularly helpful to eliminate the discriminate fishing methods that are prevalent in riverine capture fisheries of this area and are also the causative factors for declining population.

In a nutshell, the highest level of genetic diversities within population as well as lower between populations suggested that lower differentiation rate between populations might be taking place due to high rate of gene flow. Approaches employing several DNA marker systems may increase the accuracy of genetic studies of these populations. Thus, further genetic analyses using microsatellite and mitochondrial DNA markers will further enhance the genetic relationship among two riverine populations of *Eutropiichthys vacha* in greater detail.

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An Analysis of Genetic Diversity Among Indian Coldwater Fishes (Pisces: Cyprinidae) Using RAPD Markers

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The present investigation aims to study the genetic relatedness among the coldwater fish species and construction of phylogenetic relationships among them. Eleven random primers were employed to screen for RAPD markers in the most commonly available coldwater fish species of Uttarkhand region, viz., *Tor putitora*, *Schizothorax richadrsonii*, *Raiamas bola* and *Garra* species. The sizes of the amplified products were from 200 to 5,000 bp in all the fish species with all the primers employed. Total 188 bands were scored with the 11 primers employed, with the average numbers of bands scored being 3.54 ± 0.72, 5.18 ± 0.69, 3.64 ± 0.58 and 4.73 ± 0.78 in *Tor putitora*, *S. richadrsonii*, *R. bola* and *Garra* species respectively. The maximum numbers of scorable bands were obtained with primer OPA-03 primer in *Tor putitora* (08 ± 0.71), *Schizothorax richadrsonii* (08 ± 0.71), *Raiamas bola* (07 ± 0.71) and *Garra* species (05 ± 0.71), and minimum numbers (1 to 3) of amplified fragments were observed with primer OPA 05. Higher proportion of polymorphic bands were produced by OPY02 (7.5%) and NUSZG4 (5.75%) primers among these fish species. The highest genetic distances were observed between *T. putitora* and *R. bola* (0.60), followed by *T. putitora* with *Garra* species (0.52), and the least genetic distance was observed between the *S. richardsonii* and *Garra* species (0.36), followed by *T. putitora* and *S. richardsonii* (0.43). The phylogenic tree was constructed using TDRAW V1.4 software package, which revealed the *T. putitora* with *S. richardsonii* and *Garra* species with *R. bola* forming a separate monophyly.

Keywords: Coldwater fish, RAPD, Genetic diversity, Polymorphism, Phylogeny

Introduction

In Uttarakhand Himalayas, mainly in the Kumaon region, four species of fishes are considered to be of commercial importance, viz., *T. putitora*, *S. richardsonii*, *B. bendelisis* and *Garra* species. Genetic variability in fishes has been proved valuable for aquaculture

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and fisheries management, identification of stocks, selective breeding programs, restoration of ecology and estimating genetic contributions in stock. Thorough knowledge of genetic variability within the species is considered a prerequisite for efficient utilization of biological resources. Generally, individuals with greater genetic variability have higher growth rate, developmental stability, viability, fecundity, resistance to diseases and environmental stress. To manage any biological resources effectively, the researcher must identify the level of genetic variation within and among populations.

The Random Amplified Polymorphic DNA (RAPD) marker consists of relatively short fragment amplified via PCR by random (10 mer) arbitrary primers (Grosberg *et al.*, 1996). Very few studies have been carried out in coldwater fish species using RAPD markers for assessing the genetic relatedness and polymorphic study. Kapila and Mishra (2006) have observed 69 polymorphic loci, out of 98 RAPD loci studied in *Schizothorax richardsonii*. Barat *et al.* (2008) have studied the genetic diversity among *Mahseer* population from NE and NW Himalayan region by using 10 random primers and found higher polymorphic loci (62.20%) in NW Uttarakhand population than in NE Arunachal Pradesh (49.00%) and both the populations were in single cluster. The present study is carried out for the estimation of genetic diversity, genetic polymorphism and phylogenetic relationships among the commonly found coldwater fish species of cyprinid family, viz., *Schizothorax richardsonii*, *Tor putitora*, *Raiamas bola* and *Garra* spp in the Kumaon region of Uttarakhand.

Materials and Methods

Fish Stock

Coldwater fish species (n = 20) were collected from Kumaon Himalayan rivers using cast nets.

Isolation of DNA

Isolation of high molecular weight DNA was carried out from skeletal muscle (150 mg) using phenol-chloroform-isoamylalcohol method (Sambrook *et al.*, 1987). The quality of isolated DNA was assessed through 0.8% horizontal submarine agarose gel electrophoresis and purity and concentration were determined by spectrophotometer reading at 260 and 280 nm. The good quality of the DNA having 1.7-0.19 OD ratio at 260/280 nm were utilized for further study. The DNA samples within the species were pooled (n = 20) to a final concentration of 50 ng/μL for accurate estimation of genetic polymorphism and genetic distance within and between these fish population. The purified genomic DNA was dissolved in TE buffer and stored at 4 °C for PCR.

PCR Amplification

Eleven random primers were utilized in the present investigation, and the sequences are presented in Table 1. RAPD-PCR was performed in a total volume of 25 μL containing 50 ng of genomic DNA, 100 pM of random primer, 200 μM of each dNTP, 2.5 μL 10 × PCR reaction buffer, 1.5 mM $Mgcl_2$ and 1 U of Taq DNA polymerase. Amplification was carried

out in a programmed DNA thermal cycler (Eppendorf) which consisted of initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 36 °C for 1 min, primer extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The amplified products were resolved on 1.4% submarine agarose gel electrophoresis at a constant voltage of 3 V/cm and visualized in the Gel Doc system (Alpha imager). Molecular sizes of the amplified products were estimated through inbuilt Gel Doc system software. Only the distinct and prominent bands were scored in the RAPD profiles, which showed polymorphic patterns and the same were used for estimation of genetic distances.

Table 1: List of Operon Series Primers Used in the Present Study

S. No.	Primer Name	Primer Sequence	Length	G+C Content (%)
1.	OPA-02	5'-TGCCGAGCTG-3'	10 mer	70
2.	NUSZG4	5'-GGAGCTGGC-3'	9 mer	77
3.	OPA-03	5'-AGTCAGCCAC-3'	10 mer	60
4.	OPA-04	5'-AATCGGGCTG-3'	10 mer	60
5.	OPY-02	5'-CATCGCCGCA-3'	10 mer	70
6.	OPY-19	5'-TGAGGGTCCC-3'	10 mer	70
7.	OPA-02	5'-TGCCGAGCTG-3'	10 mer	70
8.	OPF-05	5'-CCGAATTCCC-3'	10 mer	60
9.	OPY-04	5'-GGCTGCAATG-3'	10 mer	60
10.	OPY-11	5'-AGACGATGGG-3'	10 mer	60
11.	OPA-05	5'-AGGGGTCTTG-3'	10 mer	60

Statistical Analysis

Data was scored manually based on the presence or absence of band of identical molecular size. Gels were scored for the presence or absence of amplicon in each lane and the data was recorded as a binary matrix. If a band was present, it was recorded as "1", and if absent, as '0'. The molecular sizes of the RAPD product were estimated by their comparison with standard molecular size marker (Lambda DNA-Hind III/Eco RI digest and/ or 100 bp DNA ladder), which was run parallel to the amplified products in the gel. Comparisons were carried out between samples amplified by the same primer in a pairwise manner. Standard statistical analysis (Kuhnlein *et al.*, 1990; and Lynch, 1990) was carried out to estimate the genetic distance.

Results

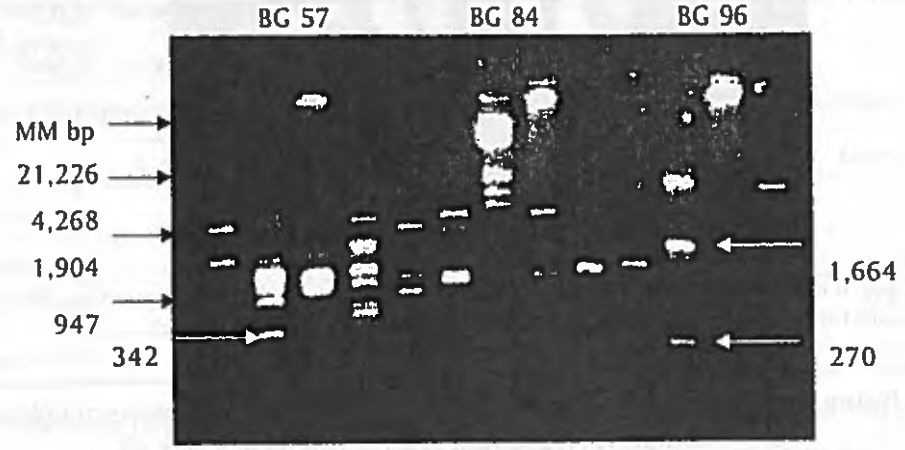
RAPD-PCR analysis of coldwater fish species, viz., *Tor putitora*, *Schizothorax richadrsonii*, *Raiamas bola* and *Garra* species, using 11 random primers was resolved; 188 bands with the average number of scorable bands ranged from 1 to 8 (3.54 ± 0.72), 2 to 8

Table 2: Numbers of Bands, Bands/Species, Number of Polymorphic Bands, Proportion of Polymorphic Bands, Band Frequency and Molecular Size, Range of Amplified in the Pooled DNA Samples with Different Primers

S. No.	Primer	Total No. of Bands Amplified				Total No. of Bands Amplified	No. of Polymorphic Bands Amplified				Total	% of Polymorphic Bands Produced	Band Frequency
		T. p	*S. r*	*R. b*	*G. s*		*T. p*	*S. r*	*R. b*	*G. s*			
1.	BG 53	4	6	5	8	23	2	2	2	2	8	2.87	0.17-0.35
2.	BG 54	5	8	5	5	23	1	3	0	0	4	5.75	0.22-0.35
3.	BG 56	3	2	5	3	13	1	1	1	0	3	4.33	0.15-0.38
4.	BG 57	2	7	3	8	20	1	1	0	2	4	5.00	0.10-0.40
5.	BG 84	3	4	3	5	15	0	1	1	0	2	7.50	0.20-0.33
6.	BG 96	1	4	1	1	07	0	1	0	1	2	3.50	0.14-0.57
7.	BG 89	3	4	5	8	20	0	1	0	4	5	4.00	0.15-0.40
8.	OPA 05	0	3	1	1	05	0	0	0	0	0	0	0.00-0.60
9.	BG 95	7	8	3	3	21	1	2	1	1	5	4.20	0.14-0.38
10.	BG 93	3	3	2	5	13	1	0	0	3	4	3.25	0.15-0.38
11.	OPA 03	8	8	7	5	28	0	3	4	2	9	3.11	0.18-0.29
	Total	39	57	40	52	188	7	15	9	15	56	3.35	0.21-0.30

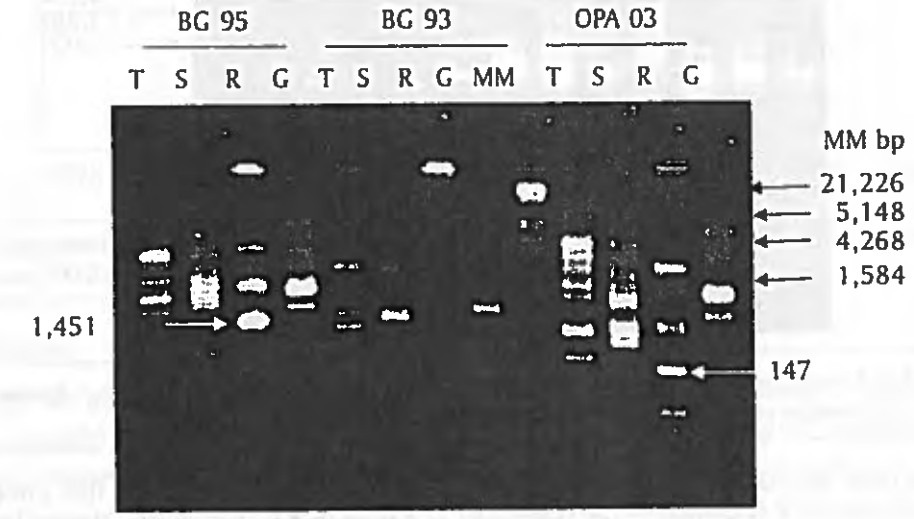
(5.18 ± 0.69), 1 to 7 (3.64 ± 0.58) and 1 to 8 (4.73 ± 0.78) in all the four species, respectively (Table 2). The sizes of the amplified products were from 200 to 5,000 bp with all the primers employed (Figures 1 to 4). The number of bands amplified by primers ranged from 1 to 8, with a mean of 17.09. The maximum numbers of scorable bands were obtained with primer OPA-03 primer in *Tor putitora* (08 ± 0.71), *Schizothorax richadrsonii* (08 ± 0.71), *Raiamas bola* (07± 0.71) and *Garra* species (05 ± 0.71) and minimum numbers (1 to 3) of amplified fragments were observed with primer OPA 05.

Figure 1: RAPD-PCR Amplification Patterns in Pooled DNA Samples of Coldwater Fish Species with Primers BG 57, BG 84 and BG 96



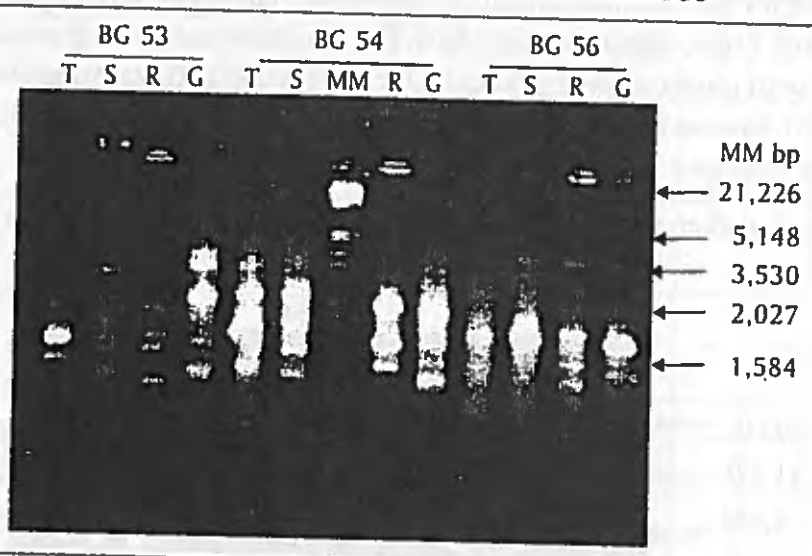
Note: T, S, R and G represent the DNA samples from *T. putitora*, *S. richardsonii*, *R. bola* and *Garra* species respectively; MM: λ-DNA Ecor I Hind III double digest marker.

Figure 2: RAPD-PCR Amplification Patterns in Pooled DNA Samples of Coldwater Fish Species with Primers BG 95, BG 93 and OPA 03



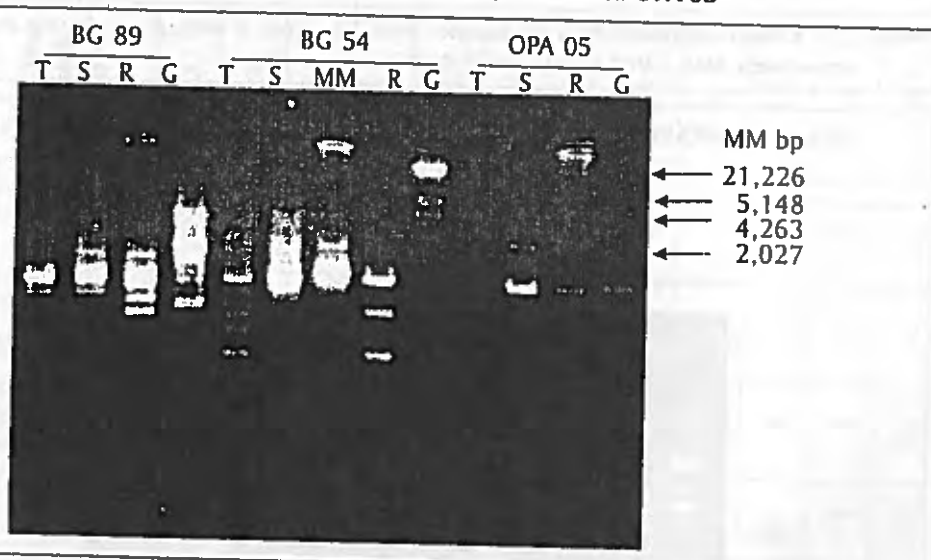
Note: T, S, R and G represent the DNA samples from *T. putitora*, *S. richardsonii*, *R. bola* and *Garra* species respectively; MM: λ-DNA Ecor I Hind III double digest marker.

Figure 3: RAPD-PCR Amplification Patterns in Pooled DNA Samples of Coldwater Fish Species with Primers BG 53, BG 54 and BG 56



Note: T, S, R and G represent the DNA samples from *Tor putitora*, *Schizothorax richardsonii*, *Raiamas bola* and *Garra* species respectively; MM: λ-DNA Ecor I Hind III double digest marker.

Figure 4: RAPD-PCR Amplification Patterns in Pooled DNA Samples of Coldwater Fish Species with Primers BG 89, BG 54 and OPA 05



Note: T, S, R and G represent the DNA samples from *Tor putitora*, *Schizothorax richardsonii*, *Raiamas bola* and *Garra* species respectively; MM: λ-DNA Ecor I Hind III double digest marker.

The level of polymorphism ranged from 0 to 7.50% among these fish species, with these 11 primers employed with the means of 5.57, 3.80, 4.44 and 3.47% in *T. putitora*, *S. richardsonii*, *R. bola* and *Garra* spp, respectively. The proportion of polymorphic bands produced pooled over primers was 3.36. Higher proportion of polymorphic bands was

produced by OPY 02 (7.5%) and NUSZG4 (5.75%) primers among these fish species. The primers OPA 04 and OPY 04 produced 342 and 1,664 and 270 bp in *S. richardsonii* and BG 95 and OPA 03 in *R. bola* of 1,451 and 147 bp specific bands. This series of primers utilized in the present investigation was found to be effective in developing species-specific RAPD markers in *S. richardsonii* and *R. bola* fish population.

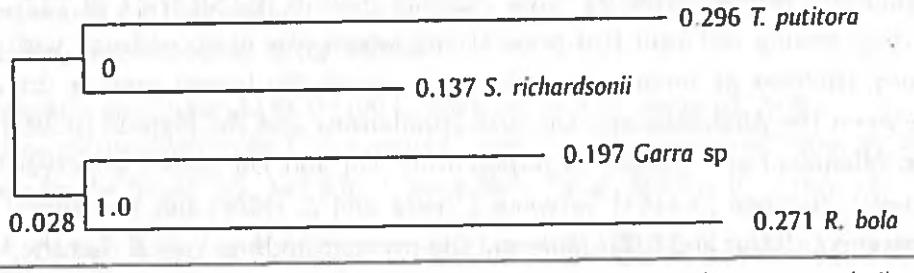
The genetic distances among the fishes were calculated by using RAPD distance, which shows the highest genetic distance between *T. putitora* and *R. bola* of 0.60, followed by *T. putitora* with *Garra* species of 0.52 (Table 3). The least genetic distance was observed between the *S. richardsonii* and *Garra* species (0.36), followed by *T. putitora* and *S. richardsonii* (0.43). The estimates of genetic distance ranged from 0.36 to 0.60 with different primers.

Table 3: Estimation of Genetic Distances Between Fish Species Using RAPD Distance

	T. putitora	*S. richardsonii*	*R. bola*	*Garra* sp
T. putitora	–	0.43	0.60	0.52
S. richardsonii	–	–	0.44	0.36
R. bola	–	–	–	0.46
Garra sp	–	–	–	–

The phylogenic tree was constructed using TDRAW V1.4 software package and showed two common clusters consisting of *T. putitora* with *S. richardsonii* and *Garra* species with *R. bola* by forming a separate monophyly (Figure 5).

Figure 5: Construction of Phylogenetic Tree (TDRAW V1.14)



Note: Unweighted pair group method of arithmetic mean dendrogram based on Nei's (1978) genetic distance using RAPD data.

Discussion

Similar to the present study, Kuusipalo (1999), Hatanka and Galetti (2003), Dergam *et al.* (2002), Aranishi and Okimoto (2004) and Grapputo *et al.* (2006) have also employed 3 to 5 random primers in different fish species and found that the total numbers of amplified fragments varied from 31 to 74, with a size ranging from 300 bp-1,500 bp, whereas Das *et al.* (2005) have used a maximum of 15 random primers and observed more numbers

of amplified fragments (270 and 449) in fish species. However, Yoon and Kim (2001) employed only 5 random primers and observed a maximum of 1,344 numbers of amplicon, while Liu *et al.* (1999) observed 462 amplified fragments (200-1,500 bp) by using 75 primers. The presence of more numbers of fragments might be due to the presence of more priming sites at the template DNA with the particular series of operon primers employed in their study.

Das *et al.* (2005) observed the varied range of 42.6%, 31.7%, 30%, 19.2%, 16.8% and 14.3% polymorphic loci in different carp species, which was similar to the present investigation, whereas El-Zaeem and Ahmed (2006) observed higher proportion of polymorphisms with an average of 55.76%. Barat *et al.* (2008) also found higher polymorphic loci (33, 62.20%) in Uttarakhand *T. putitora* population as compared to Arunachal Pradesh (26, 49.00%). Similarly, Kapila and Mishra (2006) observed 29 monomorphic species-specific, 4 location-specific, 8 unique (individual-specific) and other 57 polymorphic loci in snow trout (*S. richardsonii*) during studies of genetic stock of four geographically isolated locations of river Gola, Kosi, Chirapani and Ladhyia of Kumaon Himalayas. Similar to the present findings, Barman *et al.* (2003), Callejas and Ochando (2002), and Yoon and Kim (2001) have also found 406 (0.25-1.50 kbp), 19 (413-1155 bp) and 36 (0.19 kb-1.35 kbp) species-specific markers by employing 4 to 34 primers in their study respectively, in different fish species. Further, it is suggested that large-scale screening of random primers should be employed in order to develop specific markers and should be confirmed either by repeating the PCR reaction or by sequencing.

Genetic Distance

Lui and Chu-Wu (2007), Bardakci *et al.* (2004) and Grapputo *et al.* (2006) have also found the genetic diversity index ranging from 0.1022-0.1634 (0.122), 0.0579 and 0.1563 among different fish populations, which was in accordance with the present study, whereas Brahmane *et al.* (2006) observed the lowest genetic distance (0.213) between the Allahabad and Lal gola populations and the highest (0.394) in between the Allahabad and Bhadbhud populations. Liu and Liu (2007) observed the highest genetic distance (0.4167) between *L. vitta* and *L. sebae*, and the lowest (0.3612) in between *L. fulvus* and *L. fulviflamma*. The present findings reveal that the low levels of polymorphic loci within these species might be due to the low rate of gene flow within these populations as well as less migration rate.

Phylogenic Tree

The present investigation is similar to the report of Barat *et al.* (2008), who observed among NW and NE, *T. putitora* population forming a single cluster by using 10 random primers, and further they suggested that the distribution of genetic diversity of *T. putitora* population is the resultant of both ancient events related to drainage system formation and recent human activities.

Conclusion

The findings of the present study clearly demonstrate that the RAPD markers could be successfully employed to study the genetic polymorphism and genetic diversity among the coldwater fish species. ❋

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Phylogenetic Analysis of Coldwater Fish Species (Cyprinids) of India Using Targeted MtDNA and RAPD-PCR Markers

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In the Kumaon region of Uttarakhand Himalayas, coldwater fish species (Cyprinidae) is widespread and endemic in the natural waterbodies system. In order to test the genetic relationship among the four predominant fish species, viz.,*Tor putitora* (golden mahseer), *Schizothorax richardsonii* (snow trout), *Raiamus bola* (Indian trout), and *Garra gotyla (garra)*, an investigation was carried out using RAPD-PCR and targeted mitochondrial DNA- PCR analysis. The phylogenetic trees based on RAPD-PCR, by using 11 randon primers, targeted mitochondrial DNA-PCR, and 12S rRNA sequence showed a consistent result of forming two separate monophyletic groups consisting of *T. putitora* with *S. richardsonii* and *Garra* species with *R. bola*. The present investigation concluded that both the methods could be a valuable tool for studying molecular systematics and establishing the taxonomic position among cyprinids coldwater fish species of India.

Keywords: Coldwater Fish, RAPD, Mitochondrial DNA, Phylogeny, 12S rRNA

Introduction

In Uttarakhand Himalayas, mainly in the Kumaon region, four species of fishes are considered to be of commercial importance, viz., *T. putitora*, *S. richardsonii*, *B. bendelisis* and *Garra* species. The trouts, mahseers, schizothoracids, minor carps and exotic carps widely distributed in the Himalayan and peninsular regions of India are important both for food and recreation (Sehgal, 1987; and Tripathi, 2005). Although similarity of scales, fins, fin rays, length and weight patterns among these fish species is present, the taxonomic positions of these four coldwater fish species vary according to different sources, and no comparative studies on genetic variability and phylogenetic relationship are studied. Even though these fish species are classified under the family Cyprinidae, at subfamily level, the classification is ambiguous. Various authors have classified them under different subfamilies,

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leading to improper taxonomy of the species (Berg, 1940; Munro, 1982; Kapoor *et al.*, 2002; and The Catalogue Fishes On-line, 2007).

So keeping these in view, the present investigation was planned to study the molecular systematics at DNA level by employing RAPD-PCR and targeted mtDNA sequence analysis. The application of RAPD technique has greatly increased the ability to understand the genetic diversity within and between the species at the molecular level in pelagic fish populations (Kuusipalo, 1999), in aquarium fishes (Koh *et al.*, 1999), in cultured catfish (Yoon and Kim, 2001), in cyprinidae fish (Callejas and Ochando, 2002), in Indian major carps (Barman *et al.*, 2003), in three species of the genus Gobio (Cellajas *et al.*, 2004), in six *Labeo* species (Das *et al.*, 2005), in mosquito fish population (Grapputo *et al.*, 2006) and in *Salminus brasiliensis* groups (Lopes *et al.*, 2007)). A few studies were carried out on these coldwater fish species for assessing the genetic diversity and phylogeny (Kapila and Mishra, 2006; Sivaraman *et al.*, 2009; Sivaraman *et al.*, 2010 and Barat *et al.*, 2008).

The mitochondrial DNA (mtDNA) being more conserved is found to be one of the most valuable markers for taxonomic studies. They are particularly useful for estimating genetic distances among the species (Brown *et al.*, 1979) and inferring systematic position of fishes at species level (Kocher *et al.*, 1989; Barlett and Davidson, 1991; Orti *et al.*, 1994; Zardoya *et al.*, 1995; Brito *et al.*, 1997; Dergam *et al.*, 2002; and Shikano and Taniguchi, 2003). The 12S rRNA gene has been the most widely used targeted gene for phylogenetic analysis of different taxas, such as families (mitochondrial genome has species-specific information and has been used in phylogeny) (Alves *et al.*, 1995; Douzery and Catzeflis, 1995; and Ledje and Amason, 1996) and genera (Gatesy *et al.*, 1997; and Murphy and Collier, 1996). In the present study, the 12S rRNA gene sequence, using universal primer pairs and RAPD markers, has been selected for assessing the molecular systematic position of coldwater fish species.

Materials and Methods

Fish Samples

Samples of *S. richardsonii*, *T. putitora*, *R. bola* and *G. gotyla* were collected (n = 20) using gill nets and traps from the lakes and streams of the Kumaon region of Uttarakhand, India.

Extraction of DNA

The extraction of DNA was undertaken by using Wizard® Genome DNA purification kit (Promega), according to the manufacturer's instructions, from the muscle tissues. The concentration of the DNA was estimated by measuring the absorbance at 260 and 280 nm in a UV-visible spectrophotometer (Merck, USA), and the good quality DNA having the OD ratio at 1.7 to 1.9 was subjected to PCR amplifications. The DNA samples were pooled within the species of having 50 ng/μL for PCR study.

Polymerase Chain Reaction (PCR)

RAPD-PCR was performed in a total volume of 25 μL containing 50 ng of genomic DNA, 100 pM of random primer, 200 μM of each dNTP, 2.5 μL 10 × PCR reaction buffer, 1.5 mM

$Mgcl_2$ and 1 U of Taq DNA polymerase. Amplification was carried out in a programmed DNA thermal cycler (Eppendorf) which consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 36 °C for 1 min, primer extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The amplified products were resolved on 1.4% submarine agarose gel electrophoresis and visualized in the Gel Doc system (Alpha imager, USA).

The universal primer pairs based on the published sequences of highly conserved regions of 12S rRNA of the mitochondrial genome from the GenBank for mammal (Anderson *et al.*, 1981 and 1982) were used to amplify the partial 12S rRNA genes from the samples. The PCR was set up in 50 μL reaction volume. Based on the initial trial, the reaction mixture was optimized as follows: 5 μL of 10 × Assay buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM $MgCl_2$), 1 μL (200 μM each) of dNTP mix (sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water, pH 7.5), 1 μL or 20 Pico moles each of forward (5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3' 26 mer) and reverse (5'- GAG GGT GAC GGG CGG TGT GT-3' 20 mer) primers (Bangalore Genei, India), 1.66 U Taq DNA polymerase, 50 ng of purified DNA and autoclaved distilled water to make up the volume. The cycling conditions involved an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 45 sec denaturation at 94 °C, 45 sec annealing at 60 °C, and 1 min elongation at 72 °C. The amplification patterns were analyzed on 1.4% agarose gel. The amplicons on the gel were visualized in the Gel Doc system (Alpha Imager, USA) after staining with ethidium bromide. The amplified products of 456 bp were purified by the QIA quick gel extraction kit (USA) and were used for direct sequencing without cloning using ABI Prism 377 DNA sequencer at DNA sequencing facility (Bangalore Genie, Bangalore).

RAPD Analysis

The RAPD banding pattern was scored manually based on the presence as 1 or absence as 0 and recorded as a binary matrix of identical molecular size. Standard statistical analysis (Kuhnlein *et al.*, 1990; and Lynch, 1990) was carried out to estimate the genetic distance, and the phylogenic tree was constructed using TDRAW V1.4 software package.

Sequence Alignment

The DNA sequences were aligned and comparison was done by Clustal × (Thompson *et al.*, 1997) using MegAlign™ software package (DNA STAR, Inc.), followed by manual editing. The mt12S rRNA gene sequences of related fish species were retrieved from GenBank nucleotide sequence database (www.ncbi.nlm.nih.gov/entrez) and compared. The sequence alignment, phylogenetic tree construction, and sequence base pair distances were calculated by using Clustal X method with weighted residue weight table. The sequences were submitted to GenBank under the accession numbers of AM778102, AM778103, AM778104 and AM778106.

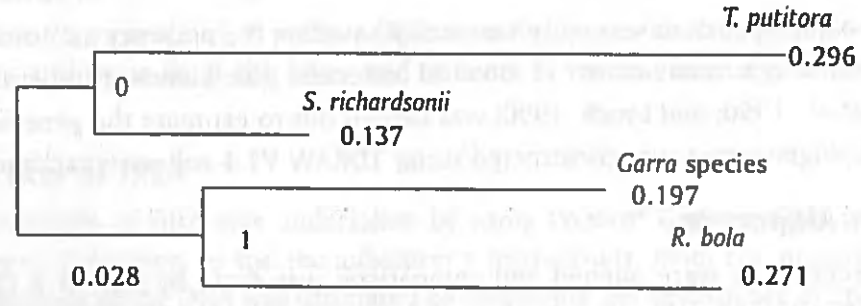
Results and Discussion

The commercially important fish species *S. richardsonii*, *T. putitora*, *R. bola* and *G. gotyla* were selected for the study because of their ambiguity in the taxonomic position (Berg, 1940; Munro, 1982; Kapoor *et al.*, 2002; and The Catalogue Fishes On-line, 2007). According to the conventional classification, all these species are grouped under the family Cyprinidae of the order Cypriniformes (Berg, 1940; Kapoor *et al.*, 2002; and The Catalogue Fishes On-line, 2007). Whereas according to Jhingran (1991), based on classification by Berg (1940), *S. richardsonii* and *T. putitora* belong to Schizothoracinae, *R. bola* (Previous name *Barilius bola*) to Rasborinae and *G. gotyla* to Cyprininae subfamilies respectively. In the subsequent classification (Talwar and Jhingan, 1991; and The Catalogue Fishes On-line, 2007) *S. richardsonii* is classified in the Schizothoracinae and *T. putitora* in the Cyprininae subfamily. In all the classifications, *G. gotyla* was put under Cyprininae and *R. bola* in Rasborinae. According to Munro (1982), the family Cyprinidae is having only two subfamilies, viz., Cyprininae and Rasborinae. In the study, RAPD-PCR and targeted mtDNA 12S rRNA sequences of the four coldwater fish species were obtained and compared with each other for further clarifying the phylogenetic relationship among them.

RAPD-PCR Analysis for Construction of Phylogenetic Tree

The phylogenic tree was constructed using TDRAW V1.4 software package, which showed two common clusters consisting of *T. putitora* with *S. richardsonii* and *Garra* species with *R. bola* by forming a separate monophyly (Figure 1). Whereas Barat *et al.* (2008) observed between northwest and northeast *T. putitora* population forming a single cluster by using 10 random primers, and further they suggested that the distribution of genetic diversity of *T. putitora* population is the result of both ancient events related to the drainage system formation and recent human activities.

Figure 1: Phylogenetic Tree Among the Fish Species Using RAPD Analysis



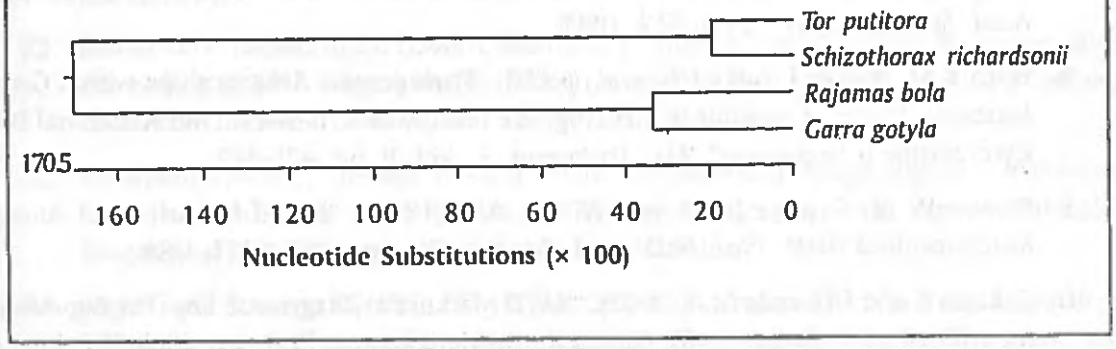
Note: Unweighted pair group method of arithmetic mean dendrogram based on Nei and Li (1978) genetic distance using RAPD data.

MtDNA Gene Sequences, Genetic Distance and Phylogenetic Tree Construction

The mtDNA 12S rRNA partial sequences of the coldwater fish species were amplified with PCR technique using universal primer and sequenced. The alignments of sequences were

compared in all the species, and the presence of a common conserved core region in all the four fish 12S rRNA genes indicates that all these species belong to the same family (Cyprinidae). It was further confirmed on the basis of homology with previously published sequences from other fish species from NCBI GenBank. Phylogenetic tree based on mtDNA showed that *T. putitora* clustered with *S. richardsonii* and *Garra* spp. with *R. bola*. The phylogenetic trees from both the PCR techniques indicated that two separate monophyly consist of *T. putitora* clustered with *S. richardsonii* and *Garra* spp. with *R. bola*. Wang *et al.* (2002) also amplified the 12S rRNA genes of different vertebrates by the same universal primers and compared with other available gene sequences. The phylogenetic tree indicates the possible occurrence of two subfamilies among these four fish species studied as Schizothoracinae/Cyprininae and Rasborinae (Figure 2). The species *S. richardsonii* and *T. putitora* showed the least genetic divergence (0.43 and 38.2%) with RAPD and mtDNA analysis and showed parallel branches of the phylogenetic tree, indicating that they can be included under the same subfamily Schizothoracinae/Cyprininae or can be included under the subfamily of Rasborinae because of the existing morphometric differences. The conventional classification by Berg (1940) and Kapoor *et al.* (2002) also suggested that the *S. richardsonii* and *T. putitora* species can be placed under the same subfamily (Cyprininae). The present results based on the RAPD and targeted 12S rRNA sequences absolutely match with the most widely accepted classification given by Berg (1940).

Figure 2: Phylogenetic Tree Among the Fish Species Using 12S rRNA Gene Sequences



Conclusion

The targeted mtDNA 12S rRNA sequences and RAPD-PCR analysis could be a valuable tool for establishing the status of molecular systematics and phylogenetic tree construction even at the subfamily level. The present study further suggests that the universal primers for more numbers of mtDNA genes as well as with more numbers of RAPD primers may provide accurate assessment of molecular systematic of fish species even at the species level. ❋

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PERFORMANCE OF THREE PRONGED CHINESE CARP FARMING IN MID HIMALAYAS OF WEST KAMENG DISTRICT, ARUNACHAL PRADESH

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The performance of three pronged carp farming was evaluated in 10 mid Himalayan fish ponds located at 1450-1500 m asl in the west Kameng district of Arunachal Pradesh. The fishes were stocked with a density 3 fishes/m^3. The performance of 3 fish species (Silver carp-*Hypophthalmichthys molitrix*, Grass carp-*Ctenopharyngodon idella* and Common carp-*Cyprinus carpio*) in terms of growth, survival and contribution to total biomass were studied and analyzed. The achieved average production was 78.6 kg, 76.5 kg and 137.9 kg for silver carp, grass carp and common carp, respectively. Among the 3 species the production was higher for common carp (47.1%) followed by silver carp (26.8%) and grass carp (26.1%). The production figures indicate that culture of Chinese carps in mid Himalaya can be suitably adopted and can contribute substantial income to the tribal fishers of hilly region.

Keywords: Chinese carp farming, mid Himalayas, Arunachal Pradesh, production, cost-benefit

Introduction

Arunachal Pradesh is situated on the extreme North Eastern tip of India in trans Himalayan region between 91°31' and 97°30' East Longitude and 26°28' and 29°33' North Latitude. The state having an area of 83,743sq.km with a total population of 1,091,117 (2001) is a mosaic of composite culture and tradition. A land inhabited by 28 major tribes and 110 sub-tribes, it is the 12th mega biodiversity region of the world and is the richest biotic province of Republic of India. The topography of the region varies from few meters from the sea level to Snow Line Mountains and has different agro-climatic zones. This area receives heavy rainfall during prolong rainy season from March to October resulting in vast and varied aquatic resources in the form of 2,000 km of rivers, 2,500 ha of wetlands and lakes, 1,250 ha of ponds and mini barrages and 2,925 ha of area suitable for paddy cum-fish culture. The state is also home to rich fish fauna, representing about 167 species including some coldwater species. However, in spite of having vast aquatic resources and fish diversity, the region is yet to catch up with the rest of the country in developing its water resources for fisheries. Although fish production from the state has increased from 1.25 lakhs tonnes during 1990-91 to 2.65 lakhs tonnes during 2003-04, there still remains a good gap between the production and demand necessitating import of fish from other states like Andhra Pradesh, Bihar, and West Bengal. The per capita availability of fish in the state is low 2.19 kg yr^{-1} against the national requirement of 11kg yr^{-1}. The present investigation deals with successful attempts made on fish production by the rural tribe by adopting appropriate three-pronged composite carp farming technology suitable for their natural environment and to create self-employment. The study also embodies to observe the performance of 3 different fish species under culture conditions of Mid Himalayas in West Kameng district, Arunachal Pradesh.

Materials and methods

The study was conducted in Chug village situated about 10 km away from Krishi Vigyan Kendra in Dirang Block of West Kameng district, Arunachal Pradesh during March to November 2009. Three species of Chinese carps *viz.*, silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*) and common carp (*Cyprinus carpio*) were stocked together at a density of 3 fishes/m^3 and recommended supplementary diet was provided under low temperature conditions. The performance of each fish species in terms of growth, survival and contribution to total biomass were studied and analyzed based on the methodology of Biswas, 1993.

10 ponds located at 1500 m asl were selected to demonstrate Chinese carp farming developed for hills, based on the data generated through survey conducted in the village on the status of the ponds and socio-economic conditions of the beneficiaries. The methods adopted in the demonstration programme on Chinese carp farming can broadly be divided into the following three phases:

Pre-stocking management - Pond bottom is dried until soil cracks followed by ploughing for eradication of undesired animals and the removal of obnoxious gases from the bottom soil. Manuring of pond with cow dung was done for production of natural fish food organisms. Liming of pond bottom was done 7 days after manuring for correction of soil and water pH as well as for pond disinfection. The pond is then filled with fresh clean water.

Stocking management - Fish seeds (60 mm in length) were stocked @ 3 nos/m^2. A proportion of 30% silver carp, 35% grass carp and 35% common carp were stocked.

Post-stocking management - The post-stocking pond management primarily involves the aspects of intermittent liming and fertilization, supplementary feeding, water management and health care. Organic manure in the form of raw cow dung was applied every fortnight. Inorganic fertilizers were not used. Supplementary feed comprising rice bran and mustard oil cake was given @ 3% of the body weight of the stocked fish biomass per day. Grass carp were fed on terrestrial vegetation, fodder grasses and vegetable wastes. Liming and manuring was done once in a month, 1-2 days after the application of organic manure. Raking of pond bottom was done after liming, for proper mixing of lime and release of obnoxious gases.

Trainings were imparted and demonstration programmes were conducted initially by the Dirang based Krishi Vigyan Kendra with a view to develop aquaculture activities in Chug village and beneficiaries including farmers, farm women and school drop-outs of the village were motivated to start composite fish farming of Chinese carps. The demonstrations comprised feeding techniques, stocking procedure, liming and manuring techniques, water quality and hygiene management *etc.* Fish seeds and feeds were provided free of cost to the beneficiaries from the KVK.

Results and discussion

The work calendar for each activity for the three-pronged Chinese carp farming technology during the period was checked out before starting the program. The work done during the calendar year was recorded in Table 1. The information on agro-climatic conditions of the study area were collected from the indigenous source as well as secondary source and recorded in Table 2. The level of management practice for adopting the

Table 1. Monthwise activity for chinese carp farming system

Period	Work done
15 March - 31 March 2009	Drying of pond bottom till soil cracks.
1 April - 30 April 2009	Removal of unwanted materials from the pond.
	Removal of marginal weeds.
	Ploughing of pond bottom soil.
	Manuring of pond bottom with raw cowdung.
	Liming of pond bottom.
	Filling of pond with fresh stream water.
1st Week of May 2009	Stocking management
	Stocking of pond with Chinese carps.
May - October	Culture period
	Post-stocking management.
1st Week of Nov 09	Harvesting of stocked fish.

Table 2. Composite carp farming technology and its operational calendar

Area of operation	Chug village, Dirang (1500 m asl)
Operational period	March 2009 – October 2009
Nature of pond	Earthen
Pond size (average) and shape	600 m^2, rectangular
Pond depth	1.5 m
Source of water	Rain, stream
Fish species	Silver carp, Grass carp & Common carp
Stocking density	3 Nos./ m^2
Stocking percentage	Silver carp (30%), Grass carp (35%) & Common carp (35%)
Fish size during stocking	60 mm
Feed	
(i) % of body weight	3%
(ii) Frequency	Daily
(iii) Items	A mixture of rice bran and mustard oil cake in 1:1 ratio
(iv) Feed for Grass carp	Terrestrial grasses, vegetable wastes
Liming (in installments)	
(i) Dose	250 kg/ha/yr
(ii) Frequency	Fortnightly
Manuring (in installments)	
(i) Dose	10,000 kg/ha/yr
(ii) Frequency	Weekly
Production (per 600 m^2)	293 kg/ 600 m^2/ 6 months
Production (per m^2)	0.48 kg/ m^2/ 6 months
Estimated expenditure	Rs. 33.00/ kg fish
Total income	Rs. 35,160.00 (Rs. 120.00/ kg fish)
Net income	Rs. 87.00/ kg fish

farming technology suitable under the climatic conditions of the study area was carried out and recorded in Table 2. The achieved production was 0.48 kg/m^2/6 month, with the estimated production cost of fish Rs. 33 per kg. The details has been shown in Table 2.

The yield depends on growth rate and retrieval of the stocked fishes at harvest. The growth and survival of fishes were found directly correlated to water temperature, quality and quantity of supplementary feed provided, natural food availability in ponds and the methodology followed.

Table 3. Performance of fish species under culture conditions

Species	Growth (g/month)	Production (Kg/600m²/6months)	Contributed production (%)	Rank
Silver carp	26.06	78.64	26.83	2
Grass carp	21.42	76.54	26.11	3
Common carp	39.52	137.95	47.06	1

In the upland water of low thermal regime, the Chinese carps were found to have grown well as compared to the Indian major carps (Tyagi *et al.*, 2005 and Mahanta *et al.*, 2009). Based on the results it was observed that more biomass could be obtained by stocking fish seeds @ 3 nos/m² in hill conditions as compared to 1 no/m². The growth of grass carp was found related to the quality and quantity of the grass and vegetable waste fed. Common carp feeding on unutilized feed, faecal matter of grass carp and benthic organisms contributed the bulk of the catch. In a hilly district like West Kameng, the quantity and quality of supplementary feed played very important role. From the results it was observed that feed which was given @ 3% body weight of fishes, produced an average production of 293 kg/ 600m²/ 6 months. In a span of 6 months, silver carp (30%), grass carp (35%) and common carp (35%) attained the maximum individual weight of 235g, 300g and 500g respectively. The performance of silver carp, grass carp and common carp in terms of growth and production during the culture period and its contribution is recorded in Table 3. Maximum expenditure incurred in this venture was from purchase of fish feeds (45.14%), followed by purchase of quality seeds (37.62%), renovation and maintenance cost (10.45%), manpower (5.22%) and finally purchase of necessary miscellaneous inputs (1.57%). The estimated total income was Rs. 35,160.00 @ Rs. 120.00 per kg selling price of fish. The net income of Rs. 87.00 per kg fish on estimated expenditure of Rs. 33.00 suggested the economic profitability of the technology for the participating rural tribe farmers.

Conclusion

The production figures indicated that culture of Chinese carp is easy to operate, economically viable and eco-friendly as it is able to convert/ recycle the cattle yard, agricultural, kitchen wastes into fish food. Therefore, this technology opens up the possibility of promoting exotic carp culture in hilly regions of the district where existing production of indigenous fish is very low.

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INDUCED SPAWNING OF *LABEO DYOCHEILUS* IN CAPTIVITY UNDER COLDWATER CONDITIONS

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An attempt was made to breed an indigenous carp, *Labeo dyocheilus* (McClelland) in captivity under coldwater condition at 18-22 °C temperature with optimization of synthetic hormone dose. Full maturity was observed during the 3rd week of July to the end of August. Hormone dose of 0.6 ml kg^{-1}body for females and 0.3 ml kg^{-1} body for males was found optimum for spawning with 84-98% fertilization and high rate of hatching. Successful breeding of this species in captive condition enabled to produce seeds for wild stock augmentation and species diversification in coldwater aquaculture.

Key words : *Labeo dyocheilus*, breeding, ovaprim, coldwater.

Introduction

There are 258 fish species, both indigenous and exotic, belonging to 76 genera, reported from Indian uplands, which are spread over in Himalayas and peninsular plateau. A few, such as indigenous mahseer, snow trout, exotic trout and common carp are however commercially important food fishes dwelling in uplands (Vass, 2005). A number of fish species such as *Garra gotyla*, *Crossocheilus diplochilus*, *Semiplotus semiplotus*, *Osteobrama belangiri*, *Labeo dero* and *Labeo dyocheilus* are also found distributed in the different reaches of the upland rivers (Vass, 2005). These species have high consumer preference as well as good market value (Sarkar *et al.*, 2001). These indigenous species would be candidate species for aquaculture could once their breeding, feeding and culture protocol are developed.

In the Kumaon region of Uttarakhand state *L. dyocheilus*, locally called as Kali, is an economically important coldwater fish (Joshi, 1994). This species has been categorized as threatened (Desai, 1994) and vulnerable (Dubey, 1994; Prasad, 1994). *L. dyocheilus* is a mid-distance coldwater migrant bottom feeder, inhabiting upland streams and rivers at an elevation of 400-800 m msl. This species perform upstream migration during May to June when water temperature rises in the stream. After rainy season in August-September, the spent fish starts downward migration. No distinct spawning ground was identified for the natural breeding of this species. The body of this species is ordinarily white with relatively small head. Generally, this species is herbivorous in nature consisting 80% algae and debris in the gut. Identification of sex is possible during spawning season (Singh *et al.*, 2008). Individuals grow to 91-158 g in one year in natural condition. Normal ovarian development of *L. dyocheilus* under captivity in Tarai region of Uttarakhand was reported by Singh *et al.* (2008), which indicated the possibility of the breeding of this species in captive condition. But, the ovarian maturity in cold water under captive condition is not yet reported. *L. dyocheilus* can bred in natural environment (Sarkar *et al.*, 2001). At the onset of ovulation, the female fish is chased by the males. Attempt has also been made to breed this

fish by dry stripping method (Sarkar *et al.*, 2001). Standardization of breeding protocol in coldwater climate, under captive condition, is required for the mass scale seed production of this species for stock augmentation in wild or species diversification in aquaculture. This paper ambodies result of induced breeding trials conducted on the species.

Materials and methods

The brooders of age 3+ years and body weight 300-600 g were collected using castnet from the Kosi river of Kumaon Himalayan region near Jim Corbett National Park (29° 29.038′ N and 79° 08.777′ E, Altitude 410 m msl) in February 2011 and stocked in the cemented tanks at Bhimtal (Latitude 29° 21′ N, Longitude 79° 34′ E; altitude 1370 mmsl). During brood care, fish were fed @ 3% of their body weight daily with conventional carp feed prepared by mixing rice bran and ground nut oil cake in the ratio of 1:1. Natural periphyton diet was also provided by placing bamboo splits and plastic sheets as substrate in pond water. Stock was observed weekly for maturity, health and for secondary sexual characteristics. Two breeding operation was attempted. Different doses of ovaprim (GnRH- domperidone) were injected intra muscularly. Spawning was carried out in cloth hapa and in FRP tanks. Dry stripping method was also tried with hormone treated females. Eggs were incubated in trays, placing in trough, having flowing water. Gonadosomatic index (GSI) was computed month wise as percentage of weight of ovary in relation to body weight. Fecundity was calculated by volumetric method to measure the volume of released eggs. Ova diameter was measured with the help of ocular micrometer. Rate of fertilization, hatching, incubation period and survival of larvae was observed for each operation. Physico-chemical parameters of the water were analysed following APHA (1985).

Results and discussion

The brooders having age 3+ years and body weight as 350-700 g showed full maturity with eggs and oozing milt during the 3rd week of July to the end of August in pond environment, while it was during first week of July to second week of August in wild stock. Gonadosomatic index (GSI) increased gradually from April to July and decreased in the August in females of pond and stream. GSI was significantly higher ($P<0.01$) during May to July in wild females compared to captive ones. GSI ranged from 3.436±0.236 to 14.465±1.342 in captive females and 4.254±0.325 to 15.864±1.435 in wild females. Monthly variation was also significantly different ($P<0.01$) in both type of females (Table 1).

Early maturity and comparatively higher GSI in wild females might be due to the higher temperature in stream (24-31 °C). Similar trend of GSI was also reported by Singh *et al.* (2008) in pond reared females of Tarai region. During spawning season females showed soft and bulged belly with swollen light reddish vent.

In the present study, it was observed that all females released viable eggs after 12-14 hrs. of the hormone injection. Hormone dose of 0.6 ml kg^{-1} body for females and 0.3 ml kg^{-1} body for

Table 1. Gonadosomatic index (mean± SD) of pond reared and wild females of *L. dyocheilus*

Brooder	April	May	June	July	August
Pond reared	3.436±0.236	5.462±0.463	10.214±1.140	14.465±1.342	12.823±1.321
Wild	4.254±0.325	6.263±0.386	12.468±1.241	15.864±1.435	11.326±1.325

males was found optimum for spawning with 84-98% fertilization and high rate of hatching (54-78%) (Table 2 and Table 3). There was no post spawning mortality. Breeding performance was better in the FRP tanks rather than in hapa and in dry stripping (Table 2 and 3). The optimum temperature for successful spawning and egg incubation was observed as 18-22 °C. At the time of spawning splashing of water was observed and female fish were chased by the males. Late evening was the best time for the hormone injection. Eggs of *L. dyocheilus* were creamy white in colour and the size of the ovarian eggs (Stage VI) ranged between 1.24±0.12 to 1.38±0.14 mm. The observed fecundity was 188700 kg^{-1}. The fertilized eggs were semi adhesive and settled in the bottom. Water hardening of the fertilized eggs took 4-5 hrs. The average diameter of the fertilized egg was 2.6-3.4 mm. Eggs of large sized female were larger in size than the eggs of small sized females. One liter of egg volume contained 39000 fertilized eggs. The hatching period was 20-46 hrs. Early hatching was observed at higher temperature and late and prolonged hatching was observed at low temperature (Table 4). 18-20 °C was observed optimum for the egg incubation with higher hatching rate and better recovery of larvae. Late and prolonged hatching resulted in healthy hatchlings and better recovery of spawn. At 20 °C hatching period was 24-36 hrs, which resulted in 68% survival of 4 days old spawn and 54% survival of 20 days old fry. At the same temperature (20 °C) hatching period was extended (28-42 hrs) with the aqueous wash treatment of seed powder of *Myrobolus indicus* (100 g 10 l.). Tannin content of this seed produced a bio-film over the developing embryo and protected it from fungal infection and prolonged the hatching time. In the treated eggs, 86% survival of 4 day old spawn and 66% survival of 20 day old fry was recorded. The fecundity of this species is higher tham *Tor putitora* as reported by Pandey *et al.* (1998). High relative fecundity (243000 kg^{-1}) of an indigenous coldwater minor carp, *L. dero* was also reported by Prasad *et al.* (2008). The latency period (12-14 hrs) was in agreement with Sarkar *et al.* (2001). However, it is comparatively higher than observed by Singh *et al.* (2005) and

Table 2. Spawning performance of *L.dyocheilus* with Ovaprim in cloth hapa at 22 °C

Weight (kg) Female	Weight (kg) Male	Hormone Dose (ml kg^{-1} wt.) to F	Hormone Dose (ml kg^{-1} wt.) to M	Time taken for spawning (hours)	Number of eggs released
0.540 (1 no.)	0.840 (2 nos.)	0.4	0.0	-	nil
0.480 (1 no.)	0.820 (2 nos.)	0.6	0.0	-	nil
0.430 (1 no.)	0.830 (2 nos.)	0.4	0.1	-	nil
0.440 (1 no.)	0.810 (2 nos.)	0.6	0.3	14	83020

F- Female, M- Male, b.wt- body weight.

Table 3. Spawning performance of *L. dyocheilus* with Ovaprim in FRP Tank at 20 °C

Weight (kg) Female	Weight (kg) Male	Hormone Dose (ml kg^{-1} wt.) to F	Hormone Dose (ml kg^{-1} wt.) to M	Time taken for spawning (hours)	Number of eggs released
0.427 (1 no.)	0.950 (2 nos.)	0.4	0.0	14	nil
0.580 (1 no.)	0.940 (2 nos.)	0.6	0.3	12	109450
0.340 (1 no.)	0.830 (2 nos.)	0.6	0.3	14	64160

F- Female, M- Male

Table 4. Egg incubation performance of *L.dyocheilus* at different temperature

Eggs from	Fertilization (%)	Hatching (%)	Hatching period (hours)	Survival of hatching to spawn 4D	Survival of spawn to fry 20D
Hapa (at 22 °C)	84	54	20-26	62	48
FRP (at 20 °C)	96	74	20-36	68	54
FRP (at 20 °C)*	96	78	28-42	86	66
FRP (at 18 °C)	96	70	26-46	76	58
STR (at 20 °C)	98	52	26-42	64	52

Hapa- hapa breeding, FRP- breeding in FRP tank, STR- dry stripping, * eggs were treated with aqueous extract of *Myrobolus indicus*., 4D- four days old larvae, 20D- twenty days old larvae from day of hatching

Kushwahai *et al.* (2007) at 28-32 °C. The rate of fertilization (84-98%) in the experiment was comparable with earlier reports (91-94%, 90-95%, 70-95%) of Kushwahai *et al.* (2007), Sarkar *et al.* (2001) and Singh *et al.* (2005). Basnet (2007) also observed fertilization rate as 80-95% in *L. dero*. Hatching percentage was observed in the range of 70-78%, with better results at 18-20 °C, which was comparable to the study of Basnet (2007) as 72% in *L. dero* and Hossain *et al.* (2007) as 87.3% in *L. bata*. Low percentage of hatching (52%) was observed in stripping, that might be due to the improper ratio of eggs and milt. Higher hatching percent and better survival of tinny hatchlings could be achieved in FRP tank rather than in cloth hapa. Hatching period (20-46 hrs.) for *L. dyocheilus* at 18-20 °C in the present study is higher than *L. dero* (16-18 hrs,) at 24-26 °C, which was reported by Prasad *et al.* (2008). The hatchlings were whitish in colour with sufficient yolk material. The average size of one day hatchling was 3.48±0.24 mm, weighing 0.006 g. Yolk material was absorbed in 72-78 hours. of hatching at 20 °C temperature and larvae starts external feeding at 4th day. Filtered plankton was given for first 4 days then yolk of boiled hen egg was given for the next one week. Finely powdered ground nut oil cake and rice bran was given to the 15 days old larvae with simultaneous plankton feeding. The survival percent of fry (48-66%) was comparable to survival of fry of *L. bata* (46-63%) (Hossain *et al.*, 2007) and was higher than the survival of *L. dero* (26%), Basnet (2007).

The water of the hatchery was cold (18-22 °C), alkaline in pH (8.4-8.6), high in dissolved oxygen (6.4-8 mg l^{-1}), medium alkalinity (84-142 mg l^{-1}) and low in free CO_2 (0-1 mg l^{-1}). High pH (>8.6) resulted in the burstiong of fertilized eggs in hapa with high mortality of hatchlings. It is concluded that *L. dyocheilus* is easily amenable to breed in captive condition either through hypophysation and / or stripping method and can be further reared up to stockable size.

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Proximate and Mineral Composition of Indigenous Hill Stream Fishes of Uttarakhand

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ABSTRACT

The fishes *Labeo dero, Labeo dyocheilus, Labeo pangusia, Tor putitora* and *Schizothorax rechardsonii,* predominantly found in Kosi river, contribute major proportion of the total catch from the area. Average moisture, ash, crude protein and crude fat ranged from 81.54-70.65, 8.86-6.23, 73.01-52.43 and 32.53-12.66 %, respectively. Crude protein was highest (73.01 %) in *L. pangusia* and lowest (52.43 %) in *T. putitora.* Fairly higher level of fat (32.53 %) makes the *L. dero* a moderately fatty fish. Average calcium, potassium and sodium content in fishes ranged from 263-406, 784-1246 and 100-241 mg/100g, respectively. Sodium was highest (241 mg/100g) in *T. putitora* and *S. rechardsonii* while potassium and calcium were highest in *S. rechardsonii* and values were 1246 and 406 mg/100g, respectively. Iron, zinc, magnesium and selenium content ranged from 0.263 to 1.016, 0.769 to 1.497, 0.076 to 0.221 and 0.265 to 1.311 mg/100g, respectively. The proximate and minerals compositions varied significantly ($P<0.05$) among species. Hence, all the fishes particularly *S. richardsonii, L. dero* and *L. pangusia* can be considered as relatively rich sources of protein, fat and minerals for consumers.

Keywords: *L. dero, L. dyocheilus, L. pangusia, T. putitora, S. richardsonii,* Nutrient composition.

INTRODUCTION

Labeo dero, Labeo dyocheilus, Labeo pangusia, Tor putitora and *Schizothorax richardsonii* are predominantly found in Kosi river, one of the important rivers of Uttarakhand, which originates from southern slope of the Bhatkot - Kausani range (2517 m above msl) of Almora district and enters the Bhabar near Ramnagar (346 m above msl). These hill stream fishes contribute major proportion of the total catch from the area. However, the fishery of these upland fishes is declining due to various natural and anthropogenic factors. It is noteworthy that the fishes of the hill streams are much preferred by the consumers because of their taste, which depends on protein, minerals and other nutrients (Sehgal 1999).

Fishes are known to be the less expensive source of animal protein apart from rich source of micro- and macro minerals and other nutrients (Majumdar and Basu 2009). The level of these nutrients vary from species to species and even in the same species, on the basis of its size, sex, habitat, etc. (Dempson et al. 2004). The minerals present in fishes are of biological importance as they are required for important metabolic process thereby known to be essential for human health. The fishes that are being cultured are good in taste and preferred by the consumers due to excellent nutrient composition. However, many upland riverine fishes are not preferred by the consumers and consequently kept out of aquaculture practice due to lack of information about their nutritive value (Mills 1980).

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Gopakumar (1997) and Louka et al. (2004) recorded the proximate composition of various fishes from different environments. Salam and Davies (1994), Berg et al. (2000), Dempson et al. (2004) and Majumdar and Basu (2009) made attempts to characterize nutritional quality of various freshwater and few coldwater fish species. In view of paucity of information regarding proximate and mineral composition of indigenous hill stream fishes particularly of cold Himalayan region, an attempt has been made to estimate the proximate and mineral composition of five important indigenous hill stream fishes of Kosi river of Uttarakhand.

MATERIALS AND METHODS

Fishes were collected from the Kosi river at Ramnagar and Rati ghat of district Nainital, Uttarakhand. Water quality (pH, DO, CO_2 and temperature) at the sampling site was recorded as per APHA (1995) methodology. Three *Labeo* species (*L. dero, L. dyocheilus* and *L. pangusia*), one each of Tor (T. putitora) and *Schizothorax species* (*S. richardsonii*) were collected and brought laboratory, in ice packed condition and measured their length and weight prior to analysis. After descaling and degutting, pooled muscles of each species were homogenized for the analysis of moisture, ash, crude protein and crude fat as per AOAC (1995). Ash samples, dissolved in 2 ml of concentrated acid mixture (HCl: HNO3; 1:1) were diluted with distilled water (Shearer 1984) and diluted mixture was analyzed for calcium, sodium, potassium, iron, zinc, magnesium and selenium using Atomic Absorption Spectroscopy (Thermo-Electron Corporation, FS95-Furnace Auto Sampler) in triplicate. Data were subjected to statistical analysis (one way ANOVA) and differences among the mean treatments were tested by Tukey's test using the statistical package- SPSS, version 12.01 for Windows.

RESULTS AND DISCUSSION

The water temperature ranged between 5-30°C and, DO 5.0-8.5 mg/ L, CO_2 nil and pH 7.5-8.2 at both the sampling sites. The proximate compositions of fish samples are given in Table 1. Moisture was highest (81.54 %) in *L. pangusia* and lowest (70.65 %) in *S.*

Table 1. Proximate composition of selected hill stream fishes (% on DM basis)

Composition	*L. dero*	*L. dyocheilus*	*L. pangusia*	*T. putitora*	*S. richardsonii*
Moisture	$76.92^{b} \pm 1.5$	$76.32^{b} \pm 2.67$	$81.54^{a} \pm 0.78$	$72.68^{c} \pm 0.02$	$70.65^{c} \pm 0.48$
Dry matter	$23.08^{b} \pm 1.5$	$23.61^{b} \pm 2.69$	$18.78^{c} \pm 0.59$	$27.32^{a} \pm 0.02$	$29.35^{a} \pm 0.48$
Ash	$8.86^{a} \pm 2.05$	$6.51^{b} \pm 0.15$	$7.39^{b} \pm 0.06$	$6.23^{b} \pm 0.02$	$7.38^{b} \pm 0.01$
Crude protein	$70.24^{b} \pm 2.71$	$68.53^{b} \pm 3.0$	$73.01^{a} \pm 2.83$	$52.43^{c} \pm 0.35$	$56.45^{c} \pm 2.34$
Crude fat	$32.53^{a} \pm 3.56$	$12.66^{c} \pm 2.95$	$24.19^{b} \pm 4.05$	$19.25^{c} \pm 0.31$	$18.42^{c} \pm 0.71$

Values are Mean±SD of triplicate analysis (n=3). Values with different superscript in a row varied significantly ($P<0.05$).

Table 2. Mineral composition of selected hill stream fishes (mg/100g DM basis)

Mineral	*L. dero*	*L. dyocheilus*	*L. pangusia*	*T. putitora*	*S. richardsonii*
Sodium	$138^{b} \pm 25.5$	$100^{b} \pm 4.5$	$103^{b} \pm 12.6$	$241^{a} \pm 10.5$	$241^{a} \pm 31.1$
Potassium	$1233^{a} \pm 149$	$925^{b} \pm 147$	$784^{b} \pm 23$	$1228^{a} \pm 25$	$1246^{a} \pm 59$
Calcium	$342^{b} \pm 7.39$	$263^{b} \pm 15.62$	$270^{b} \pm 14.05$	$397^{a} \pm 12.01$	$406^{a} \pm 14.05$
Iron	$1.016^{a} \pm 0.009$	$0.984^{a} \pm 0.008$	$0.263^{d} \pm 0.0003$	$0.628^{b} \pm 0.017$	$0.464^{c} \pm 0.033$
Zinc	$1.239^{b} \pm 0.057$	$0.963^{c} \pm 0.055$	$0.769^{d} \pm 0.006$	$1.407^{a} \pm 0.016$	$1.497^{b} \pm 0.005$
Magnesium	$0.213^{a} \pm 0.005$	$0.076^{d} \pm 0.002$	$0.164^{b} \pm 0.009$	$0.128^{c} \pm 0.014$	$0.221^{a} \pm 0.015$
Selenium	$0.265^{d} \pm 0.006$	$0.442^{c} \pm 0.006$	$0.565^{b} \pm 0.008$	$1.311^{a} \pm 0.017$	$0.121^{c} \pm 0.006$

Values are Mean±SD of triplicate analysis. Values with different superscript in a row varied significantly ($P<0.05$).

rechardsonii. Ash content was highest (8.86 %) in *L. dero* and lowest (6.23 %) in *T. putitora*. Crude protein content was 73.01, 70.24, 68.53, 56.45 and 52.43 % in *L. pangusia, L. dero, L. dyocheilus, S. rechardsonii* and *T. putitora*, respectively, while, crude fat content in corresponding species was 32.53, 24.19, 19.25, 18.42 and 12.66 % and the variation among species was significant (P<0.05). Muscle of L. dero contained more crude fat (32.53 %) than that of *L. pangusia* (24.12 %) and *L. dyocheilus* (12.66 %).

Ackman (1990) and Mazumder and Basu (2009) reported that *Hilsa*, a high fat fish, had higher consumer preference. Therefore, *S. rechardsonii* and *L. dero* may be the most preferred fishes in view of their higher protein and crude fat contents that may be beneficial for the health of consumers as fish fat is beneficial for arthritis, psoriasis, asthma and cardiovascular problems besides improving the immune system and blood circulation and reducing the risk of and some skin condition (Berg et al. 2000).

The mineral composition of different species of fish has been presented in Table 2. Sodium content was highest in both *T. putitora* and *S. rechardsonii* while potassium and calcium contents were highest in *S. rechardsonii*. Potassium was lowest in *L. pangusia* while, calcium and sodium was lowest in *L. dyocheilus* and variations among species were significant (P<0.05). Potassium content was significantly higher in *L. dero, T. putitora* and *S. rechardsonii* than in other species. A marked relationship between sodium and calcium was also noticed. Calcium content was directly proportional to the sodium content of fishes (Figure 1).

Highest iron content was in *L. dero* while zinc and selenium in *T. putitora* and magnesium in *S. richardsonii*. Lowest contents of iron and zinc were in *L. pangusia* and those of magnesium and selenium in *L. dyocheilus*. The levels of micro mineral contents fluctuated (Figure 2) also varied significantly (P<0.05) among species.

These results indicated that hill stream fishes are rich sources of macro- and micro-minerals. The results corroborated with those of Nurullah et al. (2003), who reported fluctuation in mineral contents in selected indigenous fish species of Bangladesh. Ekpo and Ibok (1999) and Famole et al. (2007) concluded that any fish species could be a good source of minerals when it contains an appreciable concentration of micro minerals.

It is concluded that the hill stream fishes are rich in protein, fat and minerals and their consumption in sufficient quantities can play a vital role in human nutrition.

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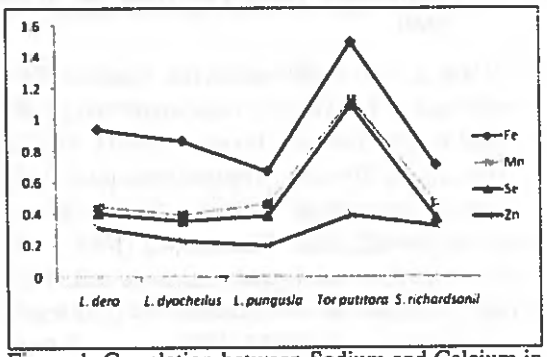

Figure 1. Co-relation between Sodium and Calcium in the five hill stream fishes.

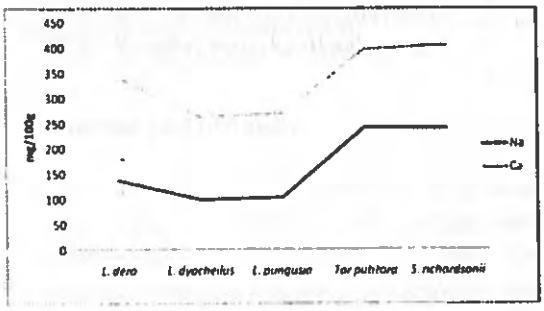

Figure 2. Micro-mineral variations among the hill stream fishes.

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SPECIES SPECIFICITY OF CHOCOLATE MAHSEER (*NEOLISSOCHILUS HEXAGONOLEPIS*) AND MALAYSIAN MAHSEER (*NEOLISSOCHILUS SOROIDES*)

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Ultrastructure of tip of the barbell and arrangement of scales were studied for differentiation of two similar looking Mahseer. The microstructural arrangement of scales of *Neolissochilus hexagonolepis*, below the focus, was smooth with the presence of spherical structures on the surface. In case of *Neolissochilus soroides* the micro-structural arrangement was coble shaped. The larval mark between the two species in respect to their size, arrangement, completeness and relative spacing of circuli showed differences. The surface scanning electron micrographs of tip of the barbell of *N. hexagonolepis* showed thick filamentous structures, while that of *N. soroides* was compartment like.

Key words: *Neolissochilus hexagonolepis*, *N. soroides*, ultrastructure, scale, barbel.

Introduction

The etymology of mahseer is highly divisive and so about the number of valid species. A number of workers have carried out detailed investigations with regard to the nomenclature of coldwater fish species in different aquatic systems of Indian subcontinent (McClelland, 1839; Talwar and Jhingran, 1991). Chocolate mahseer, *Neolissochilus hexagonolepis* is considered as a threatened species (McClelland 1939, Menon, 1994) . A similar and not easily, differentiable species is also available in Gombok river of Malaysia, *Neolissochilus soroides*.

Fish scales exhibit distinct pattern of dark and light bands corresponding to closely and widely spaced circuli. There are also numerous hidden details in their sculptural design that contribute effectively to fish identification and classification (Kaur and Dua, 2004). Circuli, radii, ctenii, lateral line canal and other structures associated with scales have been used for classification of fishes (Di Cenzo and Sellers, 1998: Hollander, 1986). More precision has been achieved through the application of scanning electron microscopy (SEM) on scale morphology to strengthen its utility in fish classification (DeLamater and Courtenay, 1974; Khemiri *et al.*, 2001). Utility of scales have been used in distinguishing the taxonomic groups (Kaur and Dua, 2004). The sense of touch seems to be highly developed in fishes and the barbels, whisker-like organs, are the most important lactive structure. The number of taste receptors in barbels is different in relation to the habitat and feeding habit of the fishes (Kapoor *et al.*, 1975; Takashi, 1982). The present communication is an attempt to throw light on the microstructural features engraved on the scales and barbels that can contribute to differentiation of the two species *N. hexagonolepis* and *N. soroides* by SEM.

Materials and methods

The specimens of *N. hexagonolepis* were collected from Umran river of Meghalaya, based on the methodology of Scott *et al.* (2000). The source of Malaysian mahseer was Gombok river, a tributary of Kelang river, Selangor, Malaysia. Both the specimens under study were similar in terms

of length and weight. The normal scales were removed from 2nd / 3rd row of scales above the lateral line. The tip of the barbels were also collected and used for analyzing. The scale and the barbels were fixed in 2.5% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer pH 7.2-7.4 for 4 hour, at 4 °C, washed in buffer overnight, post fixed in 1% osmium tetroxide for 1 hour and dehydrated through increasing concentrations of acetone. The dehydrated samples were dried in a critical point drier (Samdri Pvt, Tousimis) using acetone as the intermediate fluid and CO_2 as the transitional fluid. The samples were also dried with TMS technique (Dey *et al.*, 1989). The samples were then secured horizontally to brass stub (10 mm x 10 mm) with double coated adhesive tape connected via a patch of silver paint to ensure charge conduction. A conductive coating was applied to the sample using JFC 1100 (Jeol) ion sputter coater. A relatively low vacuum (10^{-3} tor) was established in the sputtering chamber and the "target" material used was gold. The preparations were examined with scanning electron microscope JSM-35CF (Jeol) using the secondary electron emission mode at an accelerating voltage of 15kv. The analysis of covariance (ANCOVA) was applied based on the methodology of Zar (1999) for comparison.

Results and discussion

SEM view of scales showed that the location of focus was different in the two species. The distinct larval mark was observed in the focus region of *N. hexagonolepis*, which was absent in *N. soroides*. However, the mark between the two species in respect of their size, arrangement, completeness and relative spacing of circuli showed differences. The ultrastructural arrangements were enlarged below the focus and compared. In case of chocolate mahseer, the smooth surface was observed with the presence of spherical structure on the surface. In case of Malaysian mahseer, the surface structure was not smooth and the microstructural arrangement was coble shaped (Table 1). The surface ultra structure through SEM of tip of the barbel of *N. hexagonolepis* was with thick filamentous structure. However, the arrangements had uniformity in their position from one end to the other end. In case of *N. soroides*, compartment like structures were observed on the surface. In higher magnification of the barbels of chocolate mahseer, lobe like structure was seen. However, the mosaic like structure with certain distinct boundaries was observed in case of ultra structure of Malaysian mahseer. Similar observations in case of surface architectures through scanning electron micrograph was reported in other fishes (Antoni and Hugo, 1997; Irina *et al.*, 2011). The results showed that there were differences in the scales of *N. hexagonolepis* and *N. soroides*. The size, completeness and spacing of circuli have shown some differences between the species. It was noted that the presence of larval mark and microstructure has already been worked out by earlier workers (Kaur and Dua, 2004; Tandon and Johal, 1993). The important point to be considered here is the presence and absence or the pattern of larval mark with the arrangement of circuli on the focus region that are the species specificity characters of the two species. The differences were also seen in the region below the focus regarding the size, thickness and arrangement of bony ridges (Fig. 1 and 2). The arrangement, shape and structure of the surface of the tip of the barbel clearly indicated some variation in case *N. hexagonolepis* and *N. soroides* (Fig. 3 and 4). The number of taste receptors present in the barbels are often different according to the feeding habit of the fish (Kapoor *et al.*, 1975). The two species of *N. soroides* and *N. hexagonolepis* were significantly different at 5% level of significance in

the regression line of total length on standard length (Table 2). However, no significant differences between the two species for the remaining characters were seen. This may be due to the taxonomical similarities. The summary statistics of the fitted models is presented in Table 2. The regression coefficient b of different variables (Y) on standard length (X) indicated that the rate of growth in respect to standard length was found highest in the case of total length in *N. soroides* than *N. hexagonolepis* and lowest in length of mouth cleft as shown in Table 2.

The regression equation for length-weight was fitted by 'least squares method' for each species. The slopes of the two fitted regression lines were further examined by ANCOVA and found that the two species differ significantly by (F=23.719; $p<0.001$) in length-weight. The length-weight equation for each species was fitted separately as follows. Log W=- 0.8 + 0.881 log L for *N. soroides* and Log W=- 4.547 + 2.747 log L for *N. hexagonolepis*. The study suggested the presence of species-specific characteristics engraved on the scales and barbels. Though not

Table 1. Important structural details considered in the fish scale through SEM and their comparison in two species of genus *Neolissochilus*

Character	*N. hexagonolepis*	*N. soroides*
Normal scale		
Shape	Hexagonal	Oblong
Size	Large	Medium
Focus: location	Central	Above center
Larval mark	Present	Absent
Region below focus (size/thickness/arrangement of bony ridges)	Numerous, closely spaced circuli Thick, rounded ridges	Few, widely spaced ridges Thick, coble shaped ridges
Barbels		
Shape	Needle	Needle
Size	Small	Small
Structure	Filamentous	Compartment
Arrangement	Compact linear arrangement	Nesting arrangement
Thickness	Thick	Thin
Feature	Lobular pattern with evenly spaced loops	Loosely packed zigzag loops with distinct boundaries

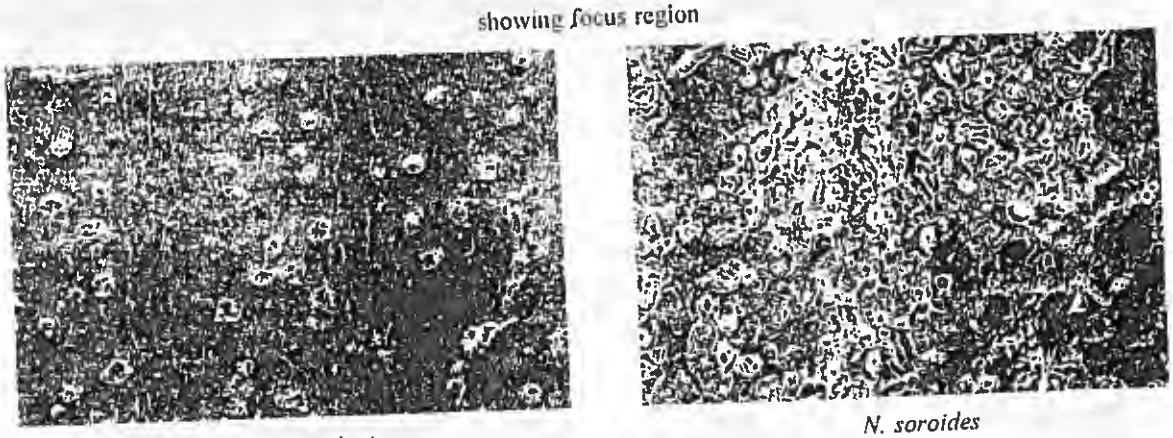
Table 2. Summary statistics of the fitted models

Regression	ANCOVA results for comparing the two fish species of *Neolissochilus*			Estimated parameters		
	-value	p-value	Comments	A		B
TL on SL	5.019	0.040	Significant	Species 1: -131.303 Species 2: -251.450		3.020 0.656
BD on SL	0.004	0.949	Not significant	14.916		0.106
BL on SL	0.173	0.683	Not significant	37.466		0.203
HL on SL	0.158	0.697	Not significant	17.147		0.056
ML on SL	0.017	0.899	Not significant	7.014		0.015

TL=Total length, BD= Body depth, BL=Body length, HL=Head length, ML=Mouth length and SL= Standard length

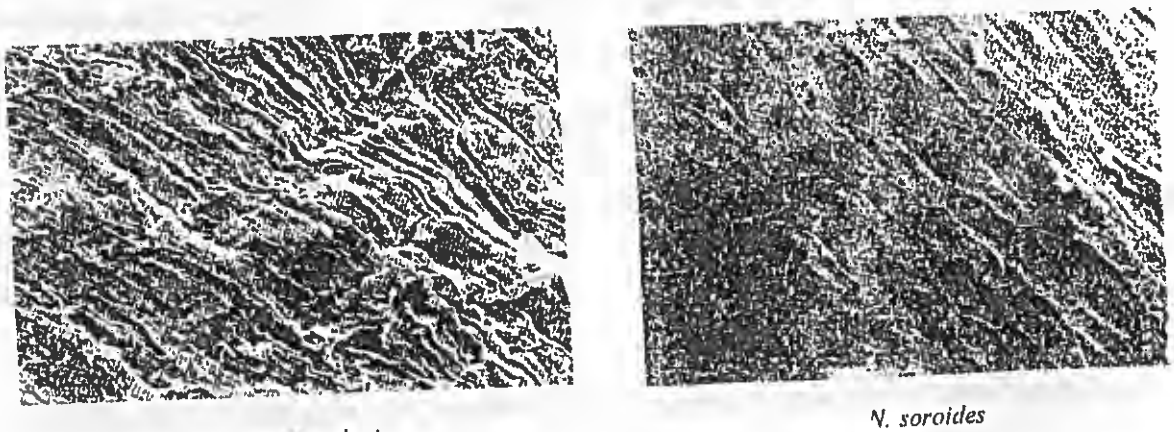
N. hexagonolepis *soroides*

Fig. 1. Scanning electron micrographs of scale showing focus region



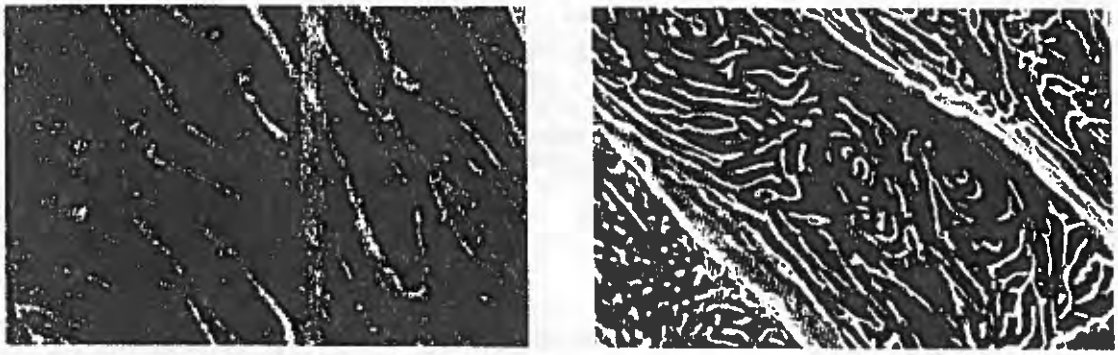
N. hexagonolepis *N. soroides*

Fig. 2. Scanning electron micrographs of scale showing region below focus



N. hexagonolepis *N. soroides*

Fig. 3. Scanning electron micrographs showing tips of barbel region



N. hexagonolepis *N. soroides*

Fig. 4. Scanning electron micrograph showing enlarge view of barbel

much work has been done-ultrastructural level through scanning electron microscopy for identifying the species specific characteristics of fish, microstructural specification could be an important criteria for identifying fish species.

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Changes in Haematological parameters of Common carp *(Cyprinus carpio)* and Rainbow trout (*Oncorhynchus mykiss)*, infected with *Saprolegnia* spp.

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Key words: Haematological parameters, *Cyprinus carpio, Oncorhynchus mykiss, Saprolegnia*

Abstract

Haematological parameters of *Cyprinus carpio* and *Oncorhynchus mykiss*, infected with *Saprolegnia* spp. were studied. All parameters, except total leucocyte count (TLC), decreased in response to infection. A significant decrease was observed in haemoglobin (Hb), total erythrocyte count (TEC), haematocrit (Hct). Among the parameters, only TLC had a significant increase in the infected fish. Results show that saprolegniosis caused anaemia and immunosuppression. The present study was conducted to investigate physiological impairment in *Saprolegnia* infected *C. carpio* and *O. mykiss*.

Introduction

Fish are important source of food and recreation, and are a key unit in many natural food webs. *Cyprinus carpio* and *Oncorhynchus mykiss* are important coldwater fish species of commercial interest in many countries. Some changes such as stress and immunosuppression allow infection to develop quickly (Frans et al., 2008; Pickering, 1994). *Saprolegnia* spp. generally termed water moulds, cause saprolegniosis, a fungal disease in cold water fish appears as cotton-like circular, crescent-shaped, or whorled pattern on the surface of the animal, and not only affects the animal itself but also infects the eggs by penetration of the egg membrane (Willoughby, 1994). The fish infected with *Saprolegnia* are easily recognised by the cotton-like white to greyish patches on the skin and gills visible to the naked eye (Stueland et al., 2005). The infection progresses very quickly and often results in mortality and can cause huge losses of both fish and ova (Stueland et al., 2005; Howe and Stehly, 1998). The study of infected fish is essential to make efforts to determine the efficacy of various antifungal treatments (Howe and Stehly, 1998), and to understand the mode of action of fungal infection and the resistance capability of fish.

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Haematology provides an index of physiological status of fish (Shah and Altindag, 2004) and its study is important for the development of health management for the rapidly growing aquaculture industry. Generally haematological examination was carried out for the purpose to examine the effect of toxic substances on the fish, to evaluate the condition of the fish, to evaluate the non-specific resistance of different fish breeds, strains and of the brood fish, to assess the suitability of feeds and feed mixture pellets and to evaluate the effect of stress situations etc. In the present investigation, alterations in important haematological parameters such as total erythrocyte count (TEC), total leucocyte count (TLC), haemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) due to fungal infection, were evaluated as a diagnostic tool for the fungus infection in the coldwater fish.

Materials and Methods

Haematological parameters were measured for 50 healthy and 50 fungal infected *C. carpio* and *O. mykiss*. Adult common carp (*C. carpio*) were collected from Directorate of Coldwater Fisheries Research (DCFR) farm, Bhimtal and *O. mykiss* were collected from state trout farm, Bairangna (Uttarakhand). Triplicates were maintained for each sample. They were fed at a balanced ration and provided with continuous aeration. Blood samples were taken at first, second and third week after appearance of infection. Sampling was also done at the same time from control group. Blood samples were collected from caudal vain using sterilized disposable 2 ml syringe. TEC and TLC were determined with haemocytometer crystalline chamber using diluting fluids. For estimating haemoglobin Sahli's haemoglobinometer was used. PCV was estimated by using microhematocrit. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to Reddy and Bashamohideen (1989). Statistical analysis was done by using one-way ANOVA at 5% level of significance.

Results

The quantitative changes of haematological parameters like, TEC, TLC, Hb, PCV, MCH, MCHC, MCV in *C. carpio* and *O. mykiss*, both in control (non infected) and fungal infected samples after first, second and third week of infection are given in Table – 1 and Table - 2.

The haematological analysis revealed a significant reduction in TEC, Hb, PCV, MCH, and MCV except TLC, in fungal infected fishes in comparison with non infected fish which was significantly high in fungal infected samples as compared to non infected one. Analyzed data on one-way ANOVA at 5% level of significance reflected that the resulting values of haematological parameters of *C. carpio* and *O. mykiss* are significantly different from the control due to the infection of *saprolegnia spp.*

Discussion

To investigate the fish blood factors and their changes, the normal rate of these factors must be initially measured in healthy fish. Quality and quantity of leukocyte cells are generally used in the determination of immune reactions and diseases (Cagirgan, 1990). It is known that leukocyte cells are normally lower in healthy fishes and could be used as a significant indicator for infectious diseases. All the blood parameters of control group of *O. mykiss* were not very different from the values reported by Atamanalp et al. (2008) for the control group of same species.

In the present study, the increases in WBC counts in infected samples were accepted as a response of cellular immune system to fungal infection. Palikova and Navratil (2001) concluded that immune system of fish displays similar responses to unfavorable conditions. Similar findings were highlighted by Jamalzadeh et al. (2009) in Caspian Salmon (*Salmo trutta caspius*) infected with *saprolegnia*. WBCs are important cells in the immune system, because of their main defensive function. Due to the infection of fungus

the WBC counts enhanced. It indicates the fish can develop a defensive mechanism to overcome the stress caused due to infection.

The lower value of TEC in fungal infected *C. carpio* and *O. mykiss* was in accordance with Caspian Salmon (*Salmo trutta caspius*)in the study of Jamalzadeh et al. (2009). The reduction of RBC can be caused due to unavailability of sufficient oxygen due to fungal infestation on gills which in turn leads to enhanced destruction of RBC or decrease in rate of formation of RBC due to non availability of Hb content in cellular medium (Chen et al., 2004).

The significance of the PCV observation is for knowing the effect of stressors and the health of the fish and for determining the oxygen carrying capacity of the blood in fishes.

The MCV, MCH and MCHC values are completely dependent on the level of PCV, TEC and haemoglobin concentration of the blood sample. In the present study, the observed data revealed that the PCV, RBC and hemoglobin concentration remain altered in the fungal infected fish. Therefore, the values of MCV, MCH and MCHC remain in lower side in the fungal infected fish which would be a useful diagnostic tool for saprolegniosis infection in the cultivable cold water fish species like common carp and rainbow trout.

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Tables

Table-1:Changes in Haematological parameters (means±SD) of *Cyprinus carpio* due to saprolegnia infection

Parameter	*Control*	*Infected group (1st week)*	*Infected group (2nd week)*	*Infected group (3rd week)*
Hb (gm %)	5.8±0.24	4.9±0.36*	4.5±0.42	4.0±0.38*
TEC (10^6/mm^3)	1.7±0.10	1.5±0.14	1.4±0.12	1.3±0.16*
TLC (10^3/mm^3)	16.2±2.1	17.8±1.8	19.6±1.6	20.4±1.9*
PCV (%)	31.70 ± 1.85	27.55 ± 1.85	25.53 ± 1.40	23.04±1.85
MCV(μm^3)	186.47±21.2	183.33±19.4	182.14±20.2	176.92±21.7
MCH(pg)	34.11±3.7	32.66±3.1	32.14±3.6	30.76±3.1
MCHC (%)	18.29±1.7	17.78±1.6	17.62±1.4	17.36±1.8

*=p<0.05, n=50

Table-2:Changes in Haematological parameters (means±SD) of *Oncorhynchus mykiss* due to saprolegnia infection

Parameter	*Control*	*Infected group (1st week)*	*Infected group (2nd week)*	*Infected group (3rd week)*
Hb (gm %)	6.4±0.47	6.1±0.23	5.8±0.32	5.5±0.21*
TEC (10^6/mm^3)	0.78±0.11	0.76±0.17	0.73±0.13	0.70±0.14*
TLC (10^3/mm^3)	73±7.2	75±7.6	78±7.1	82±7.9*
PCV (%)	44.00±4.3	42.00±3.9	40.00±4.1	38.00±3.8
MCV(μm^3)	564.10±46.2	552.63±49.5	547.94±44.3	542.85±48.9
MCH(pg)	82.05±8.6	80.26±8.1	79.45±7.5	78.57±7.6
MCHC (%)	14.54±1.2	14.52±1.6	14.50±1.4	14.47±1.3

*=p<0.05, n=50

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Application of multivariate statistical techniques for water quality characterization of Sarda Sagar Reservoir, India

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ABSTRACT

Multivariate statistical techniques were applied to water quality dataset collected from Sarda Sagar Reservoir. The results revealed the usefulness of multivariate techniques for evaluation of input water sources and interpretation of large complex water quality dataset for effective management of water resources. The largest source of variation (25%) is contributed from physical water quality parameters. Additional inputs come from the second factor accounting for about 15%. The third factor, accounting for 14% is associated with wastewater discharges and the fourth factor accounting for 12%. Finally, it suggests that a fraction of wastewater discharge is the only primary source of variations in this reservoir and there is no major threat of pollution. Cluster analysis showed that number of sampling sites as well as the sampling frequency could be consolidated.

Keywords: Multivariate techniques, Physico-chemical parameters, Sarda Sagar Reservoir, Wastewater

Introduction

Sarda Sagar Reservoir, extend from Pilibhit to Udhamsingh Nagar districts in Tarai region of India, located between 28° 40' to 28° 53' N and 80° 2' to 80° 12' E (Fig. 1). The climate is tropical, which is influenced by south-west monsoon. It is well known that physico-chemical parameters of water influence the biology of fish as well as the productivity of the reservoir. The physical and chemical characteristics of the soil and water play an instrumental role in determining the structure and composition of biotic communities of a reservoir. These characters of soil and water are influenced by climatic, morphometric and geographic factors because the water column is always in dynamic equilibrium with the soil bed and other environmental factors. These factors also provide the essential source of energy and affect the circulation of heat and nutrients (Lewis Jr., 1984 and 1987; Toth and Padisak, 1986; Jhingran, 1988: Sugunan, 1995). Other than irrigation, Sarda Sagar Reservoir is important from the fishery point of view, which harbors a number of fishes such as major carps, minor carps, catfishes and a large population of weed fishes. The fish production for the year 2006-07 was estimated at 179.3 t. There have been several studies on fish fauna of Sarda Sagar Reservoir (Motwani and Saigal, 1974; Sinha and Sharma, 2003; Kumar, 2009). However, there is paucity of information on the water quality parameters of this reservoir. Keeping in view, the importance and lack of information on physico-chemical parameters of this reservoir, an attempt has been made to characterize various water quality parameters of Sarda Sagar using multivariate statistical techniques in the present

Fig. 1. Location map of Sarda Sagar Reservoir

study. In view of the spatial and temporal variations in hydrochemistry of any waterbody, regular monitoring programmes are required for reliable estimates of water quality. This generally results in a huge and complex data matrix comprised of a large number of physico-chemical parameters (Chapman, 1992), which are often difficult to interpret for drawing meaningful conclusions (Dixon and Chiswell, 1996). The multivariate statistical techniques can be appropriately used for meaningful data reduction and interpretation of multi-constituent chemical and physical measurements (Massart *et al.*, 1988). The statistical techniques such as factor analysis and cluster analysis are most widely used in analysis of water-quality data for drawing meaningful conclusions (Vega *et al.*, 1998). Factor analysis is normally used to understand the correlation structure of collected data and to identify the most important factors contributing to the data structure (Padro *et al.*, 1993). Further, cluster analysis is used to classify entities with similar properties, in which a large number of heterogeneous entities are divided into a smaller number of homogenous groups on the basis of their correlation structure (Hartigan, 1975). These analyses will help to identify a method, which accurately represents the system and reduces the monitoring and assessment requirements of this reservoir

Materials and methods

Data collection

Sixteen sampling stations across the reservoir at a difference of one-minute interval were identified. Satellite image of IRS LISS III 1C for November 2006 and Global Positioning System were used to locate the sampling station for collecting water samples. The sampling stations are shown in Fig. 2. Sampling was carried out on monthly basis for the period from December 2006 to November 2007. Water quality parameters were studied using standard methods (AOAC, 1999; APHA, 1985). The set of output data has been analyzed using SPSS 12.0 version.

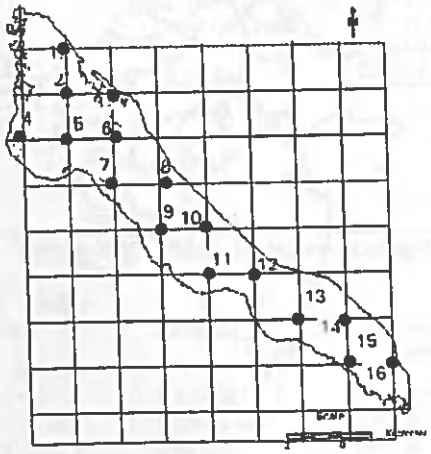

Fig. 2. Sampling stations (1-16)

Checking for adequacy of the data

Kaiser-Meyer-Olkin (KMO) sampling adequacy test and Bartlett's test of sphericity (homogeneity of variance) were applied for checking the adequacy of data. KMO sampling adequacy test less than 0.5 is probably not amenable to useful factor analysis (Kaiser, 1974).

Factor analysis

Factor analysis involves determination of adequacy of the data and estimation of the eigenvalues and factor loadings. Lower eigenvalues may contribute little to the explanatory capability of the data; only the first few factors are generally needed to account for much of the parameter variability. In this study, factor extraction is performed using the method of principal axis factoring. The most widely accepted method for deciding the number of factors to use, is the Kaiser criterion (Kaiser, 1960), which retains only those factors with eigenvalues >1. Factor loadings will be used to measure the correlation between variables and factors, the squared factor loading being the variance explained by that factor. Factor rotation is also used to facilitate interpretation by providing simpler factor structure.

Cluster analysis

Cluster analysis comprises of a series of multivariate methods, which are used to find true groups of data. In clustering, the objects are grouped such that similar objects fall into the same class (Danielsson *et al.*, 1999). Hierarchical cluster analysis is the most commonly used technique. This method joins the most similar observations, and then successively the next most similar observations. The levels of similarity at which observations are merged are used to construct a dendrogram. Some measures of similarity must be computed between every pair of objects.

In the present study, Euclidian distance, d_{ij} was used:

$$d_{ij} = \sqrt{\sum_{k=1}^{m} (X_{ik} - X_{jk})^2}$$

where X_{ik} denotes the k^{th} variable measured on object i and X_{jk} is the k^{th} variable measured on object j. A low distance shows that the two objects are similar whereas a large distance indicates dissimilarity.

Results and discussion

Factor analysis using a principal axis factoring of extraction method and varimax rotation on physico-chemical parameters of the Sarda Sagar Reservoir has been carried out. Also, correlation matrix is chosen because the

covariance method has problems when the variables are measured on widely different scales. The Kaiser-Meyer-Olkin measure of sampling adequacy is 0.534, indicating that the present data is suitable for factor analysis. However, Bartlett's test of sphericity is highly significant ($p<0.001$), indicating sufficient correlation between the variables to proceed with the analysis (Table 1). All the extracted communalities are reasonably high (>0.5) and acceptable, except slightly lower values of free carbon dioxide, dissolved oxygen and phosphorous (Table 2).

Table 1. KMO and Bartlett's Test

Kaiser-Meyer-Olkin measure of sampling adequacy		0.534
Bartlett's test of sphericity		
	Approx. chi-Square	1497.858
	Degrees of freedom	66
	Significance	0.000

Table 2. Communalities

Parameters	Initial	Extraction
Temperature	0.872	0.874
Transparency	0.884	0.810
Conductivity	0.909	0.915
Total dissolved solids	0.880	0.829
pH	0.575	0.517
Free carbon dioxide	0.683	0.390
Dissolved oxygen	0.427	0.414
Alkalinity	0.529	0.749
Hardness	0.634	0.710
Nitrate	0.626	0.663
Phosphorous	0.445	0.361
Silicate	0.667	0.703

Extraction method: Principal Axis Factoring

The first four factors in the initial solution have eigenvalues >1 that accounts for about 66% of observed variation in water quality observations (Table 3). According to Kaiser criterion, only the first four factors should be used because subsequent eigenvalues are <1.

A factor loading close to ±1 indicates a strong correlation between a variable and the factor, while a loading close to zero indicates weak correlation. The factors are rotated with varimax rotation, which is a standard rotation method (Kaiser, 1958). In the present study, only those absolute factor loadings >0.6 are considered for interpretation purposes.

Hierarchical clustering using Ward's method with Euclidian distance measure has been employed to evaluate whether sampling frequency can be reduced or not. The

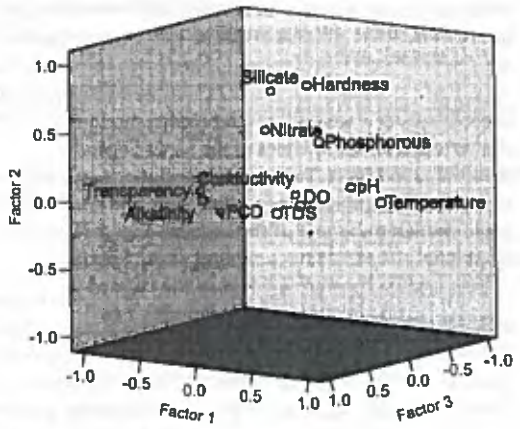

Fig. 3. Factor Plot in Rotated factor space

Table 3. Total variance explained

Factor	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %	Total	% of Variance	Cumulative %
1	3.913	32.610	32.610	3.648	30.403	30.403	3.029	25.241	25.241
2	2.248	18.733	51.343	1.903	15.862	46.265	1.766	14.713	39.953
3	1.765	14.706	66.048	1.482	12.349	58.613	1.747	14.555	54.508
4	1.259	10.489	76.537	0.901	7.512	66.125	1.394	11.617	66.125
5	0.755	6.292	82.829						
6	0.580	4.829	87.658						
7	0.536	4.470	92.127						
8	0.312	2.596	94.724						
9	0.296	2.469	97.193						
10	0.194	1.617	98.810						
11	0.106	0.883	99.693						
12	0.037	0.307	100.000						

Extraction method: Principal Axis Factoring.

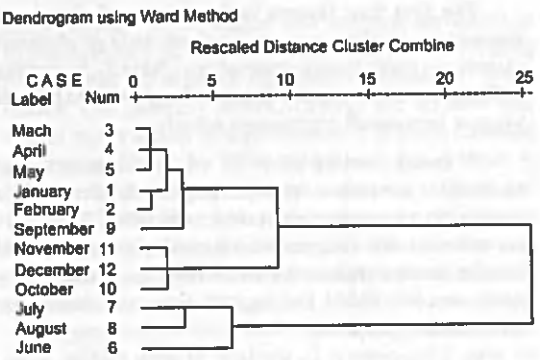

Fig. 4. Dendrogram showing sampling time clusters on Sarda Sagar Reservoir

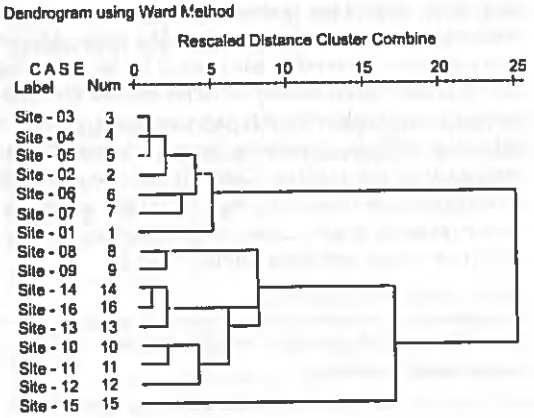

Fig. 5. Dendrogram showing sampling site clusters on Sarda Sagar Reservoir

results of cluster analysis for water quality monitoring data from different sampling times of the Sarda Sagar Reservoir are depicted in Fig. 4. Two major associations are evident – the first association included June, July and August; the second included the rest of the months. Fig. 5 shows the cluster analysis result on sampling sites. Similarly, two possible associations are evident in the case of sampling sites : the first association is between sites 1 to 7, which is most significant and a second group of associations is among the sites 8 to 16.

An interpretation of the rotated four factors in Table 4 is made by examining the factors noting the relationship to the original variables. Further, it is justified with the factor loadings plot shown in Fig. 3. The first factor explains about 25% of the total variance (Table 3) with strong positive loadings of the water temperature and strong negative loading of transparency, and moderate positive loading of pH. Thus, it basically represents the physical parameters group. Temperature affects the physical, chemical and biological processes in water bodies, and therefore, has an important role in determining various water quality variables. Thus, water temperature may be considered as an indicator variable of this factor. The second factor explains about 15% of the total variance (Table 3) that has strong positive loadings of hardness and silicate. Silicate may be considered as a key parameter of this factor as it plays a vital role in diatom production, which is the most important planktonic community of any aquatic body. Silica is found in the water body mainly in the form of orthosilicate at the normal pH (Sharma, 2000). The third factor incorporates those water quality variables that are characteristics of wastewater discharges as it explains about 14% of the total variance (Table 3) with strong positive

Table 4. Rotated Factor Matrix (a)

Parameters	Factor			
	1	2	3	4
Temperature	0.869	0.030	0.008	-0.343
Transparency	-0.863	-0.079	-0.192	0.151
pH	0.648	0.133	0.059	0.275
Free carbon dioxide	-0.542	-0.173	0.016	0.257
Hardness	0.168	0.820	-0.076	-0.065
Silicate	0.047	0.804	0.206	0.106
Phosphorous	0.362	0.434	0.050	0.195
Alkalinity	-0.123	0.075	0.832	-0.190
Total dissolved solids	0.442	0.016	0.713	0.354
Conductivity	0.615	0.078	0.657	0.315
Dissolved oxygen	0.058	0.003	-0.076	-0.636
Nitrate	-0.247	0.437	-0.137	0.627

Extraction method: Principal Axis Factoring. Rotation Method: Varimax with Kaiser Normalization
a Rotation converged in 8 iterations

loadings of alkalinity, moderate positive loadings of total dissolved solids and conductivity. Total dissolved solids may be considered as an indicator variable of this factor. Ecological degradation of reservoirs is reported on many occasions from time to time. In case of Sarda Sagar, the pollution load carried by the upstream rivers such as Lohavati and Goriganga, which pass through major townships, might have accumulated in this reservoir. The fourth factor has moderate negative loading of dissolved oxygen and moderate positive loading of nitrate. This factor explains about 12% of the total variance. Dissolved oxygen content, which plays a vital role in supporting aquatic life in water bodies, may be considered as the key parameter of this factor.

Results of the cluster analysis revealed that different seasons like, wet and dry seasons are affecting the water quality of this reservoir. The rainy season (June-August) is distinctly different from the remaining months. The sampling sites selected from upper portion of the reservoir, where excessive siltation occurs (sampling sites 1-7) have more or less similar water quality characteristics while the sampling sites chosen close to lower portion of the reservoir (sampling sites 8-16) represent the next cluster.

The results indicate that there is no major threat of pollution in this reservoir, however a small quantum of wastewater discharge is being accounted. Further, factor analysis is able to identify significant sources of water quality inputs to Sarda Sagar. The first four factors account for 66% of the total water quality variation. The largest source of variation (25%) appeared to be associated with physical parameters group. Additional inputs from the second factor account for about 15%. The third source of variation (14%) appeared to be associated with wastewater discharges; and the fourth factor, accounted for 12%. A monitoring programme could use a smaller set of variables to identify times for intensive sampling; water temperature as an indicator of physical parameters group; silicate as a key parameter of second factor, total dissolved solids as an indicator of wastewater discharges, and dissolved oxygen as an indicator of the fourth factor. Moreover, cluster analysis results showed that the number of sampling sites and the sampling months could be reduced. This reduced set of parameters could be monitored over larger areas within the watershed to provide more detailed spatial information about sources and processes.

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Prediction of Fish Growth Rate and Food Availability in the Himalayan Waterbodies by Estimation of RNA/DNA Ratios

G K Sivaraman[1], A Barat[2], S Ali[3] and P C Mahanta[4]

Recent biotechnological methods have made it possible to measure the real-time growth rate of fishes in response to changes in environmental conditions, as compared to the traditional methods. The accurate isolation and quantification from a tiny amount of tissue's DNA, RNA and protein concentration would give the physiological status of fishes in their natural environment. The DNA content of tissue/cell remains stable and provides an index of cell number, whereas RNA content changes in response to transcription-dependent protein synthesis and is directly correlated with the growth rate of fish. The RNA/DNA ratio of tissue has proven to be a reliable estimator of real-time growth and its nutritional status of fish larvae and juvenile fish and it is an indicator of the protein synthesizing potential of a cell.

Keywords: Fish growth, DNA, RNA, R/D ratio, Nutritional status

Introduction

Fisheries sector is developing at a faster rate and plays an important role in terms of food security as a cheap source of protein-rich food. India is 4th among fish producing countries and 2nd in inland fish production in the world (FAO, 2009). The report has reckoned India's farmed fish output (2004) at 2.47 million tons, and the value of total reared fisheries output is assessed at $2.93 mn. The annual per capita consumption of fish in India has steadily risen from 2.9 kg prior in 1981 to 4.7 kg in 2000 (Rath *et al.*, 2011). Vast water resources are gifted with diversified and remarkable type of Ichthyofauna and have the capability to contribute more significant to overall aquaculture production. Inland fisheries resources comprise rivers, streams, floodplains, estuaries, mangroves, upland lakes, reservoirs and ponds. Their natural habitats provide important nursery habitats during the larval and juvenile stages for a variety of fish species. Recent degradation of water quality seriously threatens the value of these habitats as well as food availability in their natural resources (Soe, 2011). In addition, physicochemical factors (temperature, altitude, water current, pH, phytoplankton, zooplankton and dissolved oxygen) have substantial effects on growth rates of fry and fingerlings (Sehgal, 1988).

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Himalayan Fishery Resources

India is blessed with abundant fish genetic resources consisting of 756 freshwater fish species and 258 fish species in the Himalayan natural waterbodies (Sehgal, 1987). The Himalayan mountains are characterized by a very low level of human development, with full exploitation or overexploitation of the natural resources. Concern over declining of fish harvests and an obvious reduction in biodiversity of fish species has led to a more holistic approach to fisheries management and research (Petr and Swar, 2002).

Prediction of Fish Growth and Food Availability

Initially researchers used a variety of morphometric, histological and biochemical indices to measure recent growth and nutritional condition of fish species (Buckley, 1979; Yin and Blaxter, 1986; Clemmesen, 1987 and 1988; Bailey *et al.*, 1995; Rooker and Holt, 1996; and Chicharo 1998). Richard *et al.* (1991) utilized these techniques for the assessment of the nutritional condition of field-caught larvae which would help in explaining larval survival and year-class fluctuations. The most commonly used indices are based on nucleic acids, viz., DNA content, protein:DNA, RNA concentration (μg/mg tissue), RNA:mg tissue, ratio of RNA to DNA (R/D) and RNA:protein ratios. RNA, which comprises much of the cell's machinery for protein biosynthesis and their numbers and activity, changes in response to the demand for protein synthesis and fluctuates in response to food availability in the natural food habitats (Islam *et al.*, 2006). DNA content is an index of cell number or biomass, and remains relatively constant even during periods of starvation and can serve to normalize the measured RNA. An increase or decrease in the R:D or RNA:mg tissue ratios at a given temperature would indicate a concomitant change in protein synthesis and growth rate. Relative changes in the R:D ratio have been used to describe the nutritional condition of larvae (Buckley *et al.*, 1999). When properly calibrated with laboratory experiments, the R:D ratio can also be used to estimate instantaneous growth rates (Buckley, 1980; Robinson and Ware, 1988; and Buckley *et al.*, 1999). Growth rate estimation of field- caught fish is a powerful tool for evaluating the survival potential of an individual and for identifying environmental variables which may affect recruitment success (Sivaraman *et al.*, 2009). One advantage of a nucleic acid-based growth estimate is rapid response to time; the R:D ratio in post yolk-sac larvae can reflect changes in growth rates and nutritional condition over a period of time. The physiological basis for these indices is that larval fish grows rapidly with increase in their body muscle mass through an increase in protein synthesis. In this direction, Directorate of Coldwater Fisheries Research has initiated the research on coldwater fish species in the Kumaon region, especially for the genetically important endangered fish species.

Recent Developments

Recent advances in molecular techniques, particularly the fluorometric quantification of nucleic acids, has made it possible to reliably measure near real-time growth rates of fishes in response to short-term changes in environmental conditions (Lemmens, 1995;

Kyle *et al.*, 2003; and Sivaraman *et al.*, 2009). The R/D has been used in several studies to estimate recent growth in fishes. While the DNA content of fish tissue remains relatively stable and provides an index of cell number, RNA content changes in response to transcription-dependent protein synthesis that is directly correlated with ribosomal activity, and thus growth rate. Three different methods, viz., spectrophotometric, fluorometric and High Performance Liquid Chromatography (HPLC) methods, for quantifying nucleic acids can be used for the precision of estimation (Dell'Anno *et al.*, 1998). More recent analytical protocols are based on the enhanced fluorescence of dyes that specifically bind to nucleic acids. The main advantage of these newer methods is a substantial increase in sensitivity and sample throughput compared with the UV spectrophotometric technique. DNA concentrations are measured spectrophotometrically and by HPLC. The RNA concentrations can be determined by fluorometric and spectrophotometric methods. However, the HPLC method provides independent accurate assessments of RNA and DNA concentrations (Dell'Anno *et al.*, 1998).

Conclusion

The fish scales and fins represent a DNA source as suitable as other tissues and offer an alternative technique, cost-effective and suitable alternative to conventional DNA extraction procedures, which is minimum lethal to fish while sampling for molecular genetic analysis purposes. The ratio of tissue R/D has proven to be a reliable estimator of recent growth and nutritional condition of larval and juvenile fish. The amount of RNA in a cell varies in proportion to protein synthesis, whereas DNA concentrations remain fairly constant even during starvation. Thus R/D is an indicator of the protein-synthesizing potential of a cell. The three procedures have common advantages such as the extraction time is relatively short, allowing the processing of the large number of samples usually collected in field studies, and quantification of DNA and RNA in the same subsamples is possible. ❄

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Successful Cross-Amplification of Few Microsatellite Loci Isolated from *Tor tambroides* for Indian Snow Trout, *Schizothorax richardsonii* (Gray, 1832) (Family: Cyprinidae)

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Abstract: Indian snow trout (*Schizothorax richardsonii*) is an important coldwater fish. A number of 8 microsatellite loci from a mahseer (*Tor tambroides*) were used to study their utility for cross species amplification in *S. richardsonii*. Five of them were successfully optimized through PCR. High levels of heterozygosity (Ho = 0.42-0.95) with allelic numbers on average 3.96 were obtained for these five loci. The present data confirmed the transferability of some heterologous microsatellite loci from one species to another. These five sets of markers would be useful to investigate a fine scale population genetic analysis in *S. richardsonii*.

Key words: *Schizothorax richardsonii* · Indian Snow Trout · Microsatellite Loci · PCR · Cross Species Amplification

INTRODUCTION

Indian snow trout (*Schizothorax richardsonii*) is widely distributed in Himalayan and sub Himalayan streams, rivers along Jammu and Kashmir, Himachal Pradesh, Assam, Sikkim, Bhutan, Nepal, Pakistan and Afghanistan [1]. This species has been considered as a valuable source of fish protein for the Hill community. In recent years, the natural population of the species in the wild resources has been dwindled considerably due to several anthropogenic activities, such as impoundment effect due to dam construction, indiscriminate killing, introduction of exotic fish etc [2]. The species follows allometric growth [3]. Although the species *S. richardsonii* has not yet been included in IUCN list either as an endangered or a threatened one, it's high time that efforts are made to increase the population size. Therefore, breeding and culture of this species was tried at the Directorate of Coldwater Fisheries Research (DCFR) farm, Champawat, Uttarakhand, by collecting the brooders from wild sources. Further refinement of these and to improve growth rate, a genetic breeding program depending on genetic variability studies using molecular markers is needed. A thorough literature survey revealed that there were few partial cytochrome b gene sequences (AF532075-AF532088) and two microsatellite sequences (FN568062 and FN568062) available for *S. richardsonii* in GenBank. However, there is lack of information related to genetic diversity, stock structure analysis using microsatellite markers in this species. Polymerase Chain Reaction (PCR) based microsatellite analysis offered the finest resolution now a day for studying genetic variation in several fish species [4, 5]. Microsatellites are simple DNA sequences that are repeated several times at various points in the organism's DNA. Such repeats are highly variable and can be used as a polymorphic marker. The occurrence of high degree of polymorphism and co-dominant inheritance has made them one of the most popular genetic markers for studies on genetic diversity, population genetic structure on fishes [6-9]. Microsatellites are tandem repeats of 1-6bp and developed from anonymous genomic sequences [5, 10]. But development of species-specific primers for PCR amplification of microsatellite loci is expensive and time consuming. It has been observed that primers developed to amplify markers in one species may amplify in related

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species as well [11, 12]. Thus, in the present paper it has been tried to study the level of transferability and validity of some microsatellite markers developed earlier by Nguyen *et al.* [13] from *Tor tambroides* under family Cyprinidae for further use in population structure analysis of *S. richardsonii.*

MATERIALS AND METHODS

Samples Collection: The fish Samples of were collected by cast net from five different rivers of Kumaon and Gharwal regions of Uttarakhand: Gola (Ranibag, n=8; 29°27'30"N, 79°28'45"E), Uttarvahini (Garampani, n=11; 29°27'30"N, 79°28'45"E), Chirapani (Champawat, n=8; 29°19'59"N, 80°6'0"E), Alaknanda, (Barangana, n=8; 30°26'03"N, 79°21'94"E) and Kosi (Katli, n=9; 29°35'50"N, 79°39'52" E). The sampling sites were selected to cover genetic variation on a wide geographical distribution range of the species. The Muscle samples were collected through dorsal part and preserved in 95% ethanol. All specimens were fixed in 10% formalin in the field as a voucher.

DNA Extraction and Pcr Amplification: Total genomic DNA was isolated from muscle tissue by using the standard phenol-chloroform extraction protocol described by Sambrook and Russel, [14]. Amplification of each DNA samples was performed in a 10µl reaction mixture containing 10x Taq assay PCR buffer A (100mM Tris, pH 9.0, 500mM Kcl, 15mM $MgCl_2$, 0.1% Gelatin) (Genei, India), 200µM of each dNTPs (Genei, India), 5pmol of each primer (Ocimum Biosolutions, India), 0.5U Taq DNA Polymerase (Genei, India) and 25ng of DNA. One negative control was performed for each set of amplifications. Amplifications were performed on ABI 9700 Thermal Cycler (ABI, USA) with the following parameters: 4 min of initial denaturation at 94°C followed by 35 cycles of 45s at 94°C, 45s at locus specific annealing temperatures (Table 1) and 60s at 72°C, ending with a final step of extension at 72°C for 7mins. After PCR, 5µl of formamide dye was added to each reaction. The samples were denatured at 95°C for 5min. and immediately placed on ice. Finally PCR products were separated through 6% denaturing polyacrylamide gel in 1xTBE buffer. Φ174 Hinf I digest (Fermentas, USA) was used as a size marker for the microsatellite alleles. Detection of alleles was carried out by silver staining [15]. The gel image was documented and analyzed in UV-Gel Documentation Unit (Alpha Imager 3400, Alpha Innotech Corporation, USA).

Statistical Analysis: Eight primers were selected (Tt1 B01, Tt1 C06, Tt1C10, Tt1 F02, Tt2 B02, Tt2 D01, Tt2 F04 and Tt2 F07) on the basis of the allelic richness reported by Nguyen *et al.* [12] for the present study on *S. richardsonii.* Out of eight primers five (Tt1 F02, Tt2 F04, Tt2 F07, Tt1 B01 and Tt1C10) could successfully amplified target fragments of the expected size. All five loci exhibited polymorphism in the individuals tested. We used Genetic Data Analysis Software GDA v 1.1 [16] to obtain number of alleles (A), observed heterozygosity (Ho), expected heterozygosity (He), to test for linkage disequilibrium and deviation from Hardy-Weinberg Equilibrium (HWE). The total number of alleles ranged from 3-4 with an average of 3.96 alleles per locus. However, number of alleles originally reported by Nguyen *et al.* [13] was in the range of 1-3. It was observed that all the eight primers mentioned above were annealed at 57°C [13], which were different in the present study Table 1. Observed and expected heterozygosity ranged from 0.42-0.95 and 0.70-0.74, respectively. The probability test did not detect any significant deviation in allele frequencies from that expected under ($P < 0.001$) Hardy-Weinberg equilibrium. A test for genetic differentiation was performed to test the hypothesis that the sample sets had genetic heterogeneity. The genetic heterogeneity was tested based on the genotype rather than on allele frequencies. The combined probability over all loci and the sample sets was found to be significant ($P > 0.0001$), indicating that sample sets differ significantly in their genotype frequencies.

RESULTS AND DISCUSSION

Primer sequences and the specific annealing temperature (Ta °C) in the resources species and *S. richardsonii* are given in Table 1. The optimum annealing temperatures to get the scorable bands in *S. richardsonii* differed from that reported for the resources species. Five out of the eight primer pairs tested yielded successful amplification in *S. richardsonii.* All primer pairs amplified only a single locus. It is evident from Table 1 that amplification success was higher when primers were from the resource species within the subfamily Cyprininae than Results suggested that certain sequences flanking tandem repeats are conserved within the subfamily Cyprininae and, to some extent, also between the subfamilies of Cyprinidae. The results of this studies demonstrate that the microsatellite marker sets published by Nguyen *et al.* [12] for *Tor tambroides* (Fam: Cyprinidae) produces quantifiable microsatellite

Table 1: Results of PCR amplifications using the microsatellite primer set developed by Nguyen *et al.* [13] on genomic DNA samples from Indian Snow Trout A, number of alleles observed; H_o, observed heterozygosity; H_e, expected heterozygosity; P, value of the exact test

Locus Name	Primer sequence (5'-3')	Acc. No.	Ann. temp	Repeat Sequence	Allele Size	A	H_o	H_e	P
Tt1 F02	F-CATGGACCAAATTACAAGGATTT R-AACCTGTGAGGGATGTCCAG	DQ778026	53°C	$(TG)_2TA(TG)_4TC(TG)_2$	240bp	4	0.706	0.734	0.077
Tt2 F04	F-ATGCCAGCTACAGGTCCAAT R-CGTGTGTATGATGCCACCTC	DQ778032	52°C	$(AC)_{11}$	150bp	4	0.865	0.702	0.271
Tt2F07	F-GAGACGACTCTAGTCGCTGACA R-GTGTGGCCAGTGTAGCTGAA	DQ778033	56°C	$(AG)_9$	153bp	4	0.950	0.747	0.092
Tt1B01	F-GAGGGGCATTTTGTTCTTGA R-GCTTCCCCTCATAAGCCTTC	DQ778019	56°C	$(AC)_8AT(AC)_7-(TC)_3$	245bp	3	0.425	0.714	0.066
Tt1C10	F-GCTGAAGCAGGTGAATCTGA R-TGATGCCTGTCAAACCTGTG	DQ778022	55°C	$(TG)_{13}$	188bp	4	0.670	0.742	0.170

fragments for the Indian Snow Trout, *S. richardsonii* (Fam: Cyprinidae) after standardizing the annealing temperature and optimizing other PCR profiles for each locus. Our results also suggest that PCR primers designed for microsatellite loci from *Tom tambroides* may be useful for genetic analysis for the present snow trout though the average number of alleles per locus is low which may be due to small sample size. The small sample size also affected the power of exact tests for conformations to Hardy-Weinberg expectations (HWE).

CONCLUSION

The development of microsatellite markers is a time consuming and highly expensive also. Hence, it was tried to use some available markers from public domain. The present study had shown the utility of five polymorphic microsatellite loci through cross-species amplification and exhibited considerable promise to evaluate stock structure, genetic variation, conservation genetics and molecular breeding of *S. richardsonii*. The information generated here using these novel microsatellite loci would be able to solve some issues, 1) potential transferability of some heterologous primers from one species to another, 2) population genetic studies of *S. richardsonii* may be initiated and 3) it may also reduce the time and cost for development of microsatellite markers.

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Length-Weight Relationship of Snow Trout (*Schizothorax richardsonii*) Based on Linear and Nonlinear Models from Hill Stream of Uttarakhand, India

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Abstract: The present study attempts to develop a comprehensive length weight relationship of snow trout (*Schizothorax richardsonii*) in the tributary of Kosi River from the Ratighat region of Uttarakhand, India. Both linear and non linear regression equations were obtained. Non-linear model fitted to the dataset has shown appropriateness, which follows allometric growth. The condition of the fish is found to be better. In length weight relationship of Snow trout, weight increases in length. Thus it is clear that these fishes maintain its shape throughout its life.

Key words: Nonlinear Model · Condition Factor · Length Weight Relationship

INTRODUCTION

According to Le Cren [1], knowledge of the length-weight relationship of a fish is essential, since various important biological aspects, viz., general well being of fish, appearance of first maturity, onset of spawning, etc., can be assessed with the help of condition factor, a derivative of this relationship. Moreover, the length-weight relationship (LWR) of fish is an important fishery management tool because they allow the estimation of the average weight of the fish of a given length group by establishing a mathematical relationship between the two [2]. As length and weight of fish are among the important morphometric characters, they can be used for the purpose of taxonomy and ultimately in fish stock assessment. In fisheries science, the condition factor or K-factor is used in order to compare the 'condition', 'fatness' or well being of fish and it is based on the hypothesis that heavier fish of a given length are in better condition [3]. Condition factor is also a useful index for the monitoring of feeding intensity, age and growth rates in fish [4]. Condition factor has been used as an index of growth and feeding intensity [5]. An extensive research on length-weight relationship of commercial freshwater fishes from different water bodies in India is already reported [6-8]. This study reports the LWR of *Schizothorax richardsonii* of Kosi River in Uttarakhand, India.

MATERIALS AND METHODS

The present study was carried out in *Schizothorax richardsonii* taken by cast net fisheries in the tributary of Kosi River from the Ratighat region (29°27.488'N, 79°28.812'E, Altitude- 1033 m asl) located in Kumaun hills of Uttarakhand, India. In this study, a total of 78 fish specimens comprises of *S. richardsonii* ranged from 70-177 mm in length and 4.7-30.0 gm in weight were studied for the length weight relationship. The species were identified by using the key provided by Jhingran [9]. The total length of the fish was measured from the tip of the anterior part of the snout to the end of caudal fin. Fish weight was measured after blot drying. Weighing was done with a tabletop weighing balance, to the nearest gram. The dataset has been analyzed by using SPSS 12.0 Version.

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The relationship between the length (L) and weight (W) of fish is expressed by following equation [10]:

$$W = aL^b \quad (1)$$

Where 'a' and 'b' are the parameters of the above non-linear model. Also, by taking logarithmic transformation on both sides of the above equation, we get the linearized model:

$$\text{Log } W = \log a + b \log L \quad (2)$$

Levenberg- Marquardt method [11] is the most widely used and reliable procedure for computing nonlinear least square estimates and thus used in the present study. However, ordinary least squares method is employed for fitting of the linearized model.

Moreover, summary statistics like R- square are also calculated.

$$R^2 = 1 - \frac{\sum_{t=1}^{n}\left(W_t - \hat{W}_t\right)^2}{\sum_{t=1}^{n}\left(W_t - \bar{W}\right)^2}$$

Where:

W_t = Observed fish weight (in gm);
$\hat{W}$ = Predicted fish weight (in gm);
$\bar{W}$ = Average fish weight (in gm);
n = Number of observations & t = 1, 2..., n.

A better model has the larger value of R-square [12]. A change in 'condition factor' or 'K-factor' or 'Ponderal index' has been calculated as follows [13, 14]:

$$K = 10^5 W / L^3$$

The relative condition factor (Kn) of samples was also calculated as suggested by Le Cren [1] and the formula is given below:

$$Kn = W / aL^b$$

RESULTS AND DISSCUSION

The two different forms of model (non-linear and linearized forms) are fitted in the length weight relationship analysis of *S. richardsonii*. The estimates of parameter and goodness of fit statistics are presented in Table 1. A better model has the larger value of R-square. The R- square value for nonlinear model is more as compared to its corresponding linearized model. From the above results, we conclude that nonlinear model appears to describe more precisely the length-weight relationship of *S. richardsonii* than the corresponding linearised model, which is illustrated in Fig. 1 along with observed values. The average condition factor (K) and relative condition factor (Kn) for *S. richardsonii* is 0.732488 ± 0.154(K±SD) and 0.753727± 0.154 (Kn±SD) respectively.

It is evident from the results that the 'b' value of length - weight relationship was found 2.68, represents fish that becomes less rotund as length increases, indicating the allometric pattern of growth in the fish. Some of this variation from isometry may be due to the very small specimens that had not yet reached adult body properties being included in the regression. According to Hile [13] and Martin [15], the value of 'b' usually ranges between 2.5 and 4.0. Allen [16] suggested that the value of 'b' remains constant for ideal fish. Hence, *Schizothorax richardsonii* can be considered as 'ideal' (b = 2.68) as per the suggestion of Allen [16]. On plotting the observed average weight of the species against the observed length with a predicted data, a parabolic curve has been obtained (Fig. 1) and a logarithmic graph prepared with the observed data of log l and log w with a predicted data showed a straight line relationship (Fig. 2).

The length weight relationship of *S. richardsonii* (Snow trout) was analyzed in present study. Both linear (R^2=0.92) and non-linear (R^2=0.95) regression equation were obtained. The exponential value for Snow trout is the hypothetical value (3), b value was '2.68' and the correlation coefficient was (>0.9). In length weight relationship of Snow trout, weight increases in length. Thus it is clear that these fishes maintain its shape throughout its life.

Table 1: Summary statistics of the models fitted to length-weight dataset of snow trout (*S. richardsonii*)

	Linearized model: Log W = log a +b log L	Non-linear model: $W = aL^b$
Parameters Estimates		
log a or, a	-4.440 (0.172)	3.11×10^{-3} (0.000)*
b	2.653 (0.085)	2.688 (0.0770)
Goodness of fit Statistics		
R^2	0.92	0.95

*The corresponding asymptotic standard errors are shown in the parentheses

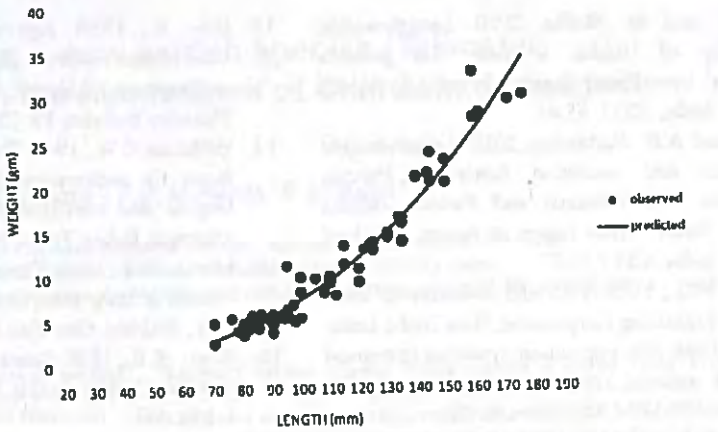

Fig. 1: Fitted Non-linear length - weight model of snow trout (*S. richardsonii*)

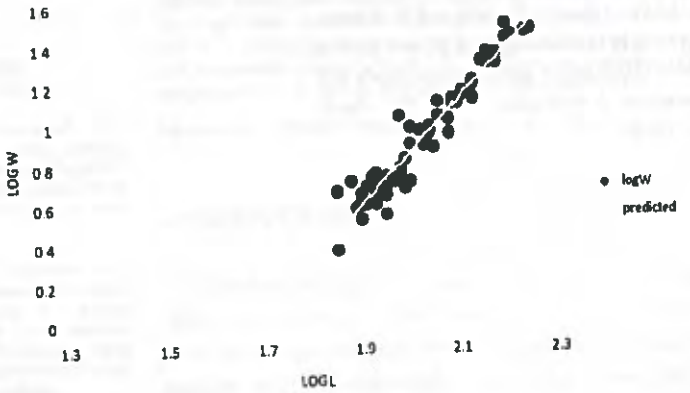

Fig. 2: Fitted Linearized length-weight model of snow trout (*S. richardsonii*)

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Mystus ngasep, a new catfish species (Teleostei: Bagridae) from the headwaters of Chindwin drainage in Manipur, India

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Abstract: *Mystus ngasep*, a new species of bagrid catfish from the headwaters of Chindwin drainage in Manipur, India, is described here. It is distinguished from its congeners in having a unique combination of the following characters: a colour pattern of the body consisting of a distinct dark tympanic spot and three brown stripes separated by pale narrow longitudinal lines, cranial fontanel reaching the base of the occipital process, a long-based adipose fin contacting the base of the last dorsal-fin ray anteriorly, 16-19 gill rakers on the first branchial arch, a slender cleithral process, pectoral spine with 9-11 serrations on the posterior edge, eye with a diameter of 16.5–19.8 % HL and prepectoral length 22.2–26.0 % SL. The new species has been compared with its congeners from Myanmar and also from northeastern India.

Keywords: Chindwin headwater, *Mystus*, new catfish.

INTRODUCTION

Fishes of the genus *Mystus* Scopoli are small to medium-sized bagrid catfishes occurring in South Asia. Roberts (1994) recognized *Mystus* to have an elongate cranial fontanel reaching up to the base of the occipital process, long maxillary barbel, very long adipose fin, 11–30 gill rakers on the first gill arch and 37–46 total vertebrae, about equally divided between abdominal and caudal regions. He included only eight species under the genus. Mo (1991) characterized the genus to have a thin needle-like first infraorbital, twisted and thickened metapterygoid loosely attached to the quadrate by means of ligament or a small extent of cartilage. Jayaram & Sanyal (2003) and Ferraris (2007) respectively listed 44 and 33 species of *Mystus* as valid.

Manipur State in the northeastern corner of India has two headwaters: that of the Brahmaputra basin in the west and of the Chindwin in the east. Hora (1921) reported *Mystus bleekeri* from the lakes and streams of Manipur Valley, including the Loktak Lake (all headwaters of the Chindwin River drainage). Hora (1936) also collected the species from the Brahmaputra basin in Nagaland and Menon (1954) from Manipur. The species was also reported from the Chindwin basin of Manipur by Menon (1953, 1954), Singh & Singh (1985), Vishwanath et al. (1998), Arunkumar & Singh (1997) and Vishwanath (2000).

Other known species of *Mystus* from the neighboring Myanmar, also drained by the Chindwin-Irrawaddy are: *Mystus cineraceus*, *M. gulio*, *M. falcarius*, *M. leucophasis*, *M. pulcher* and *M. rufescens* (Ng & Kottelat 2009). The Ganga-Brahmaputra basin in northeastern India has *M. bleekeri*, *M. dibrugarensis*, *M. tengara*, *M. cavasius* and *M. carcio*

(Vishwanath et al. 2007; Darshan et al. 2010).

Present studies reveal that the species of *Mystus* occurring abundantly in the streams, rivers and lakes (all belonging to the Chindwin drainage) in the valley of Manipur are without a name and the species has been misidentified as *M. bleekeri* after Hora (1921). The species is herein described as *Mystus ngasep* sp. nov.

MATERIAL AND METHODS

Materials examined are deposited in the Manipur University Museum of Fishes (MUMF). Measurements were made with a dial caliper to the nearest 0.1mm. Body proportions were expressed in percentage of SL and HL. Counts and measurements follow those of Ng & Dodson (1999). Dorsal fin height was measured from the base of the spinelet to the highest point of the dorsal fin. For osteological studies, clearing and staining techniques follow Hollister (1934). Methods for counting gill rakers and vertebrae follow Roberts (1992) and Roberts (1994), respectively.

Mystus ngasep sp. nov.
(Image 1, Fig. 1, Table 1)

Macrones bleekeri Hora, 1921: 165–214 (brief description of specimens from Manipur valley, Chindwin basin).

Mystus bleekeri Menon, 1953: 266 (listed from Manipur valley); Menon, 1954: 22 (in part, listed from Manipur valley); Singh & Singh, 1985: 87 (reported from Sekmai & Chakpi Rivers, Manipur); Vishwanath et al. 1998: 323 (reported from Chatrikong River, Manipur); Arunkumar & Singh, 1997: 131 (reported from Yu-River in Manipur); Jayaram & Sanyal, 2003: 42 (in part, synonymy and description).

Material examined:

Holotype: 10.xii.2007, 98.3mm SL, 24°48'N 93°55'E, Nambul River at Bijoygovinda-Polemleikai Bridge, Chindwin-Irrawaddy drainage, Manipur State, India, A. Darshan (MUMF 9500).

Paratypes: 4 ex., ii.2008, 96.5–103.0 mm SL; data as for holotype (MUMF 9501/1-9501/4); 12.viii.2000, 7ex., 87.0–71.6 mm SL, Wangoi-Ngarian Lake, (Chindwin drainage), A. Drashan (MUMF 9502/1-9502/7); 08.ix.2000, 4 ex., 79.9–108.7 mm SL, Khuga River (Chindwin drainage), Churanchanpur District, K. Santa Devi (MUMF 9503/1-9503/4); 02.xi.2006, 14 ex., 60.5–86.3 mm SL, Nambul River at Naoremthong, Imphal-west District, H. Joyshree Devi, (MUMF 9504/1-9504/14).

Non-type material: 16.v.2001, 22 ex., 70.2–96.2

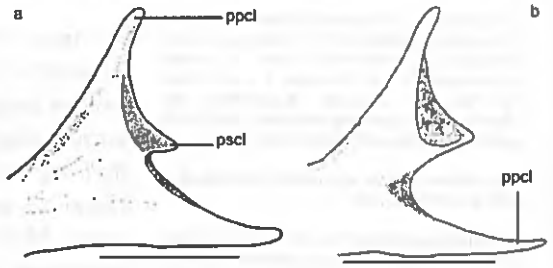

Figure1. Lateral views of cleithral process. a - *Mystus bleekeri* (MUMF 9521) 90.8mm SL; b - *Mystus ngasep* sp. nov. (MUMF 9501) paratype, 98.5mm SL. dpcl - dorsal process of cleithrum for articulation with posttemporal; pscl - posterodorsal spine of cleithrum; ppcl - posterior process of cleithrum. scale bar = 5mm

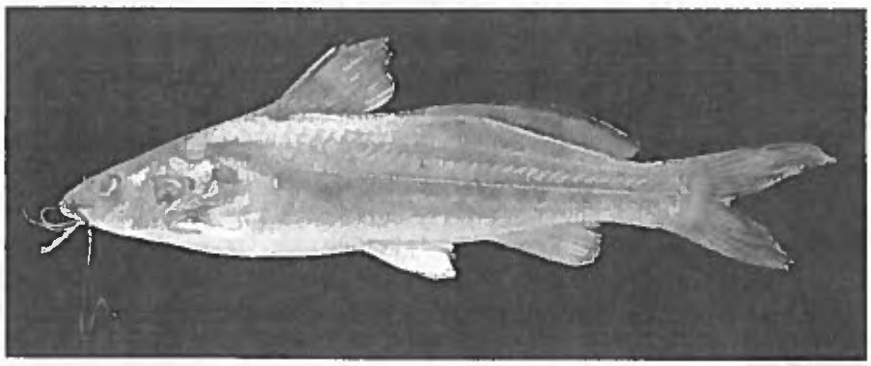

Image 1. *Mystus ngasep* sp. nov. (MUMF 9501/1) paratype, 96.5mm SL.

Table 1. Morphometric data of *Mystus ngasep* sp. nov.

	Holotype	Range	Mean±SD
In % SL			
Predorsal length	37.7	37.0–41.4	39.0±1.2
Preanal length	71.1	68.3–73.3	70.4±1.3
Prepelvic length	48.3	47.1–51.9	49.3±1.4
Prepectoral length	22.3	22.2–26.0	23.3±1.2
Height of dorsal fin	20.8	20.8–21.8	21.3±0.7
Length of dorsal-fin base	13.1	12.4–14.5	13.2±0.7
Dorsal-spine length	13.3	12.2–15.5	13.5±0.9
Anal-fin length	16.9	16.9–19.3	18.2±0.9
Pelvic-fin length	13.9	13.0–16.3	15.1±1.1
Pectoral-fin length	13.9	13.9–19.8	17.6±1.9
Pectoral-spine length	13.4	11.8–15.3	13.5±1.0
Caudal-fin length	25.9	23.0–26.9	25.2±1.2
Length of adipose-fin base	41.2	37.1–44.5	41.0±1.8
Adipose maximum height	6.2	4.4–7.0	5.9±0.7
Post-adipose distance	9.9	8.9–10.9	9.7±0.6
Caudal-peduncle length	19.5	17.9–21.4	19.4±1.1
Caudal-peduncle depth	10.8	9.2–10.8	10.1±0.5
Body depth at anus	21.9	19.2–23.2	20.8±1.2
Head length	25.1	24.6–28.6	26.4±1.3
Head width	16.3	15.7–18.3	16.9±0.8
Head depth	17.3	16.7–18.4	17.6±0.6
In % HL			
Snout length	39.7	33.8–40.7	37.8±1.6
Eye diameter	19.8	16.2–19.8	18.2±1.3
Interorbital distance	30.7	30.3–31.8	30.9±0.6
Nasal-barbel length	51.4	32.8–51.4	45.5±6.2
Maxillary-barbel length	200.0	200.0–235.0	215.4±9.7
Inner mandibular-barbel length	66.4	58.6–76.0	67.5±5.8
Outer mandibular-barbel length	101.2	94.7–118.6	106.3±8.6

mm SL, Iril River at Keibi (Chindwin River drainage), I. Linthoingambi, (MUMF 9505/1-9505/22); 06.vi.1996, 4 ex., 83.1–104.7 mm SL, Chatrickong River at Sanalok (Chindwin River drainage), Ukhrul District, K. Selim (MUMF 1096–1099).

Diagnosis

Mystus ngasep sp. nov. can be distinguished from congeners in having a unique combination of the following characters: a colour pattern consisting of a distinct dark tympanic spot and three brown stripes separated by pale narrow longitudinal lines on the sides of the body, cranial fontanel reaching the base of the occipital process, a long-based adipose fin contacting the base of the last dorsal-fin ray anteriorly, 16–19 gill rakers on the first branchial arch, a slender cleithral process (Fig. 1), pectoral spine with 9–11 serrations on the posterior edge, eye small with its diameter 16.5–19.8 % HL, pectoral and anal fins with 9–10 and 8–9 branched rays respectively and short maxillary barbel (200.0–235.0 % HL).

Description

Morphometric data are shown in Table 1. Dorsal profile rising evenly (at an angle of 20–25° to the horizontal) from tip of snout to origin of dorsal fin then goes almost horizontal to anterior third of adipose fin, then sloping gradually ventrally from there to end of caudal peduncle. Ventral profile roughly straight to end of anal-fin base, then sloping gently dorsally to the end of caudal peduncle.

Head depressed. Skin covering on dorsal surface of head thin. Anterior cranial fontanel extending from level of posterior nasal opening to posterior orbital margins, separated from posterior fontanel by epiphyseal bar. Posterior fontanel extends to the base of the supraoccipital process. Supraoccipital process long, reaching basal bone of dorsal fin, its base narrow with about one-fifth of its length, distally tapered. Eye ovoid, horizontal axis longest, located entirely in the dorsal half of the head.

Mouth sub-terminal. Oral teeth small and villiform, arranged in irregular rows. Premaxillary tooth band slightly curved backward, of equal width throughout. Tooth band on vomer continuous across midline and crescentic, slightly broader than premaxillary in middle, tapering posterolaterally, extending to level of lateral end of premaxillary tooth band. Dentary tooth band separated in the middle by thick skin, tapering laterally on each side, broader than premaxillary and vomerine tooth band at symphysis, length of one side equals lateral span of vomerine tooth band. Gill openings wide, free from isthmus. First branchial arch has 16–19 gill rakers.

Barbels in four pairs, maxillary barbel not reaching anal-fin origin, nasal reaching posterior rim of eye, outer mandibular barbel reaching base of pectoral fin and inner mandibular barbel slightly shorter. Skin smooth. Lateral line complete and midlateral in position.

Dorsal-fin origin slightly anterior to the middle of the body, with spinelet, spine, and seven branched rays. Dorsal spine three-fifths to three-fourths of dorsal-fin height, smooth on both edges. Adipose fin long, spanning most of postdorsal distance, its origin in contact with base of last dorsal-fin ray and deeply incised posterior portion. Pectoral fin with I, 9–10 rays, fin margin straight posteriorly. Pectoral spine backwardly curved with 9–11 large posterior serrations and anteriorly rough. Pelvic fin short with i,5 rays. Anal-fin origin inserted at vertical through middle of adipose-fin base, with iii-v, 8–9 rays, anterior two simple rays minute, visible in alizarin stained specimens. Caudal fin deeply forked with i,7,8,i rays, upper lobe longer.

Osteological characters: Ribs: commonly 12, rarely 11; vertebra with 40–41 (21+19=40 or 22+18=40 or 23+18=41). Haemal arches closed to form haemal canal from the 12th–14th vertebrae onwards. Branchiostegal with nine rays. Caudal skeleton composed of five hypural plates (two on lower and three on upper lobe). Parhypural free from first hypural plate. Hypurapophysis and secondary hypurapophysis fused. Epural laterally flattened and curved backward. Dorsal and ventral lobes of caudal fin with 10 and 11 Procurrent rays, respectively.

Sexual dimorphism: Males with long genital papilla reaching to the base of the second branched anal-fin ray. Females with rounded genital opening.

Colour: In life or freshly dead: dorsal portion of the head and body brownish-grey with greenish reflection; tympanic spot without distinct margin, with greenish reflection that is more pronounced in the middle; lateral surface of body silvery with brownish-golden reflection without prominent stripes, ventrally dull white.

In 10% formalin: dorsal portion of the head and body brownish-gray, tympanic spot with distinct margin, three brown lateral stripes on body separated by pale longitudinal lines, lower pale longitudinal line about twice as wide as the upper. Caudal-fin base without dark spot.

Etymology

The specific epithet is derived from the Manipuri local name of the fish: 'Ngasep'.

Distribution

Presently known from the Loktak Lake, Nambul, Manipur, Iril, Imphal, Thoubal, Khuga rivers and the tributaries of the Yu river (all belonging to the Chindwin River drainage) in Manipur.

DISCUSSION

Ng & Kottelat (2009) clarified the identity of *Mystus bleekeri* and restricted its distribution to the Ganga-Brahmaputra basin while *M. rufescens* was found to be limited to the Irrawaddy basin. Their conclusion was based on the very distant geographical origins of the type series of *M. bleekeri* (Sind, Yamuna, upper waters of Ganga and Burma) which predicted involvement of more than one species; Day's (1877) observation of a black spot at the base of the caudal fin in the Burmese specimens and Roberts's (1994) reference of Day's type material from Burma as *M. rufescens*.

As mentioned earlier, six congeners of *Mystus ngasep* sp. nov. are known from Myanmar. Among those, *M. cineraceus, M. rufescens* and *M. falcarius* are very similar to the new species in having a long-based adipose fin that contacts the base of the last dorsal-fin ray anteriorly and cranial fontanel reaching to the base of the occipital process. A diagnostic summary of the species of *Mystus* from the Chindwin-Irrawaddy and Ganga-Brahmaputra River drainages is given in Table 2.

The new species differs from *Mystus cineraceus* in having three brown stripes on the body separated by pale narrow longitudinal lines above and below the lateral line (vs. a brownish body with a midlateral stripe lacking the pale longitudinal lines). It further differs from *M. cineraceus* in having more gill rakers on the first branchial arch (16–19 vs. 13–15; Table 3), more pectoral-fin rays (9–10 vs. 7–8), more anal-fin rays (8–9 vs. 6–7) and a shorter maxillary barbel (200.0–235.0 % HL vs. 247.4–345.0).

Specimens of *Mystus rufescens* collected from the Chindwin basin in the Indo-Burma border in Manipur were examined and found to have a long-based adipose fin contacting the base of the last dorsal-fin ray anteriorly, a cranial fontanel reaching the base of the occipital process and a black spot at the base of the caudal fin. Vinciguerra's (1890) description of the species clearly states the presence of a black spot at

Table 2. Key diagnostic characters of *Mystus* with a long adipose-fin base, distributed in the Chindwin-Irrawaddy and Ganga-Brahmaputra River drainage.

	M. cineraceus	*M. ngasep* sp. nov.	*M. falcarius*	*M. rufescens*	*M. bleekeri*	*M. cavasius*
Pectoral-fin rays	7–8	9–10	7–10	7–9	9–10	6–10
Gill rakers	13–15	16–19	22–29	14–18	11–15	13–22
Anal-fin rays	6–7	8–9	8–9	7–9	8–9	6–9
Anal-fin length *	10–13.4	16.9–19.3	9.8–11.5	-	15.7–19.7	9.3–12.4
Eye diameter **	19.2–25.4	16.2–19.8	22.4–30.2	20.8–23.5	20.2–25.9	21.2–32.7
Maxillary-barbel length**	247.4–345	200–235	435.6–538	255.3–290.2	241.3–330	355.8–504.9
Humeral spot	absent	absent	present as crescent shape	absent	absent	present as ovoid shape
Black spot at caudal-fin base	absent	absent	absent	present	absent	absent
Nuchal spot	absent	absent	present very prominently	absent	absent	present but faint

* - as % SL; ** - as % HL

Table 3. Frequencies of gill rakers count in four species of *Mystus* with a long adipose fin distributed in the Chindwin-Irrawaddy River drainage.

	Total numbers of gill rakers on the first branchial arch															
	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Species																	
M. cineraceus	6	3	3														
M. ngasep sp. nov.				5	21	18	11										
M. rufescens		1	2	1		1											
M. falcarius										9	7	5	5	1	1		1

the base of the caudal fin. We have also examined a syntype of *M. bleekeri* , labelled as ZSI 781, collected from Prome (=Pyay), Myanmar. The ZSI specimen has all the diagnostic characters of *M. rufescens* and also bears a noticeably darker region at the base of the caudal fin. The new species can be easily differentiated from *M. rufescens* by the absence of a black or dark brown spot at the base of the caudal fin (vs. spot present; Image 2), shorter maxillary barbel (200.0–235.0 % HL vs. 255.3–290.2) and smaller eye (eye diameter: 16.2–19.8 % HL vs. 20.8–23.5).

Mystus ngasep sp. nov. differs from *M. falcarius* and *M. cavasius* in having (vs. lacking) brown lateral stripes on the body, a shorter maxillary barbel (200.0–235.0 % HL vs. 355.8–538.0), a lower dorsal fin (dorsal-fin height: 20.8–21.8 % SL vs. 25.7–33.6) and lacking the black spot in front of dorsal spine (vs. spot present).

Mystus ngasep sp. nov. differs from *M. leucophasis* and *M. pulcher* in having a longer cranial fontanel reaching the base of the occipital process (vs. not reaching, but extending up to half the length of supraoccipital bone); adipose-fin base in contact (vs. not in contact) with the base of the last dorsal-fin ray anteriorly, and a smooth (vs. serrated) dorsal spine. *Mystus leucophasis* further differs from the new species in having (vs. lacking) a filamentous extension of the upper principle-ray of the caudal fin. *M. ngasep* sp. nov. further differs from *M. pulcher* in having a wider vomerine tooth-band (as wide as the premaxillary tooth-band vs. about one-third of the premaxillary tooth-band), fewer vertebrae (41–42 vs. 35) and lacking (vs. having) a black spot at the base of the caudal fin.

Jayaram & Sanyal (2003) reported *Mystus armatus* from Manipur based on five specimens (92.2–125.6 mm SL), but they did not provide the exact collection site of the specimens. Ng & Kottelat (2009) found no evidence that *M. armatus* is known from the Irrawaddy River drainage and also suggested that Jayaram & Sanyal's (2003) specimens of *M. armatus* from Manipur might be a misidentification of *M. cineraceus*. We feel that Jayaram & Sanyal (2003) might have misidentified specimens of *M. rufescens* as

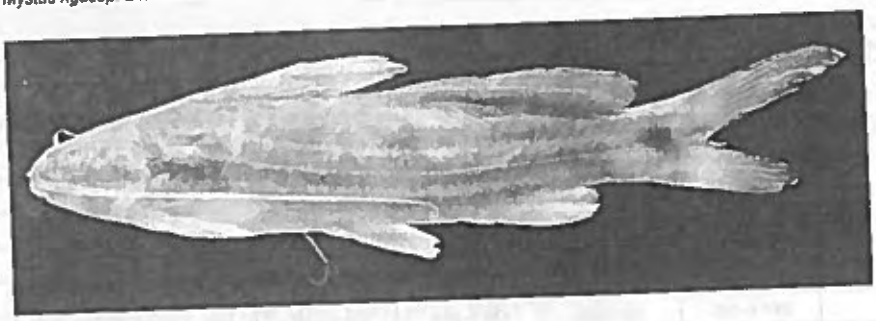

Image 2. *Mystus rufescens* (MUMF 9530) 101.1mm SL.

M. armatus, because *M. armatus* also possess a black spot at the base of the caudal fin also present in *M. rufescens*. We have also not encountered any species of *Mystus* with a black spot at the base of the caudal fin from our extensive surveys of the Brahmaputra River drainage in Manipur. However, we were unable to verify the identity of Jayaram & Sanyal's (2003) material, as we were unable to locate this material for study in the collections of the Zoological Survey of India in Kolkata. Jayaram & Sanyal (2003) also misidentified several specimens collected from the Chindwin River drainage in Manipur (ZSI 4236/2, ZSI F 4293/2, ZSI F 4346/2) as *M. bleekeri*. *Mystus ngasep* sp. nov. is very similar in colouration and meristic counts to *M. bleekeri*. However, the new species differs from *M. bleekeri* in having a slender (vs. broad) cleithral process, smaller eye (diameter 16.2–19.8 % HL vs. 20.2–25.9), shorter maxillary barbel (200.0–235.0 % HL vs. 241.3–330.0), more gill rakers on the first branchial arch (16–19 vs. 11–15), fewer pectoral spine serrations on the posterior edge (9–11 vs. 11–16) and longer prepectoral length (22.2–26.0 % SL vs. 19.5–21.8) and dorsal spine that extends to about three-fifths to three-quarters (vs. nearly half) of the fin height. It further differs from *M. bleekeri* in having a narrower base of the supraoccipital process, its width at the base being about one-fifth of its length (vs. two-fifths to half of its length); more vertebrae (40–41 vs. 37–40), with the closure of the haemal arches appearing from the 12th–14th (vs. commonly 11th or rarely 12th) vertebra onwards.

Mystus ngasep differs from *M. dibrugarensis* in having fewer gill rakers (16–19 vs. 28) on the first arch, more vertebrae (40–41 vs. 36), the absence (vs. presence) of a thin black mid-lateral line connecting the tympanic spot and the black spot at the base of the caudal fin. It differs from *M. tengara* in having a smooth (vs. with 8–9 serrations posteriorly) dorsal spine, longer adipose-fin base (37.1–44.5% SL vs. 24.0–31.7), fewer gill rakers on the first arch (16–19 vs. 31–42), 11–12 (vs. 8–9) ribs and 40–41 (vs. 34–37) vertebrae.

Mystus ngasep sp. nov. differs from *M. carcio* in having more vertebrae (40–41 vs. 32), a longer adipose-fin base (37.1–44.5 % SL vs. 8.5–11.9), vomerine tooth-band continuous (vs. interrupted in the middle), fewer gill rakers on the first arch (16–19 vs. 23–24) and lacking (vs. having) the coracoid shield below the pectoral fin. It differs from *M. gulio* in having a longer occipital process (reaching to the basal bone of dorsal fin vs. not reaching), origin of adipose-fin base in contact (vs. not in contact) with the base of the last dorsal-fin ray, and a smooth (vs. posteriorly serrated) dorsal spine.

Comparative material

Mystus bleekeri: ZSI Kolkata 1076 (lectotype), 101.5mm SL; India: Yamuna River.

MUMF 9521 (10), 85.6–108.3 mm SL; India: Ganga River at Patna. MUMF 9522 (10), 74.2–98.8 mm SL; India: Guwahati: Brahmaputra River.

Mystus rufescens: ZSI Kolkata 781 (1) [syntype of *M. bleekeri*], 95mm SL; Burma: Prome. MUMF 9530 (5), 84.5–101.1 mm SL; India: Manipur: Chandel district, Moreh market.

Mystus cavasius: MUMF 9513 (10), 74.8–109.7 mm SL; India: Guwahati: Brahmaputra River.

Mystus falcarius: MUMF 9514 and 9517 (9), 96.5–206 mm SL; India: Manipur: Lokchao River. Data of Chakrabarty & Ng (2005) are also used for comparison.

Mystus pulcher: ZSI Kolkata F 4716-19/1 (4 syntypes), 51.7–55.5 mm SL; Burma: Bhamo. MUMF 1100–1105 (6), 55.8–69.9 mm SL; India: Manipur: Ukhrul District: Chatrikong River (headwater of Chindwin River drainage).

Mystus tengara: MUMF 9520/1-9520/20 (20), 67.9–75.7 mm SL; India: West Bengal: Kolkata. MUMF 9523 (15), 52.1–77.5 mm SL; India: Brahmaputra River at Guwahati.

Mystus carcio: ZSI FF4081 (1), 47.9mm SL; India: Assam: Brahmaputra River at Guwahati. ZSI FF4080 (1), 42.9mm SL; same data as above. MUMF 9518/1 (1), 39.0mm SL; India: Assam: Brahmaputra River at Guwahati. MUMF 9518/3-9518/10 (8), 30.2–47.9 mm SL; same data as above. MUMF 9519/1-9519/17 (17), 39.0–47.0 mm SL; same data as above. MUMF 9531 (1), 36 mm SL; India: Assam: Ujan Bazar, Guwahati.

Mystus leucophasis and M. gulio: Data of Jayaram & Sanyal (2003).

M. cineraceus: Data of Ng & Kottelat (2009).

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Author Contribution: The study: AD survey, collection, morphometric and anatomic study and phylogeny of catfishes of northeastern India; WV supervision of taxonomy and phylogeny of freshwater fishes of northeastern India; PCM Inventory and cataloguing of coldwater fishes of India; AB Supervise phylogenetic study of coldwater fishes.
Current paper: AD detailed examination of *Mystus* species of northeastern India and comparison with specimens in ZSI and in other museums and preparation of drawings; WV supervision in establishing new species and discuss taxonomic status; PCM supervision in identification of coldwater fish species interpretation of the result, and discuss taxonomic status; AB Differential diagnosis, interpretation of the results, comparison with available literature and discuss taxonomic status.

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Population Structure of Indian Hill Trout (*Barilius bendelisis*) Inferred from Variation in Mitochondrial Dna Sequences

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Abstract: *Barilius bendelisis* is an important food fish and a demanding ornamental species in India. Despite its great economic importance, several wild populations have been suffering drastic reduction. The genetic variability within population is extremely useful for gathering information on individual's identity, breeding patterns, degree of relatedness and disturbance of genetic variation among them. In order to understand the genetic structure of three populations of *Barilius bendelisis*, sequences of mitochondrial gene, cytochrome b (307 bp) from three wild populations were sequenced and analysed. A total of 17 polymorphic sites and 14 parsimony informative sites were detected in cytochrome b gene (307bp) sequences in all three populations. The nucleotide diversity was 0.0237 between Saryu and Kosi river populations, 0.01831 between Saryu and Kalsa river populations and 0.01346 between Kosi and Kalsa river populations. The eight different haplotypes were detected among the three populations studied, single population specific haplotype was observed in Kosi river population. UPGMA dendrogram based on cytochrome b gene sequences of *B. bendelisis* shows that the Kosi river population make cluster with Kalsa river population rather than the Saryu river population. The genetic distance was 0.04 between Saryu and Kosi river population, 0.03 between Saryu and Kalsa river population and 0.02 between Kosi and Kalsa river population. The cytochrome b gene sequences revealed high level of genetic differentiation within and between populations of *B. bendelisis* and demonstrated the suitability of partial Cytochrome b gene (307 bp) sequence in determining the genetic diversity in *B. bendelisis* populations.

Key words: *B. bendelisis* · Indian Hill Trout · Population structure · Cytochrome b · mtDNA

INTRODUCTION

The genus *Barilius* (Hamilton-Buchanan) belongs to the family Cyprinidae, found all over Asia. So, far 20 species of *Barilius* have been reported in the Himalayan and sub-Himalayan regions. *Barilius bendelisis* (Hamilton, 1807), commonly known as Indian Hill Trout, plays significant role in the capture fishery in several parts of the Himalayan region of Uttarakhand and a demanding ornamental as well as potential food fish.

In spites of its greater importance few scattered reports on habitat characterization, feeding habits, food composition [1, 2] of *Barilius bendelisis* are available. Surprisingly, there was no information available on the genetic diversity of natural populations of this species. In recent year, different molecular techniques, using nuclear and mitochondrial DNA (mtDNA), have provided new information concerning the genetic variability of wild and cultivated populations of several fish species [3]. Mitochondrial DNA is maternally inherited without genetic recombination. The evolutionary rate as well as the genetic differentiation of mtDNA among populations is thought to be approximately 5-10 times higher than that exhibited by nuclear genes [4]. Mitochondrial DNA represents a significant marker system for use in population and phylogenetic studies. An extensive review of the advantages of mtDNA as a tool for population genetic analysis has been provided [5]. Among many mitochondrial genes, cytochrome b has been used successfully to identify genetic variation in many fish species [6]. Cytochrome b tend to show intra-specific variation mainly in 3rd position of codon which can be

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used to identify stocks. Variation in mtDNA Cytochrome b gene has been used for population studies in cyprinidae fishes [7]. There is no work done on genetic diversity of *Barilius bendelisis* previously. The aim of the present study was to present a preliminary assessment of the genetic variability of three wild populations of *B. bendelisis*, based on the nucleotide sequences of cytochrome b region of the mitochondrial genome. The results were useful not only to characterize *B. bendelisis* populations but also to give support to recovery efforts and to the biodiversity maintenance and breeding programme of this fish species.

MATERIALS AND METHODS

The sampling of *B. bendelisis* in selected rivers of Himalayan regions Saryu river near Champawat (n = 50), Kalsa river near Chanfi (n = 52) and Kosi river near Ramnagar (n = 53) were carried out using cast net. The fin tissues were cut and put immediately on 90% ethanol, than kept at -20°C until DNA extraction and the voucher fish specimens immediately fixed in 8% formalin.

Genomic DNA was isolated from all fin tissue samples by phenol-chloroform extraction method [8]. Isolated genomic DNA was precipitated with 2-2.5 volume of chilled ethanol. The DNA pellet was washed twice with 70% ethanol, air dried and re-suspended in 1X TBE (10mM Tris-Hcl, pH 8.0 and 1mM EDTA) buffer and kept at 4°C till further use. The quality of DNA was checked by 0.8% agarose gel electrophoresis and the concentration of DNA was estimated with the help of UV-VIS spectrophotometer (Thermo Scientific, England) by taking absorbance at 260nm and 280nm, as one OD_{260} equals 50 µg/ml of double stranded DNA. The intact DNA samples with absorbance ratio 1.6 to 1.8 were used for further experimental work.

The partial Cyt b gene were amplified in seven samples per population of *B. bendelisis* in 25 µL reaction volume, containing 2.5µL of 10X PCR-buffer (100mM Tris, pH 9.0, 500mM Kcl, 15 mM $Mgcl_2$, 0.1% Gelatin) (Bangalore, Genei, India), 200 µM of each dNTPs (Bangalore, Genei, India), 10 pmol of each primer "L14841" and "H15149" [9], (Table 1), 1 Unit of Taq DNA polymerase (Bangalore, Genei, India), 100 ng of genomic DNA and rest miliQ water with PCR amplification cycles as follows: preliminary denaturation step at 94°C for 4 min, 35 PCR cycles of 94°C for 45 sec (Strand denaturation), 54°C for 30 sec (annealing), 72°C for 1 min (primer extension) and final extension at 72°C for 7 min. One negative control (absence of DNA template) was included for each set of amplification. The PCR amplified products were checked in 1.2% agarose gel in 1X TBE (Tris-Hcl, boric acid, EDTA, pH 8.0) buffer at constant voltage of 3V/cm and the amplified fragment visualized with ethidium bromide staining [10], (Figure 1) under UV illumination in the Gel-Doc system (Alpha Imager 3400, Alpha Innotech Corporation, USA).

Molecular weight of target fragment was determined using 1Kb and100bp DNA ladder (Fermentas, Canada). The amplification products were purified before sequencing with Qiaquick columns (Qiagen, USA) followed by manufacturer's instructions. After purification, sequencing of PCR products of five individuals of *B. bendelisis* from each population were performed in both directions by Cycling sequencing with the primers we used for PCR amplification. Sequencing was performed in an ABI Prism 3100 automated sequencer (Applied Biosystems, USA) using Bigdye Terminator. Nucleic acid sequences were subjected to BLASTn [11] at the National Centre for Biotechnology Information (NCBI), Website (http://www.ncbi.nlm.nih.gov/blast). All the Sequences of the mtDNA gene (Cyt b) of *B. bendelisis* were submitted in Gene-Bank, with the accession number (HQ852726 to HQ852740).

The sequences were aligned using ClustalW software [12] website (htpp://www.ebi.ac.uk/clustalw). The genetic diversity (π) and haplotype diversity (h) were calculated using software DNASP version 5.10 [13]. The analysis of molecular variance (AMOVA) as implemented in Arlequin version 3.01 [14], used to assess the population structure of *B. bendelisis*. The phylogenetic relationship among three populations of *B. bendelisis* was constructed by unweighted pair group method with arithmetic mean (UPGMA), using software MEGA version 4.0 and bootstraps support was calculated using 1000 replication. The mean genetic distances between the populations were calculated using the software MEGA version 4.0 [15].

Table 1: Primers used for PCR and sequencing of mitochondrial cyt b gene in this study

Primer name	Sequence
L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA
H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA

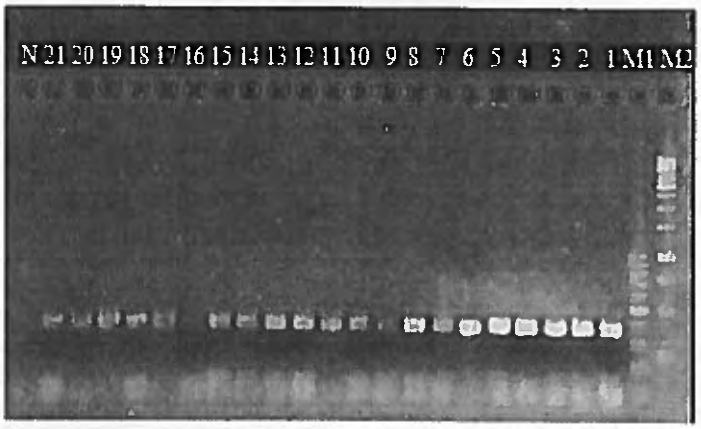

Fig. 1: Amplified Cytochrome b gene in three populations of *B. b* (1-7 Saryu, 8-14 Kosi and 9-21 Kalsa) with (M_2) 1Kb ladder, (M_1) 100bp ladder and (N) Negative control

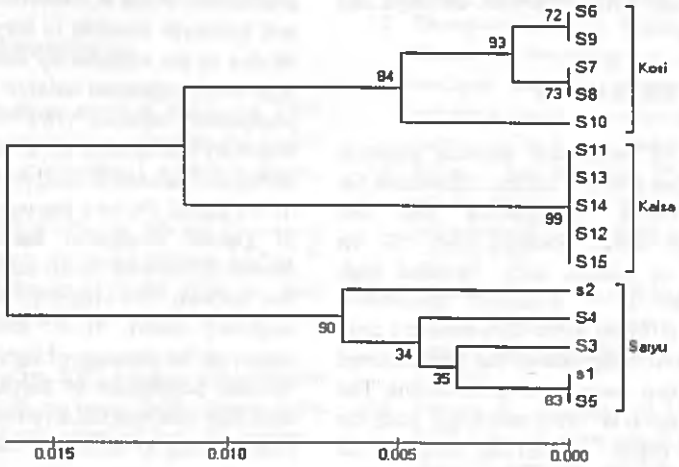

Fig. 2: UPGMA Dendrogram, based on the nucleotide divergence, showing the relationship between the three populations of *Barilius bendelisis (B. b).*

RESULTS

Cytochrome b gene amplified in this study was 307 bp long. The average nucleotide frequencies among 15 samples of three populations of *B. bendelisis* was 0.196 (A), 0.316 (T/U), 0.276 (C) and 0.212 (G). Cyt b gene revealed 17 variable sites and 14 parsimonic informative sites in 307 bp long region. The nucleotide diversity was 0.0237 between Saryu and Kosi river populations, 0.01831 between Saryu and Kalsa river populations and 0.01346 between Kosi and Kalsa river populations. A total of eight distinct cyt b mtDNA haplotypes were identified in three populations of *B. bendelisis* (Table 2). The hierarchical analysis of molecular variance (AMOVA) of population structure reveals a highly significant subdivision between populations in the total sample (F_{ST} = 0.83333; P<0.005) (Table 3), percentage of variation among population is 16.67% and within population 83.33%. Population pair wise F_{ST} value ranged from 0.79245 to 0.88235 (Table 4).

Table 2: Number of haplotypes detected in three different populations of *B.b*

Haplotype	Saryu	Kalsa	Kosi
Hap 1	2	0	0
Hap 2	1	0	0
Hap3	1	0	0
Hap 4	1	0	0
Hap 5	0	2	0
Hap 6	0	2	0
Hap 7	0	1	0
Hap 8	0	0	5

Table 3: AMOVA analysis based on cytochrome b sequences of three populations of *B. b*

Source of variation	d. f	Sum of squares	Variance of components	Percentage of variation	Fixation index	P-value
Among populations	2	38.133	3.66667 Va	16.67		
Within populations	12	8.800	0.73333 Vb	83.33	0.83333	0.00000+-0.00000

Table 4: Population pair-wise F_{ST}

	Saryu	Kalsa	Kosi
Saryu	0.00000		
Kalsa	0.79245	0.00000	
Kosi	0.84444	0.88235	0.00000

UPGMA Dendrogram based on cyt b gene sequences shows that, three different populations of *B. bendelisis* make three different clusters (Figure 2). Mean genetic distance between populations range from 0.02 to 0.04, with the highest genetic distance between the Saryu and Kosi river population.

DISCUSSION

Understanding of population genetics structure of *B. bendelisis* species provides critical information for developing conservation, management and fish production strategies. Result obtained from 307 bp mtDNA sequences in present study, revealed high genetic differentiation in *B. bendelisis* populations collected from three different rivers. Cytochrome b gene amplified in *B. bendelisis* populations has been reported to be useful in detecting variation in *B. bendelisis*. The universal primer (Kocher *et al.* 1989) used in this study for amplifying 307 bp region of mtDNA, found to be polymorphic in *B. bendelisis* populations. This region found to be polymorphic and has been used successfully for intraspecific genetic diversity analysis in various other fish species, like *Salmo trutta* [16]; *Cyprinodon variegates* [17]; *Sardina pilchardus* [18] and *Lates calcarifer* [19]. Nucleotide sequences of Cytochrome b gene in *B. bendelisis* were A+T rich (51.2%), which are similar to many fishes [20]. The nucleotide diversity was 0.0237 between Saryu and Kosi river populations, 0.01831 between Saryu and Kalsa river populations and 0.01346 between Kosi and Kalsa river populations. A total of 8 different haplotypes were found among three populations of *B. bendelisis*, from which Hap1, Hap2, Hap3 and Hap 4 were found in Saryu river population, Hap5, Hap6 and Hap7 were found in Kalsa river population and Hap 8 was found in Kosi river population. A common haplotype was not observed in any of the population of the species. The haplotype diversity for Saryu river population was 0.90000, for Kalsa river population was 0.80000 and for Kosi river population was 0.00000. The haplotype and nucleotide diversity for Kosi river population was 0.0000, as Kosi river population give a single haplotype, indicating low gene flow among these three wild populations of the *B. bendelisis*. The highest nucleotide and haplotype diversity in Saryu river population might be due to the isolation by distance. AMOVA revealed high within population variation (83.33%) and low among populations variation (16.67%). It is reported that a migratory fish species has 85% and 15% of its diversity within and between its local populations, respectively and 67.6% and 32.4% for a non migratory fish [21]. The level of genetic divergence between populations of *B. bendelisis* observed in this study was slightly lower than that reported for a migratory fishes, which indicated the migratory nature of *B. bendelisis*. F_{ST} value also supported the presence of significant genetic differences between populations of Saryu, Kosi and Kalsa rivers. Such high intra-specific diversity could be expected as the rivers belong to different river basins. Therefore, it is likely that populations under study could have evolved in isolation after fragmentation from common ancestor. It is accepted t hat high mtDNA variation, exiting in Saryu river population than Kalsa and Kosi river populations. Mean genetic distance between population 1(Saryu) and population 2 (Kosi) is 0.04, between population 1 (Saryu) and population 3 (Kalsa) is 0.03 and between population 2 (Kosi) and 3 (Kalsa) is 0.02. The Kosi river population makes a cluster with Kalsa river population which shows that *B. bendelisis* population of Kalsa and Kosi River are genetically closer to each other than Saryu river population. Result obtained demonstrated that partial cyt b fragment (307 bp) is observed to be a potential marker for studying variation within as well as among populations in *B. bendelisis*. Such information has provided useful information in case of many other fishes. The success of conservation programs and effective management policies depend on the level of

genetic divergence within and between species and developing strategies to maintain the natural genetic diversity [22]. The analysis of mtDNA (cyt b) represents an important tool for the characterization of distinct populations of *B. bendelisis* for conservation, breeding and management programmes.

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Abstract

This article documents the addition of 299 microsatellite marker loci and nine pairs of single-nucleotide polymorphism (SNP) EPIC primers to the Molecular Ecology Resources (MER) Database. Loci were developed for the following species: *Alosa pseudoharengus, Alosa aestivalis, Aphis spiraecola, Argopecten purpuratus, Coreoleuciscus splendidus, Garra gotyla, Hippodamia convergens, Linnaea borealis, Menippe mercenaria, Menippe adina, Parus major, Pinus densiflora, Portunus trituberculatus, Procontarinia mangiferae, Rhynchophorus ferrugineus, Schizothorax richardsonii, Scophthalmus rhombus, Tetraponera aethiops, Thaumetopoea pityocampa, Tuta absoluta* and *Ugni molinae*. These loci were cross-tested on the following species: *Barilius bendelisis, Chiromantes haematocheir, Eriocheir sinensis, Eucalyptus camaldulensis, Eucalyptus cladocalix, Eucalyptus globulus, Garra litaninsis vishwanath, Garra para lissorhynchus, Guindilla trinervis, Hemigrapsus sanguineus, Luma chequen. Guayaba, Myrceugenia colchagüensis, Myrceugenia correifolia, Myrceugenia exsucca, Parasesarma plicatum, Parus major, Portunus pelagicus, Psidium guayaba, Schizothorax richardsonii, Scophthalmus maximus, Tetraponera latifrons, Thaumetopoea bonjeani, Thaumetopoea ispartensis, Thaumetopoea libanotica, Thaumetopoea pinivora, Thaumetopoea pityocampa ena clade, Thaumetopoea solitaria, Thaumetopoea wilkinsoni* and *Tor putitora*. This article also documents the addition of nine EPIC primer pairs for *Euphaea decorata, Euphaea formosa, Euphaea ornate* and *Euphaea yayeyamana*.

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This article documents the addition of 299 microsatellite marker loci and nine pairs of single-nucleotide polymorphism (SNP) genotyping primers to the Molecular Ecology Resources Database. Table 1 contains information on

Table 1 Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources (MER) Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Alosa pseudoharengus, Alosa aestivalis	18	n/a	47166–47201	JN383992–JN384009	Labbe, Ellen M.; Argo, Emily E.; Schultz, Thomas F.; Palkovacs, Eric P.; Willis, Theodore V.
Aphis spiraecola	9	n/a	47081–47089	HM854169–HM854171, JN214382–JN214384, JN214386–JN214388	Mezghani-Khemakhem, M.; Kharrat, I.; Casse, N.; Bouktila, D.; Makni, M.; Makni H.

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Table 1 (Continued)

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Argopecten purpuratus	8	n/a	47373–47380	JN674552–JN674559	Haye, P. A.; Segovia, N. I.; Gallardo-Escárate, C.
Coreoleuciscus splendidus	13	n/a	47143–47155	JF972368–JF972380	Kwan, Ye-Seul; Lee, Wan-Ok; Won, Yong-Jin
Garra gotyla	28	*G. para lissorhynchus, G. litaninsis vishwanath, Barilius bendelisis, Schizothorax richardsonii, Tor putitora*	47345–47372	HQ288484, HQ288485, HQ288489–HQ288499, HQ288501, HQ288502, HQ288504, HQ288506, HQ288507, HQ288510, HQ288511, HQ288517, HQ288526, HQ288661, JF268657, JF268662, JF268664, JF268665	Matura, Rakesh; Sharma, Suresh; Barat, Ashoktaru; Pande, Veena; Mahanta, Prabin Chandra
Hippodamia convergens	12	n/a	47397–47408	JN565049–JN565060	Michel, Andy P.; Zhang, W.; Gardiner, Mary M.
Linnaea borealis	10	n/a	47156–47165	JN674504–JN674512	A'Hara, S. W.; Scobie, A. R.; Broome, A.; Long, D.; Cottrell, J. E.
Menippe mercenaria, M. adina	22	n/a	46925–46968	GU970048–GU970069	Seyoum, Seifu; Bert, Theresa M.; Puchulutegui, Cecilia; Davis, Michelle C.; Muriel-Cunha, Janice; Crawford, Charles R.; Mcmillen-Jackson, Anne I. Barbieri, Luiz
Parus major	15	n/a	47128–47142	HQ263118–HQ263132	Saladin, Verena; Richner, Heinz
Pinus densiflora	16	n/a	47381–47396	JN634766–JN634781	Lee, Kyung Mi; Kim, Yong Yul; Kim, Ki Hwan; Jeon, Ji Hyun; Cho, Kyung Jin
Portunus trituberculatus	11	*P. pelagicus, Eriocheir sinensis, Hemigrapsus sanguineus, Chiromantes haematocheir, Parasesarma plicatum*	46914–46924	JF505633–JF505643	Li, H.; Ye, N. H.; Liu, Y. G.; Zhang, Y. X.; Liu, S. S.
Procontarinia mangiferae	11	n/a	47057–47067	JF746879–JF746889	Amouroux, P.; Normand, F.; Nibouche, S.; Delatte H.
Rhynchophorus ferrugineus	15	n/a	47113–47127	JN374673–JN374687	Capdevielle-Dulac, C.; El-Mergawy, R. A. A. M.; Avand-Faghih, A.; Rochat, D.; Silvain, J.-F.

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Table 1 (Continued)

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Schizothorax richardsonii	34	n/a	47292–47325	HM591233–HM591236, HM591238, HM591240–HM591242, HM591244, HM591246–HM591256, HM591258, HM591260, HM591264–HM591266, HM591270–HM591272, HM591276, HM591278, HM591279, HM591281, HM591283	Barat, Ashoktaru; Chandra, Suresh; Matura, Rakesh
Scophthalmus rhombus	15	*S. maximus*	47090–47104	JF900344–JF900358	Vandamme, S. G.; Maes, G. E.; Van Houdt, J. K. J.; Hellemans, B.; Robbens, J.; Parmentier, K.; Volckaert, F. A. M.
Tetraponera nethiops	14	*T. latifrons*	46982–47009	JN190035–JN190048	Platscheck, F.; Djieto-Lordon, C.; Garcia, M.; Sauve, M.; Peccoud, J.; Dubois, M. P.; McKey, D.; Blatrix, R.
Thaumetopoea pityocampa	13	*T. p. ena clade, T. wilkinsoni, T. pinivora, T. libanotica, T. bonjeani, T. ispartensis, T. solitaria*	46969–46981	JN400258–JN400270	Burban, C.; Magnoux, E.; Rousselet, J.; Kerdelhué, C.
Tuta absoluta	19	n/a	47326–47344	JN680765–JN680783	Guillemaud, Thomas; Legoff, Isabelle; Blin, Aurélie; Tabone, Elisabeth; Desneux, Nicolas; Malausa, Thibaut
Ugni molinae	16	*Myrceugenia correifolia, M. colchagüensis, M. exsucca, Guindilia trinervis, Luma chequen. Guayaba, Psidium guayaba, Eucalyptus cladocalix, E. camaldulensis, E. globulus*	46809–46824	HQ917086–HQ917101	Ramos, R.; Ravest, G.; Méndez, M.A.; Hinrichsen, P.

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Table 2 Information on the focal species, the sequencing primer pairs developed, the number of single-nucleotide polymorphisms (SNPs) observed and any other species the loci were tested in. The next columns contain the number of allele specific primers and probes developed and the Molecular Ecology Resources (MER) database and GenBank accession numbers, respectively. The authors responsible for each set of loci are listed in the final column

Species	No. primer pairs	No. SNPs in sequence	Other species tested	No. Allele specific primers/ probe	Target gene(s)	MER database numbers	Genbank Accession no.	Authors
Euphaea formosa, *E. yayeyamana*, *E. ornate*, *E. decorata*	9	See Table 2 in text for details.	n/a	n/a	See Table 1 in text for details.	47048–47056	JN246927–JN247002, JN389796–JN390424	Lee, Yat-Hung; Lin, Chung-Ping

Table 3 Information on other resources recently uploaded to the Molecular Ecology Resources program wiki (http://tomato.biol.trinity.edu/programs/). The authors are listed in the final column

Species	Category	Type of resource	Authors
Oncorhynchus tshawytscha	Technique	Microsatellite allele ladder-based standardization	LaHood, Eric; Schlei, Ora; Wenburg, John; Moran, Paul

the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column. Table 2 presents information on SNP genotyping resources added to the MER database and presents data on the focal species, the number of sequencing primer pairs, the observed number of SNPs, other species the loci were tested in, and the number of allele specific primers or probes. The MER database and GenBank accession numbers and the authors responsible are also listed. A full description of the development protocol for the loci presented here can be found on the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/).

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Use of vermi-compost in rearing of *Labeo rohita* and *Cyprinus carpio* fingerlings

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Abstract: An on farm trial was conducted to evaluate the efficacy of vermi-compost in rearing of fingerlings of Labeo rohita and Cyprinus carpio at a private fish farm in Udham Singh District of the Uttarakhand state. The control pond was manured with cowdung, while experimental pond was manured with vermi-compost. The significant difference was observed for dissolved oxygen, ammonia nitrogen and in nitrite nitrogen with more conducive condition in the vermi-compost fed nursery pond. Better plankton production and GPP was found in experimental pond. The average survival rate of fishes was comparatively higher in experimental pond for both. *Labeo rohita* (78%) and for *Cyprinus carpio* (82%) with net fish production of 95.91 kg (60.6% increase from control). An uniform linear growth pattern was exhibited by both species with SGR in the range of 3.57-4.77 in experimental pond with isometric pattern of growth.

Keywords: vermi-compost, stabilization tank, dissolved oxygen, GPP, SGR

INTRODUCTION

The importance of incorporation of animal manure in fish pond in enhancing production of fish food organisms has been documented & reviewed by (Wohlfarth and Schroeder 1979, Delmendo 1980, Edwards 1980; Perkar and Olah 1990; Chauhan *et al.*, 1998 and Singh and Sharma 1999). In intensively manured fish ponds, both autotrophic and heterotrophic productions contribute to fish growth (Schroeder, 1987). In the tropical countries, generally, cow dung is used for fertilizing the fishpond. Efficacy of any animal waste depends on its nutrient profile or potential to make available all necessary nutrients into pond water. Vermi-compositing is easily adoptable and simple techniques even in rural regime to produce vermi-compost. A provable study is needed for application of vermi-compost in rearing ponds of fish.

MATERIALS AND METHODS

Field experiment (90 days) was carried out at a private fish farm in Udham Singh District of the Uttarakhand state. Most of the laboratory analytical work was conducted with two ponds of the same area (0.012 ha. each) were selected for the study purpose. Vermi-composting unit was located on pond dykes. Both the ponds were drained out completely by pumping out the water and dried for 10-15 days. These ponds were limed @ 200 kg/ha (2.4 kg each pond) initially and filled with water upto 1.2 mt. The experimental and controlled pond were manured with vermi-compost and raw cow dung @ 5 t/ha respectively i.e. 60 kg each (Ismail, 1994). Half of the total quantity of the total manure was applied after two days of liming (30 kg in each pond) and rest quantity was applied in two subsequent installments (15 kg each) after 30 and 60 days of experiment. Two fish species (*Labeo rohita, Cyprinus carpio*) were selected for polyculture under composite fish farming system according to the package of practices described by Anon (1993). Seed of the fry stage (1month old) of *Labeo rohita* (Rohu) and advanced fry stage (1.5 month old) of *Cyprinus carpio* (Common carp) was procured from the private fish hatchery and acclimatized for 4 hours in conditioning hapa and stocking was done after the 10 days of manuring on 25h May, 2005 @ 20,000/ha. or 2 advanced fry/sqm. i.e. 240 fry in each pond in the ratio 1:1 (120 advanced fry of Labeo rohita and 120 advanced fry of *Cyprinus carpio*). Initial length and weight of individual seed fish was recorded. Pond soil was analysed for pH (Black, 1965), organic carbon (Jackson, 1958) total nitrogen and total available phosphorus. Physico-chemical parameters of pond water (APHA 1985) and plankton level were recorded initially before stocking and in subsequent fortnights at 15 days interval. The primary production of phytoplankton was measured by "light and dark bottle method" (Gaarder and Gran, 1927). Supplementary feed with rice bran and mustard oil cake including fishmeal was given @10% of the total fish biomass.

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Fish sampling was done for estimation of healthr status and growth of fingerlings on 30, 60, 90, 120, 150,160 and 180th days of experiment. Total fish were harvested by drag netting. Specific growth rate was estimated by formula given below:

$$SGR = \frac{\text{Log final weight} - \text{log initial weight} \times 100}{\text{Time}}$$

The length weight relationship of the experimental fish was worked out as per cube law given by Le Gren (1951).

The condition factor (K) of the fishes was calculated as per formula given below:

$$K = \frac{W \times 100}{L^3}$$

Where,

W = Weight of the fish (g)

L = Length of the fish (cm)

$$\text{Survival rate} = \frac{\text{Number of fishes recovered}}{\text{Numer of fishes stocked}} \times 100$$

Proximate composition of organic manures and fish carcass was analyzed (AOAC 1988) at the end of the experiment.

RESULTS AND DISCUSSION

The pH of pond soil was slightly alkaline (7.2 - 7.4), suggesting the productive nature of pond bottom. The total organic carbon content was higher in control pond than the experimental pond, which may be due to the use of raw cow dung. Nitrogen concentration ranged from 41.00-50.30mg/100g soil in experimental pond and 42.37-47.66 mg/100 soil in control pond. There is a gradual increase in phosphorus content from beginning to end of experiment. It was in the range of 5.99-6.99 mg/100g soil in experimental and 5.42-6.62 mg/100g soil in control pond.

Physico-chemical parameters of water in both ponds were found seasonally fluctuated. In experimental pond, water temperature had been found in the range of 16.6-28.2°C, while pH values varied from 7.0 to 7.4. Dissolved oxygen level was observed in sub optimal level (6.086±0.179 mg/l) with lower level (5.2 mg/l) during the month of June. Free CO_2 in experimental pond water was observed in the range of 2.2-6.0 mg/l with an average of 4.357±0.562 mg/l. Fluctuation in total alkalinity found in the range of 91-177 (155.3 ± 24.7) mg/l. Almost similar pattern of fluctuation was experienced in control pond. Concentrations of ammonia-nitrogen, nitrite-nitrogen and nitrate-nitrogen were in the range of 0.145-0.256 mg/l, 0.032-0.039 mg/l and 0.24-0.69 mg/l, respectively in experimental pond. Fluctuating pattern was also found in phosphorus content with mean value 0.23±0.08 mg/l. Significant difference was found in the concentration of dissolved oxygen, ammonia-nitrogen and nitrite-nitrogen in the control and experimental pond and these values were more conducive for the growth of fish in vermi-compost fed pond (Table 1).

Total phytoplankton density was lower during the month of September-November with mean value 1238.143± 239.0 nos./l and it was higher in the month of June- July with average plankton volume (1.671 ml/50 l) in experimental pond. A total of 16 genera of phytoplankton were recorded with maximum composition of green algae (58.2%) followed by diatoms in experimental pond, in which dominance was occurred by green algae in August- September, by diatoms and dinoflagellates in July-September, by blue green algae in September-October and by Euglenophytes in September-October. A total of 6 species of zooplankton were observed with abundance in the month of July in both ponds. Total density varied from 196-562 nos./l with almost equal occurrence of rotifers and Cladocerans. Gross primary productivity (GPP), Net primary productivity (NPP) and Community Respiration (CR) showed wide seasonal fluctuation with mean values 0.702±0.087, 0.331±0.035 and 0.386±0.036g cm3/ hr, respectively. Period of highest GPP values (July - September) coincides with the highest NPP values and plankton density in both ponds. Low values of CR were observed in initial of experiment and coincide with higher concentration of zooplankton. The average survival rate of fishes was comparatively higher in experimental pond for both, *Labeo rohita* (78%) and for *Cyprinus carpio* (82%). Average net weight was highest for *Cyprinus carpio* (612.620 g). A net fish production of 95.91 kg (7992.5 kg/ha/yr), was obtained from Experimental pond, while it was 59.68 kg (4973.3 kg/ha/yr) in control pond. Thus, an increase of 60.6% in total production of experimental pond was experienced with vermicompost manuring in place of cowdung.

Table 1. Mean values (SD) of physico-chemical characteristics of water in experimental (E) and control (C) pond.

Parameters	Pond	
	E	C
Water temperature (°C)	$27.314^{a} \pm 0.797$	$27.314^{b} \pm 0.815$
pH	$7.257^{a} \pm 0.538$	$7.214^{b} \pm 0.046$
Dissolved oxygen (mg/l)	$6.086^{a} \pm 0.179$	$5.500^{a} \pm 0.141$
Free carbon dioxide (mg/l)	$4.357^{a} \pm 0.562$	$5.586^{b} \pm 0.604$
Total alkalinity (mg/l)	$150.714^{a} \pm 4.664$	$151.7146^{b} \pm 0.357$
Ammonia-nitrogen (mg/l)	$0.1667^{a} \pm 0.019$	$0.191^{a} \pm 0.014$
Nitrite-nitrogen (mg/l)	$0.035^{a} \pm 0.001$	$0.043^{a} \pm 0.002$
Nitrate-nitrogen (mg/l)	$0.573^{a} \pm 0.050$	$0.423^{b} \pm 0.054$
Phosphate-phosphorus (mg/l)	$0.257^{a} \pm 0.021$	$0.224^{b} \pm 0.028$

Table 2: Mean values (SD) of different biotic parameters in experimental (E) and control (C) pond.

Parameters	Pond	
	E	C
Plankton (ml 50 l)	$1.671^{a} \pm 0.224$	$1.600^{b} \pm 0.229$
Phytoplankton (Nos l)	$1238.143^{a} \pm 239.0$	$1083.14^{b} \pm 219.24$
Zooplankton (Nos l)	$335.857^{a} \pm 50.64$	$316.71^{b} \pm 55.99$
GPP (gc m^{-2} hr)	$0.702^{a} \pm 0.087$	$0.683^{b} \pm 0.065$
NPP (gc m^{-2} hr)	$0.331^{a} \pm 0.035$	$0.306^{b} \pm 0.033$
CR (gc m^{-2} hr)	$0.380^{a} \pm 0.036$	$0.377^{b} \pm 0.038$

Figures are significantly different ($P < 0.05$)

Table 3. Length-Weight relationship of fish reared in Control & experimental pond

Species	Exponential equation	Logarithmic equation	Correlation coefficient 'r'
Labeo rohita (C)	$W = -3.8735L^{2.6450}$	Log W = 1.4119+2.6450 log L	0.9919
Labeo rohita (E)	$W = -3.9779\ L^{2.7539}$	Log W = 1.4003+2.7539 log L	0.9875
Cyprinus carpio(C)	$W = -3.8399\ L^{2.6766}$	Log W = 1.4157+2.6766 log L	0.9209
Cyprinus carpio(E)	$W = -4.2319L^{2.8411}$	Log W = 1.3735+2.8411 log L	0.9761

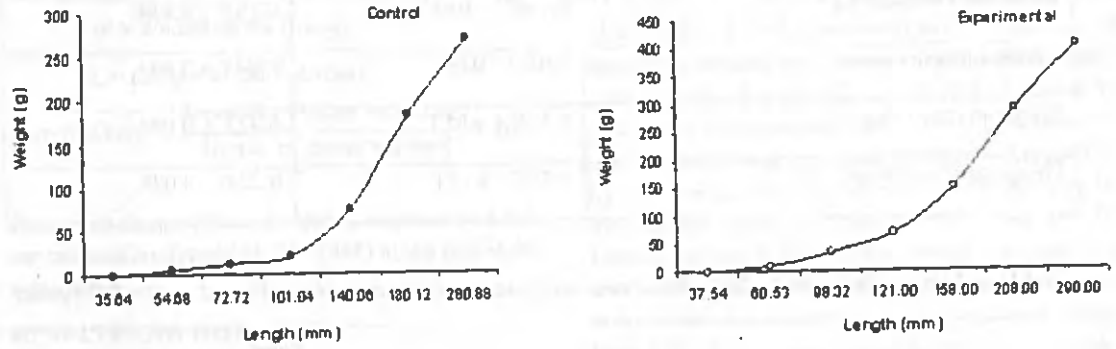

Fig. 1. Length-weight relationship of *Labeo rohita* in control/experimental ponds.

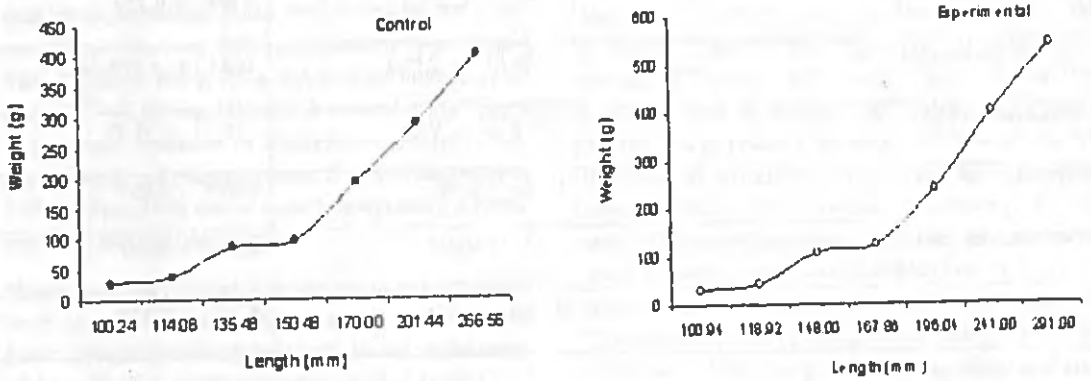

Fig. 2. Length-weight relationship of *Cyprinus carpio* in control/ experimental ponds.

An uniform linear growth pattern was exhibited by both species with SGR in the range of 3.57- 4.77 in experimental pond. The values of regression coefficient 'n' were in the range of 2.739 to 2.8411 with condition factor 'k' in the range of 1.53-1.65, showed isometric pattern of growth and better favourable conditions in experimental pond. Over all high protein, content in both fish species (19.32-19.94) indicates better protein utilization by group of fish raised with vermicompost.

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DNA Bar-Coding of Indian Coldwater Fishes of Genus *Schizothorax* (Family: *Cyprinidae*) from Western Himalaya

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Abstract: The rapid and accurate characterization of species using morphological data is a critical constraint. To overcome this, species identification using molecular tools has been supplemented in many studies in present era. The present study was designed to test the utility of Cytochrome Oxidase I (COI) DNA barcodes for the identification of two commercially important coldwater species of Genus *Schizothorax* (Snow trout), Family *Cyprinidae*, from Uttarakhand Himalayas. COI gene (655 bp) was amplified using PCR and sequenced. The mean intra-specific nucleotide sequence divergence was 1.75% (range 0.00-3.50%) and inter-specific divergence of *S. richardsonii* is 0.00% (range 0.00040-0.00080%) and *S. progastus* is 0.00% (range 0.00036-0.000206), respectively. Although, DNA bar-coding aims to develop species identification systems, some phylogenetic signal was apparent in the data. It was concluded that COI sequencing or 'bar-coding', was found to be suitable for the identification of coldwater fish species.

Key words: Cytochrome Oxidase Subunit I · Bar-coding · Fish · MtDNA · *Schizothorax*

INTRODUCTION

Accurate and unambiguous identification of fish and fish products, from eggs to adult, is important in many areas. It would enable retail substitutions of species to be detected, assist in managing fisheries for long-term sustainability and improve ecosystem research and conservation. Hitherto, a wide variety of protein and DNA based methods have been used for the genetic identification of fish species [1-4]. DNA sequence analysis has been used for 30 years to assist species identifications, but different sequences have been used for different taxonomic groups and in different laboratories [5]. Proposed that a single gene sequence would be sufficient to differentiate all, or at least the vast majority of, animal species and proposed the use of the mitochondrial DNA gene cytochrome oxidase subunit I (COI) as a global bio-identification system for animals. The sequence was likened to a barcode, with species being delineated by a particular sequence or by a tight cluster of very similar sequences.

Species identification by DNA bar-coding is based on sequencing a short standardized genomic region of the target specimen and comparing this information to a sequence library from known species. The proposed standard barcode sequence for animal species is a 650-bp fragment of the mitochondrial gene cytochrome *c* oxidase I (COI). Many benefits of DNA bar-coding for species identification and discovery have been discussed [6], although the concept continues to be hotly debated [7]. In addition to species identification, the construction of barcode database could expose novel DNA barcodes that may indicate provisional new species [8]. Genetic interrelationships of Cyprinid subfamilies have been extensively investigated from morphological, anatomical and molecular perspectives [9-12]. Two studies mainly based on morphological and anatomical characters have investigated phylogenetic relationships among genera and species and explored the taxonomic status of these

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fishes [13, 14] but, the molecular identification based on mtDNA COI gene are somewhat understudied for the highly specialized *Schizothorax* species in the Garhwal Himalaya [15].

The fishes of genus *Schizothorax*, members of the family Cyprinidae, commonly known as snow trout, consist of 15 genera and over 100 species all over the world [16]. In India, these species are distributed in the cold waters from Jammu and Kashmir [17], to Assam and Eastern Himalayas through Bhutan and Sikkim at an altitude of 1180-3000m [18]. So, far 28 species of snow trout have been reported in the Himalayan and Sub-Himalayan regions. Their inherent biological features, such as short growth period and slow growth to maturity, are the main constraints hindering their resources and population increase [19]. This genus consists of a group of species that are remarkably similar in general morphology. The species of *Schizothorax* are often the most difficult to distinguish based on external morphological characters. A DNA bar-coding approach may be useful for the identification of taxa. For these reasons, the utility of the COI barcode sequence for the identification of snow trout was tested. In the present study, an attempt was made to examine COI diversity within and among two fish species, with the goal of determining whether DNA bar-coding can achieve unambiguous species recognition in fishes.

MATERIALS AND METHODS

Samples Collection: The fish Samples were collected by cast net from two different river system (Khanda and Dugadda gad) of pauri Garhwal from Uttarakhand Himalaya (Fig. 1). The Muscle samples were collected through dorsal part and preserved in 95% ethanol. All specimens were fixed in 10% formalin in the field as a voucher. The Rivers (Khanda and Dugadda gad) drain North-Western Garhwal Himalaya. It is a tributary of the River Alakananda, originates from the high altitude zone of the Distt Pauri Garhwal close the distt Chamoli. It travels distance of about 10 to 20 kilometer through the wide valley of the Distt Pauri and Tehri (Table 1).

Laboratory Procedures

DNA Extraction, Amplification and Sequencing: Total genomic DNA was isolated from muscle tissue by using the standard phenol-chloroform extraction protocol. Approx. 655 bp nucleotide was amplified from the 5' region of the COI gene from mtDNA using different combinations of two pairs of primers: F1 (5'-TCAACCAACCACAAAGACATTGGCAC-3'), R1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'), F2 (5'-TCGACTAATCATAA AGATATCGGCAC-3'), R2 (5'-ACTTCAGGGTGACCGAAGAATCAGAA-3'). Polymerase chain reaction (PCR) amplifications were

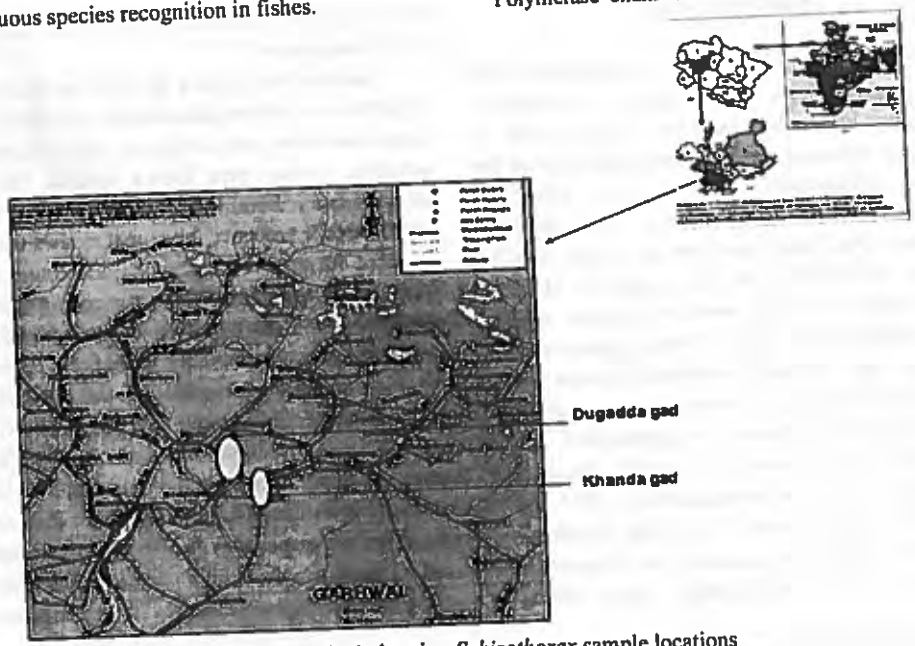

Fig. 1: Map of Uttarakhand (Garhwal region) showing *Schizothorax* sample locations

Table 1: Specimen information, collection dates and collection localities for the Himalayan Snow trout *Schizothorax* species studies

Species	Voucher ID	GenBank accession no.	Collection locality
Schizothorax progastus (Mcclellad)	SK8011-a	FJ170772	Khanda and Dugadda Gad Lat-29° 45'
	SK8011-b	FJ170773	27.0"N and Log-78° 32'22.4"E.
	SK8011-c	FJ170774	
	SK8011-d	FJ170775	
	SK8011-e	FJ170776	
Schizothorax richardsonii (Gray)	SD8012-a	FJ170777	Pauri and Tehri Garhwal,Uttarakhand
	SD8012-b	FJ170778	
	SD8012-c	FJ170779	
	SD8012-d	FJ170780	
	SD8012-e	FJ170781	

performed on a MJ research PTC-200 thermocycler in a 50µl reaction consisting of: 5µl of 10X buffer (100mM Tris, pH 9.0, 500mM KCl, 15mM $MgCl_2$, 0.1% Gelatin) (Genei, India), 200 µM each nucleotide (dNTP) (Genei, India), 5pmole of each primer (Sigma Genosys, USA), 1.5U taq polymerase (Genei, India) and 1-2µl of total genomic DNA. The thermal regime consisted of an initial step of 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 54°C and 1 min at 72°C, followed in turn by 10 min at 72 °C and then held at 4°C. All PCR products were visualized on 1.2% agarose gels and purified using the Mini Elute ™PCR Purification Kit (B. Genei, India) according to the supplier's instructions. The most intense products were selected for sequencing. Purified DNA fragments were directly sequenced using an automated sequencer (Applied Biosystems 377) following the manufacturer's instructions DYEnamic™ET Dye Terminator Cycle Sequencing Kit.

DNA Sequencing Analysis: The DNA sequences were aligned using the Editseq 5.0 and Megalign 5.0 software of the DNA Star package (DNAStar Inc.). To ensure accuracy, strands were sequenced in both directions for each individual. Both DNA strands were checked for ambiguous bases and edited manually. Sequence divergences were calculated using the Kimura two parameter (K2P) distance model [20]. Finally, several matrices were computed from the pair wise distance matrices using the package (K2P), namely the mean, minimum and maximum of the distance within species (D within species) and the distance between species (D between species). DNA sequences were confirmed and edited manually using Edit sequence (DNA STAR software). Neighbour-joining (NJ) and UPGMA trees of K2P distances were created to provide a graphic representation of the patterning of divergence between species [21]. In the two chosen subgroups of fish, bootstrapping was performed in MEGA4 [22] with 500 replications.

RESULT AND DISCUSSION

Amplification and Sequencing of the COI Barcode Region: Our analyses, based on the commonly used mitochondrial genes cytochrome *c* oxidase I (the standard DNA barcode for animal species) are capable of discriminating coldwater species with high accuracy. A total of 10 COI barcodes of 655 bp were thus obtained for 2 species of *Schizothorax* (Fig. 2). Well defined peaks and the absence of stop codons indicated that co-amplification of nuclear pseudo-genes did not occur (Zhang and Hewitt, 1996). In accordance with previous work [5], the sequences aligned with ease due to the absence of insertions and deletions. Nucleotide composition showed a CT bias within *Schizothorax* (mean C = 28.1%, T =28.0%, A = 25.6%, G = 18.2%). All sequences have been deposited in GenBank (Accession no # FJ 170777 to #FJ 170781 and #FJ 170772 to #FJ 170776). Accession numbers for the barcodes, specimen and collection data, sequences, trace files and primers

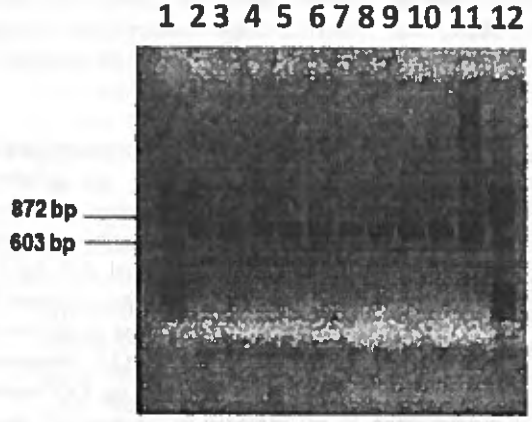

Fig. 2: CO-I mitochondrial gene PCR amplified products Lane-1and12: Ö×174 DNA marker (*Hae III* digested) Lane-2-6: *S. progastus*, Lane- 7- 11: *S. richardsonii*

Table 2: Percentage sequence divergences (K2P) between and within the *Schizothorax* species for the cytochrome oxidase 1 (COI) barcode region.

Species	Minimum	Mean	Maximum
Schizothorax progastus (With species)	0.00036	0.00121	0.00206
Schizothorax richardsonii (With species)	0.00000	0.00040	0.00080
Schizothorax progastus VS Schizothorax richardsonii (Between species)	0.00000	1.75000	3.50000

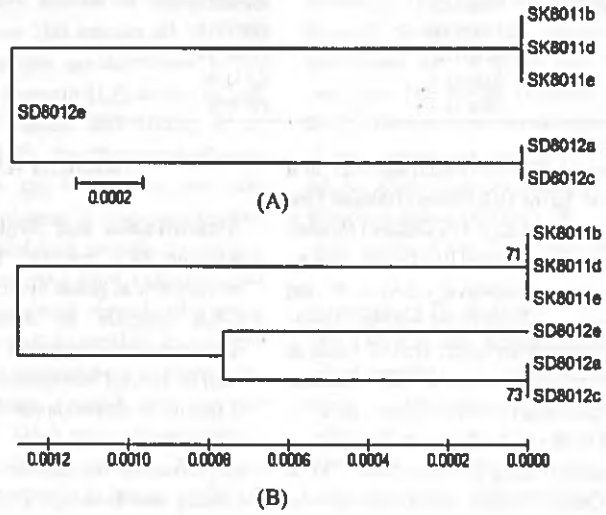

Fig. 3: Neighbour-joining and UPGMA tree of Kimura two-parameter (K2P) distances. Numbers above branches refer to bootstrap proportions among 500 bootstrap replicates. Specimen voucher codes referred to in (Table 1) are shown in parentheses following species names

details were available in NCBI genbank. The average percentage divergence (K2P) distance of individual's species of *S. progastus* is 0.00121% and for *S. richardsonii* is 0.00040%. Furthermore, the sequence divergence between these two species is 1.75000% (Table 2). There is high inter-specific sequence divergence for *Schizothorax* species as compared to intra-specific sequence divergence.

Neighbour-Joining Analysis of COI Barcode Sequences: The purpose of this study was to investigate whether the COI barcode provided sufficient resolution to identify snowtrout of the genus *Schizothorax*. The NJ analysis showed that the COI barcode is an effective tool for identification purposes [5]. All *Schizothorax* species were resolved as reciprocally monophyletic groups, despite low COI divergences between some individuals. Although the COI barcode region alone is not intended to be used to resolve taxonomic relationships, it appears to contain enough phylogenetic signals to delineate close relationships within *Schizothorax* from the Garhwal region of Uttarakhand. Both tree-building methods (NJ and UPGMA) recovered each *Schizothorax* species as a monophyletic group (Fig. 3). The NJ analysis of the COI barcode region placed *S. richardsonii* as a sister to *S. progastus*.

The NJ method has been promoted as the analysis tool of choice for the construction of bar-coding databases, due to its advantage of speed and its performance when sequence divergences are low [5, 23]. However, a comparison of tree-building methods is vital during the development of the bar-coding method, particularly as the suitability of this method for species delineation has been questioned in the past [24]. In some cases, an oversimplified or inadequate phylogenetic analysis may fail to distinguish reciprocally monophyletic groups, whereas an analysis that more realistically models the history of molecular evolution for the COI gene may perform better [25, 26]. To assess this, we compared the NJ tree with UPGMA trees generated from the COI data. Two complications were encountered in this study. In the, two specimens which had been preliminarily identified in the field as *S. richardsonii* (SD8012 b and d from khand,

pauri gharwal) were recovered with *S. progastus* in the COI NJ tree. The second complication concerned two specimen (SK 8011 a and c) identified morphologically as *S. progastus,* but recovered with its closest relative, *S. richarsonii,* in the NJ tree. All the four samples of two species were excluded from the both the trees to maintain the uniformity. These problems may be due to (a) inadequate phylogenetic analysis; (b) an inability of COI to resolve these species and (c) incorrect specimen identification, due to the significant morphological similarities shared by each set of sister species. Because of these close relationships, the possibility of high levels of intra-specific variation, perhaps due to retained ancestral polymorphisms and hybridization, were considered [27].

CONCLUSION

This study has strongly validated the efficacy of COI barcodes for identifying *Schizothorax* species from western Himalaya. The COI barcode region proved straight forward to amplify and sequence, which would facilitate the rapid generation of a barcode database and subsequent identification of specimens. Further, adequate resolution was provided by the COI barcodes to separate coldwater *Schizothorax* species. High COI sequence divergences existed between the species. Mean intra-specific and inter-specific COI sequence divergences differed by more than an order of magnitude. Extremely low sequence divergences between sister species and among species complexes are believed to be indicative of their recent origin. Bar-coding is a technique that could aid the prompt and accurate identification of species that would be enormously beneficial in the application of molecular taxonomy evidence. Based on the results for *Schizothorax*, it is foreseeable that DNA bar-coding could be effective in the identification of other snow trout species. Further investigations should confirm this feasibility and establish the reliability of the technique for routine application in identification cases and other circumstances featuring fishes of applied importance.

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Effect of spirulina (*Spirulina platensis*) and marigold (*Tagetes erecta*) fortified diets on growth, body composition and total carotenoid content of *Barilius bendelisis*

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ABSTRACT

The effect of *Spirulina platensis* and *Tagetes erecta* at different incorporation levels of 0, 3, 5, 7, and 10% were tested on growth, body composition and total carotenoid content in *Barilius bendelisis* (an endangered upland fish species) for 60 days in triplicate. An overall reduction from 3.29 (control, T_0) to 1.57 (7% marigold, T_3), 1.49 (5% spirulina, T_6) and 1.34 (10% spirulina, T_8) in FCR was observed with increase in either spirulina or marigold in the diet. Further, RGR (%) increased from 93.37 (T_0) to 191.17 (T_3), 203.09 (T_6) and 224.359 (T_8) while SGR (%) from 1.09 (T_0) to 1.77 (10% marigold-T_4), 1.84 (T_6) and 1.96 (T_8) for both the additives. Crude protein increased from 14.9% (T_0) to 16.93% (for both T_6 and T_8) while, potassium from 1115 mg 100^{-1}g (T_0) to 1276 mg 100^{-1}g (T_6). Proximate and mineral composition of the fish was significantly affected by Spirulina while marigold has less significant impact. On the other hand, total carotenoids were significantly affected by marigold and it improved from 2.09 μg g^{-1} (T_0) to 4.58 μg g^{-1} (T_4) than the Spirulina, which has less impact on it. *B. bendelisis* fed on 5% spirulina fortified diet not only improve the growth but also enhanced its total carotenoid content and body composition. However, marigold was found to improve the total carotenoid content of the fish with least significant impact on growth and body composition.

Key word: *Barilius bendelisis*, Marigold, Proximate composition, Spirulina, Total carotenoid

Barilius bendelisis is widely distributed in hilly regions of India (Sahoo *et al.* 2009). Inadequate pigmentation, retarded growth with degraded muscle composition is always noticed in this type of cultured fishes due to lack of carotenoid and other nutrient content in their artificial diets (Huner and Meyers 1979, Harpaz *et al.* 1998, Jha *et al.* 2010).

Fish skin colour is primarily dependent on the presence of chromatophores containing pigments, pteridines and purines (Chatzifotis *et al.* 2005). Fish can modify alimentary carotenoids and store them in the integument and other tissues (Halten *et al.* 1997). Farmed fish (particularly, in intensive culture system) have no access to carotenoids-rich feed and therefore, the necessary carotenoids must be added to the diet (Gupta *et al.* 2007). The effectiveness of carotenoid source in terms of deposition and pigmentation is species specific (Ha *et al.* 1993). The loss of pigments, due to inadequate carotenoid reached diets, can be overcome by their addition to the artificial diet. Several studies addressed this issue and have evaluated different potential pigment sources like red pepper (*Capsicum annum*), marigold flower-*Tagetes erecta* and Spirulina-*Spirulina platensis* (Boonyaratpalin and Lovell 1977, Alagappan *et al.* 2004, Buyukcapar *et al.* 2007). These additives, besides pigments, influence muscle quality, survival, resistance to diseases and growth (Harpaz *et al.* 1998, Boonyaratpalin *et al.* 2001, Rema and Gouveia 2005, Ezhil *et al.* 2008).

Therefore, the purpose of the present study is to investigate the effect of different levels of *S. platensis* and *T. erecta* incorporated diet on the growth, body composition and total carotenoid content of *B. bendelisis* in order to increase their production with enhanced pigmentation and muscle composition.

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MATERIALS AND METHODS

Experimental animal

Fingerlings of *B. bendelisis* (5.63g, average weight) were collected from a local stream of Nainital (Uttarakhand), transported in a circular container (500 L) with sufficient aeration, to the experimental site at hatchery complex of Directorate of Coldwater Fisheries Research, Bhimtal and were acclimatized to the experimental rearing conditions for one week. During acclimation, fish were fed with control diet. After acclimatization, fishes were transferred to 27 uniform size experimental fiberglass tanks of 100 l capacity and reared for 60 days.

Experimental design and feeding

Nine iso-nitrogenous (35.25±0.9% crude protein) diets were prepared with graded levels (0, 3, 5, 7 and 10% of diet) of either marigold (MG) or spirulina (SP) meal. Fishes (275) were randomly distributed in 9 experimental groups in triplicates following a completely randomized design (CRD). There were 9 treatment groups, viz. T_0 (control), T_1 (3%MG), T_2 (5%MG), T_3 (7% MG), T_4 (10% MG), T_5 (3%SP), T_6 (5% SP), T_7 (7% SP) and T_8 (10% SP). The physicochemical parameters of water were analyzed by APHA (1995) methodology, and were within the optimum range (dissolved oxygen: 6.0–8.5 mg/l, pH: 7.3–8.2 and temperature: 18–20 °C) throughout the experimental period. All the groups were fed their respective diets. Feeding was done at 5% of the body weight. Daily ration was divided into 2 split doses: about 2/3rd of total ration was given at 09:00 h and the rest at 18:00 h. The fecal matters were removed by siphoning and a constant water flow (2-3 $lmin^{-1}$) was maintained by providing inlet at one and outlet at the other end to ensure optimum dissolved oxygen throughout the experimental period. A very few accidental mortality (10%) was observed during the 60 day experimental feeding trial.

Proximate composition analysis

The proximate composition of the experimental diets and fish muscle was determined following the standard methods of AOAC (1995). The moisture content was determined by drying at 105 °C to a constant weight. Nitrogen content was estimated by Kjeldahl method and crude protein was estimated by multiplying nitrogen % by 6.25. Crude fat (ether extract) was measured by solvent extraction method using diethyl ether (boiling point, 40–60 °C) as a solvent and ash content was determined by incinerating the samples in a muffle furnace at 600 °C for 6 h.

Total carotenoids and minerals

Total carotenoids were determined following the methodology given by Olson (1979). The samples (skin and muscles) were gently meshed with a glass rod against the side of the vial and then 5 ml of chloroform was added and left overnight at 0°C. When the chloroform formed a clear 1–2 cm layer above the caked residue, the optical density was read at 380, 450, 470 and 500 nm, in a spectrophotometer taking 0.3 ml aliquots of chloroform diluted to 3 ml with absolute ethanol. A blank prepared in a similar manner was used for comparison. The wavelength, at which maximum absorption observed, was used for the calculation. Calcium, sodium and potassium were analyzed by atomic absorption spectroscopy using AOAC (1995) methods. Ashed samples were dissolved in 2 ml of concentrated acid (HCl: HNO_3, 1:1), diluted with distilled water (Shearer 1984). The diluted mixture was analyzed for calcium, sodium and potassium.

Growth study

Growth rate of fish was measured in terms of weight gain/ absolute growth rate (AGR,%), specific growth rate (SGR) and feed conversion ratio (FCR) using the following equations.

Relative growth rate (RGR)% = [(final weight – initial weight) / initial weight] × 100

SGR (%) = [(Log_e final weight – Log_e initial weight) / number of experimental days] × 100

AGR (g) = final mean weight – initial mean weight

FCR= feed given (dry weight) / body weight gain (wet weight)

Statistical analysis

Mean values of all parameters were subjected to one way ANOVA to study the treatment effect and Tukey's test (HSD) were used to determine the significant differences between two means. Comparisons were made at 5% probability level. All the data were analyzed using statistical package SPSS (Version 12.01).

RESULTS AND DISCUSSION

The quantities of ingredients used for diet preparation and the proximate composition along with total carotenoids of the diets are given in the Table 1. The initial average weight, final weight, SGR, AGR, RGR and FCR for the experimental fishes and tested diets were calculated and recorded in Table 2. Amongst the marigold fortified diets, T_3 showed highest RGR (191.17%) and lowest FCR (1.57), while, lowest RGR (93.37%) and highest FCR (3.29) were recorded for T_0 (Table 2). SGR was observed highest (1.77%) for T_4 and lowest for T_0 (1.09%). In the present study, improved growth rate and decreased FCR were noticed with the diet containing higher quantity of carotenoids in the form of marigold. These findings are in conformity with those of Amar *et al.* (2001) who reported that utilization of carotenoids through marigold flower led to improved growth and decreased FCR. Marigold flower contains carotenoids and carotenoids have a positive role in metabolism in fish as reported by Tacon (1981). In resemblance with the present observation, the growth of fish and prawn were achieved higher when carotenoids from different sources were added in their diets by Harpaz *et al.* (1998) and Ezhil *et al.* (2008).

FCR reduced from 3.29 (T_0) to 2.03 (T_5) and 1.34 (T_8) with the increase in spirulina % in the diet. Christiansen *et al.* (1996) used astaxanthin as a source of carotenoid and observed lower FCR in Atlantic salmon juveniles, similarly, lower FCR was matter-of-fact in *Penaeus monodon* fed on the diet containing spirulina as a source of carotenoid (Boonyaratpalin *et al.* 2001). Spirulina in the diet augmented RGR from 93.37 (T_0) to 203.09% (T_6) and 224.35% (T_8) and SGR from 1.09 to 1.84 and 1.96 respectively, while AGR increased from 5.76 to 10.15 and 11.65 respectively. The consequences of the present study are in conformity with those of Peimin *et al.* (1999), who reported that more quantity of *S. platensis* in diet increased the length and weight of the fish.

Table 1. Diet composition (%) and its proximate analysis (% dry matter basis)

Ingredients	Diets								
	1	2	3	4	5	6	7	8	9
Fish meal	20.27	20.27	20.27	20.27	20.27	17.27	15.27	13.27	10.27
Soybean meal	20.27	20.27	20.27	20.27	20.27	20.27	20.27	20.27	20.27
Rice bran	29.73	26.73	24.73	22.73	19.73	29.73	29.73	29.73	29.73
Wheat bran	21.73	21.73	21.73	21.73	21.73	21.73	21.73	21.73	21.73
Vitamin-Mineral Mix[1]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vegetable oil[2]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Fish oil[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Sodium alginate[4]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Marigold flower meal	0	3.00	5.00	7.00	10.00	0	0	0	0
Spirulina meal	0	0	0	0	0	3.00	5.00	7.00	10.00
Total	100	100	100	100	100	100	100	100	100
Proximate analyses									
Moisture	13.28	12.89	12.50	12.85	12.69	11.85	12.15	12.54	11.90
	±0.02	±0.03	±0.01	±0.05	±0.02	±0.02	±0.02	±0.03	±0.02
Crude protein	35.21	35.40	35.95	36.25	36.70	34.72	34.51	34.35	34.09
	±0.08	±0.05	±0.02	±0.01	±0.02	±0.04	±0.03	±0.04	±0.01
Ether extract	6.65±0.02	6.23±0.01	6.45±0.02	6.15±0.03	6.20±0.02	5.96±0.02	6.05±0.01	6.27±0.03	6.33±0.03
Ash	10.85±0.05	12.05±0.02	12.96±0.04	13.89±0.03	14.90±0.05	10.05±0.07	10.70±0.03	10.25±0.05	10.18±0.04
Dry matter	86.72±0.25	87.11±0.41	87.50±0.23	87.15±0.33	87.31±0.54	88.15±0.02	87.85±0.02	87.46±0.03	88.1±0.02
TC[3] (µg/g diet)	29.1±0.94	110.12±2.32	178.74±3.13	292.32±7.84	411.28±6.98	100.23±3.41	170.24±4.63	250.14±3.74	350.25±5.51

[1]Agrimin forte (Virbac Animal Health India Pvt. Ltd., Mumbai-59, India). Each kg contains- Vitamin A-7, 00,000 I.U., Vitamin D_3–70, 000 I.U., Vitamin E-250 mg, Nicotinamide-1000 mg, Cobalt-150 mg, Copper-1200 mg, Iodine-325 mg, Iron-1500 mg, Magnesium-6000 mg, Manganese-1500 mg, Potassium-100 mg, Selenium-10 mg, Sodium-5.9 mg, Sulphur-0.72%, Zinc-9600 mg, Calcium-25.5%, Phosphorus-12.75%.

[2]Pvt. ltd. industry, India. [3]Total carotenoids procured from local market, [4]Commercial purchase, India.
Data expressed as Mean±SE, n=3.

Table 2. Growth performance of *B. bendelisis* fingerlings fed with graded levels of marigold flower and spirulina meal

Treatment	Initial Weight (g)	Final Weight (g)	AGR (g)	SGR (%)	RGR (%)	FCR
T_0	6.26±0.8	12.03±0.76	5.76±0.55^d	1.09±0.15^d	93.37±17.12^c	3.29±0.65^c
T_1	5.66±0.35	13.53±0.85	7.86±0.55^c	1.45±0.04^c	138.87±6.38^c	2.16±0.10^b
T_2	6.13±0.3	14.83±1.0	8.7±0.7^c	1.47±0.03^c	141.7±4.62^c	2.11±0.06^b
T_3	5.8±0.81	16.8±1.41	11±0.6^b	1.78±0.10^b	191.17±16.61^b	1.57±0.13^a
T_4	6±0.1	17.36±0.55	11.36±0.45^a	1.77±0.03^b	189.39±4.38^b	1.58±0.03^a
T_5	5.17±0.1	12.8±0.56	7.63±0.46^c	1.51±0.04^c	147.501±5.98^b	2.03±0.08^b
T_6	5.01±0.24	15.16±0.71	10.15±0.83^b	1.84±0.13^b	203.096±23.57^a	1.49±0.18^a
T_7	5.2±0.56	14.12±0.46	8.91±0.94^c	1.67±0.23^b	174.358±37.14^b	1.77±0.4^b
T_8	5.2±0.31	16.85±0.27	11.65±0.1^a	1.96±0.08^a	224.359±15.11^a	1.34±0.08^a

Values in the same column with different superscripts (a, b, c) differ significantly (P<0.05).
Data expressed as mean±SD, n = 3 (each replicate had 10 fish).

Proximate compositions of fish muscles (on wet weight basis) are specified in Table 3. The protein content in fish muscle was observed as 14.9% (T_0), 14.56% (T_1), 14.72% (T_2), 15.58 (T_3) and 15.91% (T_4). The lipid content ranged between 3.56% (T_0) to 3.93% (T_4). The results of the present study revealed that the protein and lipid contents did not vary significantly (P>0.05) among the fishes fed on T_0, T_1, T_2 and T_3 diets whereas, only protein content was observed to vary significantly for T_4. In case of Spirulina fortified diets, a significant difference was noticed for protein content. Which increased from 14.9% (T_0) to 16.89% (for T_7) and 16.93% (for both T_6 and T_8). An increase in carotenoid source in the diet, significantly affected the proximate composition of the fish when spirulina was the source, while marigold did not have any significant impact on carcass composition except T_4 (10% marigold). The findings are in concurrence with others (Boonyaratpalin *et al.* 2001 and Gurung *et al.* 2005) who reported that different sources of carotenoids

Table 3. Effect of marigold flower and spirulina meal on body composition (% wet weight basis) of *B. bendelisis* fingerlings

Treatment	Moisture	Dry matter	Ash	Crude protein	Crude fat
T_0	79.23±0.28	20.76±0.28	2.5±0.07	14.9±0.12^d	3.56±0.35^b
T_1	78.27±0.26	21.72±0.26	2.53±0.06	14.56±0.07^d	3.06±0.06^b
T_2	78.23±0.25	21.76±0.25	2.53±0.06	14.72±0.03^d	3.14±0.04^b
T_3	78.9±0.71	21.09±0.71	2.34±0.05	15.58±0.04^d	3.65±0.08^b
T_4	77.99±0.28	22±0.28	2.49±0.11	15.91±0.03^c	3.93±0.04^b
T_5	75.99±0.14	24±0.14	2.17±0.11	16.57±0.13^b	4.29±0.46^a
T_6	76.57±0.16	23.43±0.16	2.22±0.14	16.93±0.10^a	4.13±0.30^b
T_7	76.31±0.21	23.68±0.22	2.2±0.11	16.89±0.20^a	4.27±0.30^b
T_8	76.24±0.21	23.75±0.21	2.24±0.15	16.93±0.24^a	4.11±0.12^b

Values in the same column with different superscripts (a, b, c) differ significantly (P<0.05); Data expressed as Mean±SD; n = 3 (each replicate had 10 fish).

might have different impact on proximate composition of fish and prawn.

The total carotenoids and mineral composition of fishes exists in Table 4. Marigold improved carotenoids in the fish tissue from 2.09 μg g^{-1} (T_0) to 4.58μg g^{-1} (T_4). Matsuno *et al.* (1980) reported that dietary preference of different species plays an important role in their colouration. Hata and Hata (1972) also suggested that the occurrence of hydroxyl group within molecular configuration of carotenoids enhances its absorption by the digestive epithelium. Animals can not synthesize carotenoids by their own, so they depend on plants and algae to obtain these pigments (Britton *et al.* 1995). The pigments found in fishes are gained from these sources or from their prey. These absorbed carotenoids are transported through the blood to the muscles and skin where they are deposited. Due to higher concentration of carotenoids in the experimental diets, fish, utilized experimental diets better than the control one, which may be a ground for better pigmentation. The outcome of the analysis are also in agreement with those of Boonyaratpalin and Lovell (1977) who experimented with marigold petal meal on tiger barb (*Puntius tetrazona*) and colour was found improved significantly than the control fed fish and with Ezhil *et al.* (2008) also used marigold as a carotenoid source on pigmentation and growth of red sword tail (*Xiphophorus helleri*).

Table 4. Effect of marigold flower and Spirulina meal on total carotenoids (μg g-1) and mineral composition (mg 100^{-1} g) of muscles of *B. bendelisis* fingerlings

Treatments	Total Carotenoids	Na	K	Ca
T_0	2.09±0.041^d	236±10^b	1115±13.4^d	419±28.5^a
T_1	2.42±0.160^d	256±7^b	1093±14.5^d	376±12.0^b
T_2	3.11±0.198^c	243±4^b	1049±14.5^e	395±7.6^a
T_3	3.62±0.251^b	204±4^b	1014±19.5^f	381±28.4^a
T_4	4.58±0.317^a	225±19^b	1024±7.6^f	387±18.1^a
T_5	2.53±0.096^d	242±7^b	1196±8.1^c	346±4.0^b
T_6	3.73±0.175^b	244±9^b	1276±11.5^a	365±5.6^b
T_7	3.72±0.420^b	277±11^a	1256±11.0^a	395±5.0^a
T_8	3.94±0.270^b	266±7^b	1216±23.2^b	451±6.5^a

Values in the same column with different superscripts (a, b, c) differ significantly (P<0.05).

Data expressed as Mean±SD, n = 3 (each replicate had 10 fish).

On the other hand, spirulina fortified diet, improved carotenoids content in the muscles from 2.09 μg g^{-1} (T_0) to 3.73 μg g^{-1} (T_6) and 3.94 μg g^{-1} (T_8). It is to be noted here that total carotenoid levels in the skin of rainbow trout, *Oncorhynchus mykiss* increased significantly with increase in green algae, *Haematococcus pluvialis* in their diet (Sommer *et al.* 1992), while, Choubert (1979) reported that the quantity of total pigments in the skin was greater in trout receiving the feed supplemented with increased amount of spirulina.

Sodium, potassium and calcium were also significantly affected even with increase in either marigold (potassium was negatively significant in marigold added diets) or Spirulina content in the diets. However, our results showed that the fishes fed with spirulina improved the potassium content from 1115 mg 100^{-1}g for T_0 to 1276 mg 100^{-1}g for T_6.

It can be concluded from the study that Spirulina fortified diet has more impact on growth and nutrient profile with moderately less impact on pigmentation, while marigold has superior impact on pigmentation with less impact on the growth and nutrient profile of the fishes.

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Phylogenetic analysis of fishes of the subfamily Schizothoracinae (Teleostei: Cyprinidae) from Indian Himalayas using *cytochrome b* gene

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ABSTRACT

Molecular phylogeny of two genera containing five species fish of the subfamily Schizothoracinae distributed in the north and north-east Himalayas was investigated based on the partial 307 bp *cytochrome b* gene sequences. The sequence analysis data showed that 48 sites out of 307 (16%) were variable without any insertion or deletion. Rate of transition (4.8%) was higher than transversion (0.65%). A total of 12 haplotypes (h) were identified. No haplotype was shared by the five species. The nucleotide diversity (π) ranged from 0.00561 to 0.06073 with least between *Schizothorax richardsonii* and *Schizothorax progastus*. The phylogenetic tree, constructed by neighbour-joining, minimum evolution and maximum parsimony methods revealed similar results suggesting that *S. richardsonii* and *S. progastus* were more closely related to each other than the other species in the subfamily, which was also confirmed by the genetic distance data. The results indicate that *cytochrome b* gene is useful in analysing genetic variation as well as in unravelling phylogenetic relationship in the subfamily Schizothoracinae.

Keywords: *Cytochrome b*, Genetic distance, Genetic diversity, Mitochondrial DNA, Phylogenetic relationship, Schizothoracinae

Introduction

Snow trouts belong to the subfamily Schizothoracinae (Family: Cyprinidae) which consists of 15 genera and over hundred species distributed all over the world (Mirza, 1991). The Indian snow trouts fall under seven genera, majority of which constitute an important part of coldwater fishery in the Himalayan region (Tilak, 1987). These are economically important fishes which inhabit fast flowing snow fed streams and lakes. Due to overexploitation, many of the species were listed as 'endangered' by the National Environmental Protection Agency and Endangered Species Scientific Commission (Yue and Chen, 1998). Classification of snow trouts at species level is generally based on classical, morphological and osteological methods. However, accurate identification of Schizothoracine fishes using morphological characters (*e.g.*, dorsal and caudal fin rays count, length and weight, structure of scales, structure of jaws and lips *etc.*) is difficult due to intraspecific morphological variability and therefore sometimes causes error in proper identification of closely related species. Although, all the species of snow trouts are classified under the subfamily Schizothoracinae, ambiguity remains under the genus level. The taxonomic positions of these species vary according to different sources leading to improper identification of the species.

There are several studies on classification of fishes under Schizothoracinae (Wu, 1984; Chen, 1998; Wu and Tan, 1991). Phylogenetic relationships among genera and species under Schizothoracinae have been investigated based on morphological characters, RAPD analysis (Chen and Chen, 2000; 2001) and mitochondrial *cytochrome b* gene sequence analysis (Dekui *et al.*, 2004; Qi *et al.*, 2005). Mitochondrial DNA (mtDNA) has been one of the most widely used molecular markers for studying intraspecific and interspecies variation in animals because of its simple genomic structure, high nucleotide substitution rate, lack of recombination and maternal inheritance (Avise, 1986; Billington and Hebert, 1988). The availability of mtDNA data has provided new perspectives on taxonomically debatable taxa and confusing questions of phylogeny (Groves and Shields, 1996). Among many mitochondrial genes, the mitochondrial *cytochrome b* gene has been widely used to study genetic variation (McVeigh and Davidson, 1991), phylogenetic relationships (Groves and Shields, 1996; Gilles *et al.*, 1998; Xiao *et al.*, 2001; Perdices *et al.*, 2004; Bajpai and Tewari, 2010; Kumar *et al.*, 2011), biogeographical patterns (Gilles *et al.*, 2001; Xiao *et al.*, 2001; Durand *et al.*, 2002) and taxonomy (Burridge, 1999; Xiao *et al.*, 2001) in many fishes and higher nvertebrates. The rate of evolution of the *cytochrome b* gene is appropriate for investigating events that have occurred within the last 20 million years, such as the evolution of the Cyprinidae (Irwin *et al.*, 1991). In the present study, we analysed the *cytochrome b* sequences of five species of

two genera of the subfamily Schizothoracinae, to infer the phylogenetic relationship among these species.

Materials and methods

A total of 20 individuals of 5 species belonging to two genera *viz.*, *Schizothorax* (*S. richardsonii, S. progastus, S. esocinus* and *S. plagiostomus*) and *Schizopyge* (*S. niger*) were collected from north and north-east Himalayas during October 2009 to March 2010. Fin tissues were preserved in absolute ethanol in the field. The details of collection are given in Table 1.

Analysis of nucleotide composition, overall transition: transversion rate (including *cytochrome b* sequences of *S, richardsonii, S. progastus, S. esocinus, S. plagiostomus* and *S, niger*) and pairwise genetic distance with Kimura 2 parameter method (Kimura, 1980) of sequences were estimated by MEGA 4 (Tamura *et al.*, 2007). Numbers of invariable, variable, singleton variable and parsimoniously informative sites of *cytochrome b* sequences were calculated using DnaSP *vers*. 4.50.2 (Rozas *et al.*, 2003). The haplotype number, haplotype diversity (h) and nucleotide diversity (π) were also performed using DnaSP software.

Table 1. Species, drainages, collection sites, number of haplotypes and GenBank Accession nos. of specimens used in the study

Species	No. of specimens	Drainages, collection site	No. of haplotypes	Genbank Accession no.
Schizothorax richardsonii	4	Bhagirathi, Uttarkashi	3	JN600500- JN600503
S. progastus	5	Bhagirathi, Uttarkashi	3	JN600504- JN600508
S. esocinus	4	Indus, Leh	2	JN600512- JN600515
S. plagiostomus	4	Upper Siang River, Arunachal Pradesh	1	JN600516-JN600519
Schizopyge niger	3	Jhelum, Jammu & Kashmir	3	JN600509- JN600511

Total genomic DNA was isolated from 50 mg fin tissue samples preserved in absolute ethanol using phenol chloroform method (Sambrook *et al.*, 1989). Partial sequence of the *cytochrome b* gene was amplified by PCR (Eppendorf, Mastercycler gradient) using universal Primers CytBF: 5'-AAAAAGCTTCCATCCAACATCTCAGC ATGATGAAA-3' and CytBR: 5'-AAACTGCAGC CCCTCAGAATGATATTTGTCCTCA-3' (Kocher *et al.*, 1989). Amplification was done in 50 µl volume containing 5 µl of 10x PCR buffer (100 mM Tris, pH 9.0, 500 mM KCl, 15 mM $MgCl_2$, 0.1% Gelatin) (B-Genei, India), and 1 unit of Taq DNA polymerase (B-Genei, India), 200 µM of each dNTPs (dATPs, dCTPs, dGTP, dTTPs) (B-Genei, India), 25 pmol of each primer and 50 ng of genomic DNA. The thermal profile used to amplify *cytochrome b* consisted of an initial denaturation of 95 °C for 5 min; followed by 34 cycles of 94 °C for 30 sec, 54 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 7 min . PCR products were stored at 4 °C. For each sample, 3 µl of PCR product was electrophoresed on 1.2 % agarose gel followed by ethidium bromide staining, and visualized under UV illumination in the Gel-Doc system (Alpha Imager 3400, Alpha Innotech Corporation, USA). Molecular weights were determined using 100 bp DNA markers (Fermentas, Canada) and the PCR products were sequenced (Chromas Biotech, Bangalore). A total of 307 base pairs of the *cytochrome b* gene fragment were successfully sequenced for 20 individuals representing five species of subfamily Schizothoracinae. The sequence data were aligned using BioEdit version 5.0.9 (Hall, 1999). All sequences representing *cytochrome b* gene were submitted to the GenBank (Accs. No: JN600500-JN600519).

Phylogenetic relationships among five species of two genera were constructed using nucleotide sequences of *cytochrome b* gene estimated by neighbour-joining, maximum-parsimony and minimum evolution. Phylogenetic analysis was conducted using MEGA 4. Bootstrap support was calculated from 1000 replications.

Results and discussion

The alignment of *cytochrome b* gene sequences showed the presence of a common conserved region in all the five species indicating that these species belong to the same subfamily. This was also confirmed on the basis of homology with previously published sequences from other fish species through NCBI Genbank. The nucleotide sequence alignment is shown in Fig. 1. The alignment data showed that 48 sites (16%) out of 307 bp were variable without any insertion or deletion. Among these 48 variable sites, 44 sites (92%) were Parsimony information polymorphic while 4 sites (8%) were singleton variable sites. Rate of transition (4.8%) was higher than transversion (0.65%). A high transition bias is well known in vertebrate mtDNA (Meyer, 1993). The majority of variable and phylogenetically informative sites of *cytochrome b* were found on first codon position and the rate of transition/ transversion (R=Si/Sv) was also higher in first codon position (R= 11.0). It indicated several million years of evolution involved in the genetic evolution of different cyprinid species (Springer and Douzery, 1996; Wang *et al.*, 2002; Sivaraman *et al.*, 2009).

Among 20 individuals of 5 species belonging to two genera *viz.*, *Schizothorax* and *Schizopyge*, 12 haplotypes

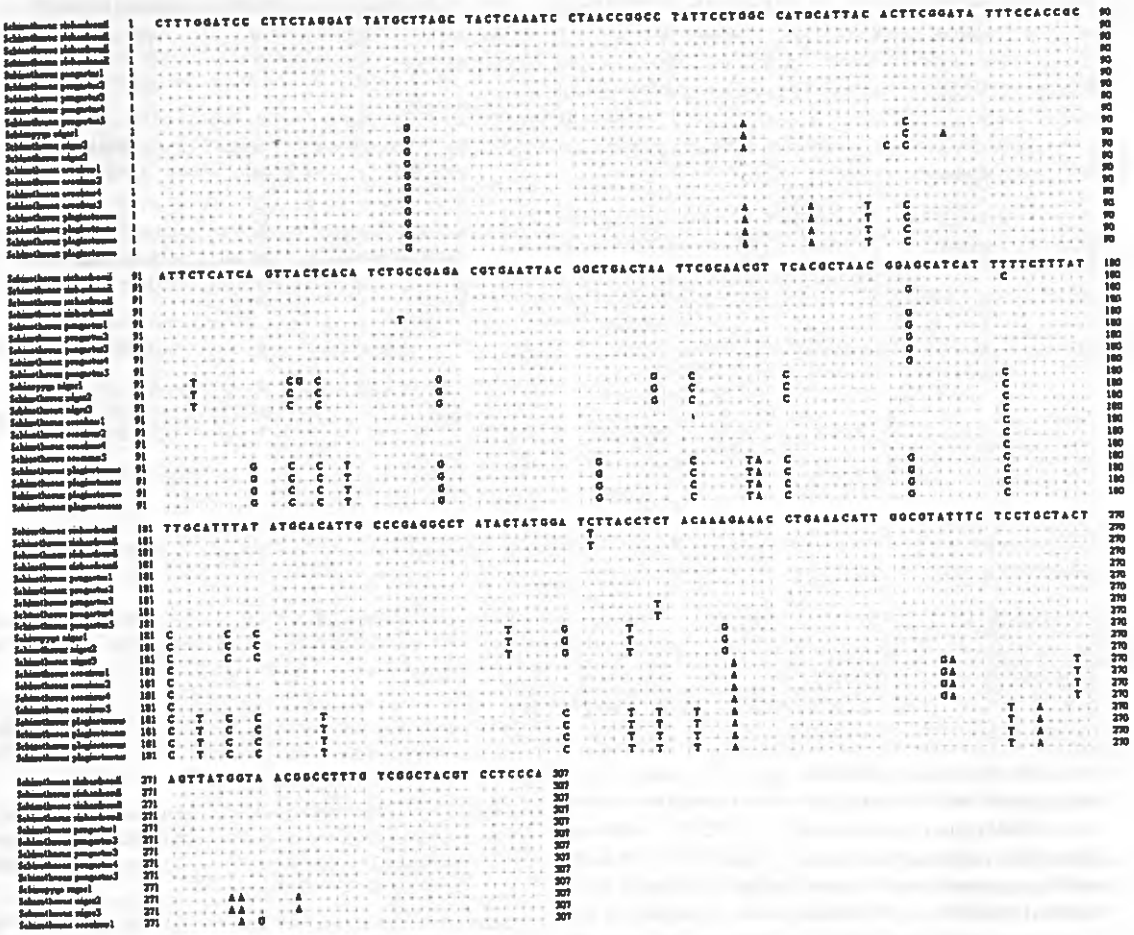

Fig. 1. Aligned sequences for the 307-nucleotide segment of *cytochrome b* gene in five species of subfamily Schizothoracinae (variable positions are indicated with nucleotide).

were observed. Among them 3, 3, 2, 1, 3 haplotype were detected in *S. richardsonii, S. progastus, S. esocinus, S. plagiostomus* and *S. niger* respectively and no haplotype was shared by the five species. Haplotype diversity (h) ranged from 0.000 to 1.000. The haplotype diversity was lowest in *S. plagiostomus* (0.000) and highest in *S. niger* (1.000). Haplotype diversity value (h) was 0.0833 for *S. richardsonii*, 0.8000 for *S. progastus* and 0.667 for *S. esocinus*. Nucleotide diversity (π) was 0.005429, 0.003257, 0.002172, 0.000000 and 0.013029 for *S. richardsonii, S. progastus, S. esocinus, S. plagiostomus* and *S. niger* respectively. The nucleotide diversity was lowest between *S. richardsonii* and *S. progastus* (π=0.00561) and highest between *S. richadsonii* and *S. plagiostomus* (π=0.06073).

The nucleotide sequences of *cytochrome b* gene were aligned in order to determine the phylogenetic relationships among the five species. The phylogenetic tree, generated using three methods (Neighbour-joining, Minimum evolution, Maximum parsimony) was similar. The Neighbour-joining (NJ) tree (Fig. 2) of *cytochrome b* gene sequences of the five species showed that *S. richardsonii* and *S. progastus* formed a monophyletic group (a result

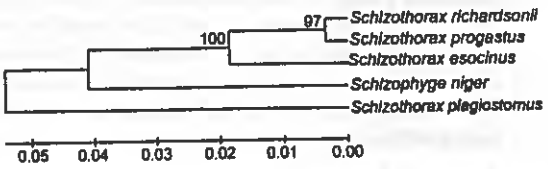

Fig. 2. Phylogenetic tree (Neighbour-joining tree) based on 307 bp mitochondrial *cytochrome b* DNA sequences for five species of Schizothoracinae

strongly supported by high bootstrap value of 97%) and then constitute one clade with *S. ecosinus*; further they constitute another clade with *S. niger* while *S. plagiostomous* formed a different cluster. It was found that the species belonging to the northern Himalayas grouped together while species from north-eastern Himalayas remained separate.

Pairwise genetic distance between the species is presented in Table 2. The mean genetic distance among five species of Schizothoracinae ranged from 0.006-0.116. The lowest pairwise genetic distance was observed between *S. richardsonii* and *S. progastus* while the maximum divergences were observed between *S. richardsonii* and *S. plagiostomous*. This revealed a closer phylogenetic relationship between *S. richardsonii* and *S. progastus* than other species under Schizothoracinae.

Table 2. Pairwise genetic distance (nucleotide Kimura 2 parameter) for *cytochrome b* gene sequences of five species of *Schizothoracinae* fishes

Species	*S. richardsonii*	*S. progastus*	*S. esocinus*	*S. plagiostomus*	*Schizopyge niger*
S. richardsonii	-	-	-	-	-
S. progastus	0.006	-	-	-	-
S. esocinus	0.034	0.037	-	-	-
S. plagiostomus	0.116	0.110	0.113	-	-
Schizopyge niger	0.076	0.079	0.081	0.087	-

The nucleotide diversity, genetic distance and phylogenetic relationship data showed distinct association with similar geographical distribution. The species that are distributed in the same drainage system (*S. richardsonii* and *S. progastus*) as well as the species distributed in the northern Himalayas (*S. richardsonii, S. progastus* and *S. esocinus*) exhibited closer phylogenetic relationship. Dekui *et al.* (2004) also observed that there was close relationship among the species that were distributed in the same drainage system. The present study did not completely resolve the phylogenetic relationships among the five species of subfamily Schizothoracinae, but study of *cytochrome b* sequences in these five species provides useful insights into the taxonomic status of these fishes and sets the stage for future investigations dealing with phylogenetic, taxonomic and conservation issues in this important group. Further studies are required on the phylogenetic relationships of these fishes based on more mtDNA genes, as the recent developmentsin molecular techniques based on these genes are very much useful for establishing taxonomical and phylogenetic relationships among different species.

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Effect of Marigold Flower and Beetroot Meals on Growth Performance, Carcass Composition, and Total Carotenoids of Snow Trout (*Schizothorax richardsonii*)

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Key words: *Beta vulgaris*, carotenoids, FCR, growth, SGR, *Schizothorax richardsonii*, *Tagetes erecta*

Abstract

A 60-day experiment was carried out to elucidate the effect of marigold (*Tagetes erecta)* flower and beetroot (*Beta vulgaris)* meals on growth performance, carcass composition, and total carotenoids of snow trout, *Schizothorax richardsonii*. Two hundred and seventy fingerlings (9.19±0.29 g) were randomly distributed into nine treatments in triplicate (10 fish per tank). Nine isonitrogenous (35.25±0.9% crude protein) diets were prepared with graded levels (3%, 5%, 7%, 10%) of either marigold flower meal or beetroot meal; the control diet contained neither marigold flower nor beetroot meal. Weight gain and specific growth rate were significantly ($p<0.05$) higher in fish fed the diet containing 10% beetroot meal than in those fed the control diet. Body carotenoid was significantly enhanced ($p<0.05$) by the dietary supplements and increased linearly with the increase of marigold flower meal ($Y = 0.532x + 1.126$, $R^2 = 0.9803$) and beetroot meal ($Y = 0.491x + 1.341$, $R^2 = 0.9376$). Results indicate that inexpensive and readily available natural carotenoid sources such as marigold flower and beetroot meals can be incorporated into diets for *S. richardsonii* to enhance pigmentation and ornamental value.

Introduction

Snow trout, *Schizothorax richardsonii*, is a small indigenous coldwater fish locally known as asela. It belongs to the family Cyprinidae, subfamily Schizothoracinae, that is widely distributed in Himalayan and sub-Himalayan regions. *Schizothorax richardsonii* thrives well in coldwater streams, lakes, and rivers and is commercially important due to its ornamental and food value. Hence, it is widely cultured in the hilly regions of Himalaya.

The food value of fish is determined by the quality and quantity of protein and other nutrients in the muscle while the ornamental value is associated with coloration due to carotenoids or pigment-bearing substances in the tissues (Halten et al., 1997). Carotenoids are most conspicuous in petals, pollen, fruit, tomatoes, citrus fruits, and some roots (Tacon, 1981). Higher animals, including fish, are unable to synthesize carotenoids *de novo* (Goodwin, 1984), and are dependent on dietary sources (Hata and Hata, 1972). Therefore, there is a direct relationship between dietary carotenoids and pigmentation in fish (Halten et al., 1997). Fishes in the wild obtain the food of the quality required for proper growth, pigmentation, and nutrient profile. But in captive conditions, a lack of nutrients and pigment-bearing substances can result in retarded growth, faded coloration, and a degraded nutrient profile of the fish. Diets suitable for pigmentation, nutrient quality, and growth have been determined for Atlantic salmon (Storebakken et al., 1987), rainbow trout (Choubert and Storebakken, 1989; Bjerkeng et al., 1992), and ornamental dwarf cichlids (Harpaz, 2007).

Carotenoid pigments can be produced commercially and are commonly used for pigmentation of fish including salmonids (Yanar et al., 2007). However, because of public concerns about the use of synthetic additives, alternative natural carotenoid sources have also been studied. In the aquaculture industry, feed additives such as carrots, red peppers, marigold flowers, rose petals, China roses, chestnut flowers, spirulina, crustacean waste, yeast, synthetic astaxanthin, vitamin C, and vitamin E have long been used to obtain the desired quality of fish (Ellis, 1979; Kim et al., 1999; Ezhil et al., 2008; Yeşilayer et al. 2011; Yilmaz and Ergün, 2011). Red pepper and marigold flower can be used to enhance color in rainbow trout (Yanar et al., 2007). Marigold flower and beetroot are readily available sources of pigmentation in the lower stretches of the Himalayan region. Thus, in this experiment we elucidate the effects of marigold flower and beetroot, which are inexpensive, abundant, and rich in carotenoids, on growth performance, carcass composition, and total carotenoids in snow trout fingerlings.

Materials and Methods

Experimental fish. Fingerlings of *Schizothorax richardsonii* (9.19±0.29 g) were collected from the local Nainital stream in Uttarakhand and transported in a 500-l circular container with sufficient aeration to the experimental facilities at the hatchery complex of the Directorate of Coldwater Fisheries Research in Bhimtal, India. Fish were acclimated to the experimental rearing conditions for one week. During acclimation, fish were fed the control diet.

Experimental design and feeding. Nine isonitrogenous diets (35.25±0.9% crude protein) were prepared with graded levels (3%, 5%, 7%, or 10%) of either marigold flower or beetroot meal; the control diet contained neither marigold flower or beetroot meal (Table 1). After acclimation, the 270 fingerlings were randomly distributed into twenty-seven 100-l fiberglass tanks (nine diet groups in triplicate) following a completely randomized design. Groups were fed their respective diets for 60 days at 5% of the body weight daily, divided into two doses; two thirds of the daily ration was given at 09:00 and one third at 18:00. Fecal matter was removed by siphoning. The flow-through system had a water flow of 2-3 l/min through an inlet at one and an outlet at the opposite end of each tank. Water quality was within optimum ranges throughout the experiment: dissolved oxygen 6.0-8.5 mg/l, pH 7.3-8.2, temperature 18-20°C.

Proximate analysis of feed. The proximate composition of the experimental diets was determined following standard methods of AOAC (1995). Moisture was determined by drying at 105°C to a constant weight. Ash was determined by incinerating the samples in a muffle furnace at 600°C for 6 h. Nitrogen was estimated by the Kjeldahl method (2200

Kjeltec Auto distillation, Foss Tecator, Sweden) and crude protein was estimated by multiplying the percent nitrogen by 6.25. Ether extract was measured by the solvent extraction method (1045 Soxtec extraction unit, Tecator, Sweden) using diethyl ether (boiling point 40-60°C) as a solvent.

Table 1. Diet composition (%) and proximate analysis (% dry matter basis), means±SE, n = 3.

	Diet								
		Marigold flower meal (%)				*Beetroot meal (%)*			
	Control	*3*	*5*	*7*	*10*	*3*	*5*	*7*	*10*
Fishmeal	20.27	20.27	20.27	20.27	20.27	20.27	20.27	20.27	20.27
Soybean meal	20.27	20.27	20.27	20.27	20.27	20.27	20.27	20.27	20.27
Wheat bran	21.73	21.73	21.73	21.73	21.73	21.73	21.73	21.73	21.73
Rice bran	29.73	26.73	24.73	22.73	19.73	26.73	24.73	22.73	19.73
Fish oil[1]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vegetable oil[2]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin-mineral mix[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Sodium alginate[4]	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Marigold flower meal	-	3.00	5.00	7.00	10.00	-	-	-	-
Beetroot meal	-	-	-	-	-	3.00	5.00	7.00	10.00
Proximate analysis (%)									
Moisture	13.28±0.02	12.89±0.03	12.50±0.01	12.85±0.05	12.69±0.02	11.85±0.02	12.15±0.02	12.54±0.03	11.90±0.02
Dry matter	86.72±0.25	87.11±0.41	87.50±0.23	87.15±0.33	87.31±0.54	88.15±0.02	87.85±0.02	87.46±0.03	88.1±9.02
Ash	10.85±0.05	12.05±0.02	12.96±0.04	13.89±0.03	14.90±0.05	10.05±0.07	10.70±0.03	10.25±0.05	10.18±0.04
Crude protein	35.21±0.08	35.40±0.05	35.95±0.02	36.25±0.01	36.70±0.02	34.72±0.04	34.51±0.03	34.35±0.04	34.09±0.01
Ether extract	6.65±0.02	6.23±0.01	6.45±0.02	6.15±0.03	6.20±0.02	5.96±0.02	6.05±0.01	6.27±0.03	6.33±0.03
Carotenoids (µg/g diet)	29.1±0.94	110.12±2.32	178.74±3.13	292.32±7.84	411.28±6.98	120.23±5.41	183.24±6.63	304.14±8.74	407.25±7.51

[1] Procured from local market
[2] Ruchi Soya Industries Ltd., Raigad, India
[3] Agrimin Forte (Virbac Animal Health India Pvt. Ltd., Mumbai 59, India); contains (per kg): Vitamin A 700,000 IU, Vitamin D_3 70,000 IU, Vitamin E 250 mg, nicotinamide 1000 mg, cobalt 150 mg, copper 1200 mg, iodine 325 mg, iron 1500 mg, magnesium 6000 mg, manganese 1500 mg, potassium 100 mg, selenium 10 mg, sodium 5.9 mg, sulfur 0.72%, zinc 9600 mg, calcium 25.5%, phosphorus 12.75%
[4] Himedia Ltd., India

Total carotenoids. Total carotenoids were determined as described by Olsan (1979). Feed samples were gently mashed with a glass rod against the side of a vial, then 5 ml chloroform was added and the vial was left overnight at 0°C. When the chloroform formed a clear 1-2 cm layer above the caked residue, 0.3-ml aliquots of chloroform were diluted to 3 ml with absolute ethanol, and optical density was read in a spectrophotometer at 380, 450, 470, and 500 nm. A blank prepared in a similar fashion was used for comparison. The wavelength at which the maximum absorption was obtained was used to calculate total carotenoids, expressed as µg/g.

Growth study. Growth performance of the fish was measured in terms of weight gain (%), specific growth rate (SGR, %/day), and feed conversion ratio (FCR) using the following equations: wt gain = 100(final wt - initial wt)/initial wt, SGR = 100(Log_e final wt - Log_e initial wt)/no. experimental days, FCR= feed given/wet body wt gain.

Statistical analysis. Mean values of all parameters were subjected to one-way ANOVA to study the treatment effects and Duncan's Multiple Range Tests to determine the significance of differences between any two means. Comparisons were made at 5% probability. All data were analyzed using statistical package SPSS (Version 16.0).

Results

There were very few accidental mortalities during the 60-day trial. There were no significant effects of marigold meal supplementation on weight gain, specific growth rate, or feed conversion ratio (Table 2). Weight gain, specific growth rate, and feed conversion ratio were significantly improved in fish fed the 10% beetroot diet. There was a third order polynomial relationship between percent weight gain and marigold flower meal supplementation, $Y = 6.1258x^3 - 56.893x^2 + 158.35x - 0.546$, $R^2 = 0.9281$ and a second

order polynomial relationship between percent weight gain and beetroot meal supplementation, $Y = 4.5464x^2 - 12.718x + 119.15$, $R^2 = 0.9476$ (Fig. 1a). Crude protein increased with dietary supplementation of marigold flower as well as beetroot meal. There was an inverse correlation ($R^2 = 0.98$) between moisture content and crude fat. Body carotenoid significantly rose with dietary supplementation of marigold as well as beetroot (Fig. 1b). The highest carotenoid was obtained in fish fed the 10% marigold diet, followed by those fed the 10% beetroot diet, which did not significantly differ from fish fed the 7% beetroot diet. Body carotenoid increased linearly with the increasing supplementations.

Table 2. Growth performance and carcass nutrient composition (%wet weight) of *Schizothorax richardsonii* fingerlings fed diets with graded levels of marigold flower meal or beetroot meal, means±SE, n = 3 (10 fish per replicate).

Diet	*Specific growth rate (%)*	*Feed conversion ratio*	*Moisture (%)*	*Dry matter (%)*	*Ash (%)*	*Crude protein (%)*	*Crude fat (%)*
Control	1.22±0.05^{a}	2.80±0.18^{b}	76.98±0.10^{c}	23.02±0.10^{a}	2.74±0.06^{e}	14.86±0.05^{a}	3.39±0.06^{b}
Marigold flower (%)							
3	1.40±0.18^{ab}	2.41±0.49^{ab}	75.75±0.14^{a}	24.25±0.14^{c}	2.16±0.03^{a}	15.00±0.04^{b}	4.27±0.03^{d}
5	1.39±0.19^{ab}	2.44±0.47^{ab}	75.58±0.05^{a}	24.42±0.05^{c}	2.23±0.02^{ab}	15.16±0.02^{c}	4.28±0.02^{d}
7	1.25±0.03^{a}	2.71±0.08^{b}	75.66±0.11^{a}	24.34±0.11^{c}	2.28±0.05^{abc}	15.51±0.02^{e}	4.36±0.02^{d}
10	1.42±0.11^{ab}	2.27±0.27^{ab}	77.04±0.07^{c}	22.96±0.07^{a}	2.38±0.04^{cd}	15.58±0.04^{e}	3.19±0.05^{a}
Beetroot (%)							
3	1.29±0.01^{a}	2.56±0.04^{ab}	76.80±0.09^{c}	23.20±0.09^{a}	2.32±0.03^{bc}	15.27±0.04^{d}	3.41±0.06^{b}
5	1.35±0.02^{a}	2.42±0.05^{ab}	76.14±0.11^{b}	23.86±0.11^{b}	2.33±0.01^{bc}	15.49±0.05^{e}	3.98±0.02^{c}
7	1.40±0.03^{ab}	2.27±0.09^{ab}	77.02±0.09^{c}	22.98±0.09^{a}	2.35±0.05^{bc}	15.72±0.02^{f}	3.36±0.08^{b}
10	1.67±0.02^{b}	1.73±0.04^{a}	76.73±0.08^{c}	23.27±0.08^{a}	2.47±0.04^{d}	15.95±0.04^{g}	3.43±0.01^{b}

Values in a column with different superscripts differ significantly ($p<0.05$).

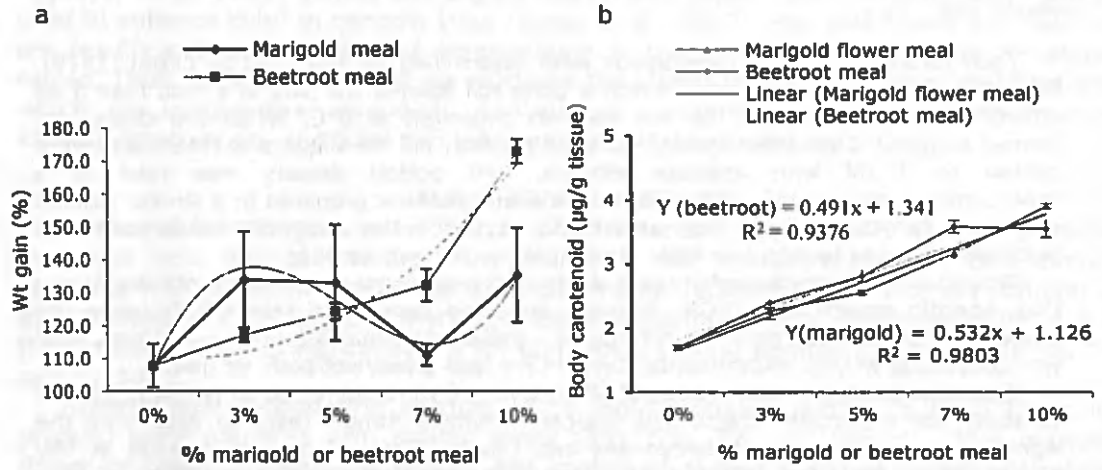

Fig. 1. (a) Growth and (b) total body carotenoids of snow trout (*Schizothorax richardsonii*) fingerlings fed diets containing marigold flower meal or beetroot meal for 60 days.

Discussion

Fish skin color is primarily dependent on chromatophores (melanophores, xanthophores, erythrophores, iridophores, leucophores, cyanophores) containing pigments such as melanins, carotenoids (e.g., astaxanthin, canthaxanthin, lutein, zeaxanthin), pteridines, and purines (Chatzifotis et al., 2005). In common with other animals, fish are unable to biosynthesize carotenoids *de novo,* but they can modify alimentary carotenoids and store them in the integument and other tissues. Farmed fish have no access to natural carotenoid-rich feeds and, therefore, the necessary carotenoids must be added to their

diet. The effectiveness of a carotenoid source in terms of deposition and pigmentation is species specific (Ha et al., 1993).

Marigold flower has color-enhancing effects on fishes (Vernon et al., 1996; Buyukcapar et al., 2007; Yanar et al., 2007; Ezhil et al., 2008). The present investigation shows that carotenoid contents of fish fed diets supplemented with the carotenoid sources (marigold flower or beetroot) were significantly higher than those of the control group at the end of the 60 days, similar to results in rainbow trout fed marigold flower meal and red pepper as carotenoid sources (Yanar et al., 2007). In our previous study we also observed a linear relationship between dietary supplementation of carotenoids and growth (Sarma and Jha, 2010). Red pepper also improves pigmentation in salmonids (Carter et al., 1994; Yanar et al., 1997).

The effects of carotenoids on growth and survival of aquatic organisms are controversial. The specific growth rate and skin coloration improved in *Silurus glanis* fed carotenoid-rich microalgal biomass (Zaťková et al., 2011). Likewise, the growth rate of rainbow trout improved by dietary supplementation of 3.2% marigold flower meal (Buyukcapar et al., 2007). Similarly, in our study, weight gain improved when beetroot was used as a source of carotenoids at higher levels. However, in a study of *Xiphophorus helleri*, the SGR was higher in the unsupplemented control groups than in fish fed carotenoid-supplemented diets (Ezhil et al., 2008). Some studies on crustaceans report non-significant effects of dietary carotenoid on both growth and survival (Yamada et al., 1990; Harpaz et al., 1998) and growth (Yilmaz and Ergün, 2011). In contrast, dietary supplementation of carotenoids resulted in no differences in growth or survival of characins, *Hyphessobrycon callistus* (Wang et al., 2006).

The increased crude protein content of fish fed supplementary marigold flower meal agrees with the significant increase in meat protein obtained in rainbow trout fed marigold flower meal (Buyukcapar et al., 2007). We are unable to find literature with which to compare the effects of beetroot meal supplementation on whole body composition.

In conclusion, this study shows that dietary supplementation of either marigold flower meal or beetroot meal can enhance growth of *S. richardsonii*. Dietary supplementation of marigold flower meal at 10% produced the highest carotenoid accumulation in the flesh. Since synthetic carotenoids are expensive, cheap and readily available natural carotenoid sources such as marigold flower and beetroot can be incorporated into snow trout diets to obtain greater pigmentation and market value. This will help farmers and other stakeholders realize greater profits in the culture of this species.

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The microcystins-induced DNA damage in the liver and the heart of zebrafish, *Danio rerio*

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Microcystins (MCYST) are the freshwater cyanobacterial toxins, known to induce hepatocellular carcinoma, necrosis, intrahepatic bleeding, as well as human and livestock mortality. Within hepatocytes, MCYST selectively bind to protein phosphatases 1 and 2A, resulting in severe liver damage. The toxicology of MCYST in mice and rats has been well studied, but little is known regarding genotoxicity in aquatic animals. In this study, the zebrafish, *Danio rerio* was exposed to crude extract of *Microcystis aeruginosa* bloom. Liver and heart were examined for MCYST-induced toxicity. Light microscopy at 36 h revealed severe, widespread apoptotic necrosis of the majority of hepatocytes, and cytoskeletal deformation in myocardiocytes. Hepatocytes were dissociated with cell shrinkage and margination of nuclear chromatin. Laddering of genomic DNA from the liver and heart of the exposed fish in an increment of 180–200 bp was consistent with apoptosis. Fluorimetric analysis of DNA unwinding was carried out to determine the DNA strand breakage. After 36 h exposure, the % double-stranded DNA was significantly reduced in hepatocytes and myocardiocytes. In conclusion, the results obtained in this study indicate that, the extract of *M. aeruginosa* bloom is genotoxic to fish. The DNA damage observed in this study may be attributed to the activation of DNA endonucleases. This model of DNA damage may contribute for identifying novel molecular mechanisms of interest for therapeutic application.

Keywords: microcystins; zebrafish; genotoxicity; DNA damage; apoptosis

Introduction

Accelerating eutrophication of waters results in blooms of planktonic algae, especially cyanobacteria (Krüger et al. 2010; Žegura, Strase, and Filipic 2011). Cyanobacteria form dense surface films, and scums are often observed when wind blows the algal cells together. The death of humans (Bell and Codd 1994; Peuthert, Chakrabarti, and Pflugmacher 2007; Pflugmacher, Hoffmann, and Hubner 2007), domestic, and aquatic animals (Gunn, Rafferty, and Rafferty 1992; Negri, Jones, and Hindmarsh 1995) from ingesting cyanobacterial cells has been often reported. Among the toxic cyanobacteria, the water bloom of *Microcystis aeruginosa* is commonly observed in highly eutrophic lakes and reservoirs. Poisoning by *M. aeruginosa* occurred in many countries globally (Falconer, Beresford, and Runnegar 1983; Carmichael 1994; Chorus et al. 2000).

Microcystis aeruginosa produces a family of potent heptapeptide toxins, called microcystins (MCYST). MCYST are the largest and most diverse group of cyanotoxins, consisting of over 80 congeners (Gupta et al. 2003). After ingestion, MCYST are readily taken up by bile acid carrier proteins and transported to liver (Eriksson et al. 1990). MCYST functions as a protein phosphatase (PP1/PP2A) inhibitor (Runnegar et al. 1999), and its presence in the liver results in hyperphosphorylation of many types of functional proteins. Hyperphosphorylation produces cytoskeletal deformation in hepatocytes, hemorrhage, and necrosis (Carmichael 1992; Falconer and Yeung 1992).

The toxicology of MCYST in mice and rats has been well studied (Runnegar, Kong, and Berndt 1993; Rudolph-Bohner, Mierke, and Moroder 1994). Bioaccumulation of cyanotoxins by aquatic animals, including fish, mollusks, and zooplankton, was reported (Williams et al. 1997; Amorin and Vasconcelos 1999; Freitas de Magalhães, Moares Soares, and Azevedo 2001). Indications that MCYST exert adverse effects on fish under field conditions were observed (Anderson et al. 1993; Rodger, Turnbull, and Richards 1994; Zurawell et al. 2005). The effects of MCYST in seen fish include impaired liver function, reduced growth rate, inhibition of gill ion transport, and changes in blood chemistry (Kotak et al. 1996; Zambrano and Canelo 1996; Liu et al. 2002; Paliková et al. 2007). Some information is available on the effects of MCYST on the embryonic development of aquatic animals (Dvorakova et al. 2002; Liu et al. 2002; Jacquet et al. 2004; Wang et al. 2005), but little is known regarding genotoxicity. There are evidence suggesting that MCYST induces the formation of reactive oxygen species (ROS), which induce DNA damage, *in vitro* (Žegura, Sedmak, and Filipic 2003; Žegura, Lah, and Filipiè 2004; Palus et al. 2007) and *in vivo* (Rao and Bhattacharya 1996; Gupta et al. 2003). The genotoxicity of MCYST was reported for rodents and mammalian cell lines (Repavich et al. 1990; Hooser 2000; Žegura, Lah, and Filipiè 2004; Žegura, Straser, and Filipic 2011), but not for freshwater fish. Thus, the purpose of this study was to characterize DNA damage in the liver and heart of zebrafish using histochemistry, DNA laddering, and fluorimetric analysis of DNA unwinding (FADU) assays.

Materials and methods

Fish husbandry

Adult zebrafish (*Danio rerio*) were procured from the Vikrant hatchery, Mumbai (India). Fishes were raised in 30 L tanks at 26°C, with a 12 h light/dark cycle.

Sample material

Microcystis aeruginosa samples were taken in June 2010 with a conical net of 25 µm mesh size from surface waters of Bhimtal Lake (Uttarakhand, India). Bhimtal Lake, one of the largest cold water lakes in the northern Himalaya of India, experience sporadic cyanobacterial bloom formations due to its eutrophicated status, especially during summer months. This lake is popular for tourism, Mahseer fishery (*Tor* spp.), and cage culture of cyprinids. *Microcystis aeruginosa* cells were harvested via centrifugation (6000 × *g*, 3 min) and frozen at −20°C until the extraction of MYCST.

Extraction of cyanobacterial toxins

The MYCST extract was prepared by the method of Ding et al. (1998). The lyophilized algae cells (100 mg) were briefly suspended in 10 mL *n*-butanol : methanol : water (1 : 4 : 15, v/v/v) with high-speed stirring at room temperature for 1 h. The suspension was centrifuged at 12,000 × *g* for 30 min. The collected supernatant was dried at 56°C, and dissolved in 5 mL of 20% methanol. The extracted fraction was then passed through a preconditioned SepPak C18 cartridge and eluted with 10 mL methanol. The elute was evaporated to dryness at 56°C and dissolved in 4 mL of distilled and deionized water. The extract was kept at 4°C for subsequent tests.

Toxicity testing

Zebrafish were exposed to *M. aeruginosa* bloom extract, at a concentration of 30 mg biomass dry weight L^{-1} in three replicates. Each exposure group contained 12 adult zebrafish, which were maintained in a glass aquarium. The total duration of the exposure was 36 h with no water exchange. After 36 h exposure, fish were dissected under a dissecting microscope, and liver and heart were aseptically excised for histochemistry, DNA laddering, and FADU assay.

Histochemistry

Histochemistry was done on polyformaldehyde fixed tissues to determine damage induced by MCYST. The fixed tissues were processed in a standard fashion using an automated processor and then embedded in paraffin from which, 3 mm sections were cut. These 3 mm sections were placed on charged slides and deparaffinized in three changes of xylene followed by rehydration in graded alcohol series. Slides were then stained with eosin and hematoxylin, and examined under Leica DMLS microscope.

DNA laddering Assay

The zebrafish liver and heart were rinsed with ice-cold PBS twice and lysed in buffer containing 0.1 M Tris-Cl (pH 8), 1 M NaCl, 0.01 M EDTA (pH 8) for 30 min at 55°C. RNase A was added, and the sample was incubated at 68°C for 10 min followed by centrifugation. DNA in the supernatant was extracted using phenol/chloroform/isoamyl alcohol. The extracted DNA was precipitated using cold isopropanol followed by centrifugation. The DNA pellet was washed in 70% ethanol, dissolved in TE buffer (100 mM Tris-Cl and 10 mM EDTA, pH 7.6), and quantified spectrophotometrically. The DNA samples of 3 µg each from the liver and the heart were loaded in the wells of 1.5% agarose gel containing 0.1 mg mL^{-1} ethidium bromide. The gel was photographed under ultraviolet light.

Fluorimetric analysis of DNA unwinding

The FADU was done by the method described by Birnboim and Jevcak (1981), which measures the rate of the unwinding of cellular DNA on exposure to alkaline conditions. In brief, the liver and heart cells were resuspended in basal salt solution, and distributed equally. Cells were lysed and chromatin was disrupted with a urea solution. An alkaline

solution was added, and DNA strand unwinding was allowed to occur at 15°C for 75 min. After this treatment, the amount of residual double-stranded DNA (DSD) was estimated by adding ethidium bromide in all the sample tubes. Fluorescence of the samples was measured by Shimadzu RF 5000 spectrofluorophotometer. The extent of DNA unwinding was measured at 15°C for different durations: 0, 15, 30, 45, 60, and 75 min.

Statistics

The mean value ± S.E.M from three experiments were evaluated by a Student *t*-test (SPSS statistics version 19). Values with $p < 0.05$ and $p < 0.01$ were considered statistically significant.

Results

Microscopic examination revealed that the water bloom was dominated by *M. aeruginosa* (approximately 95.5%) (Figure 1). Exposure of *M. aeruginosa* bloom extract resulted in 100% mortality of zebrafish within 36 h, preceded by signs of lethargy, slowed reflexes, and decreased swimming activity. Upon necropsy, the fish showed distended liver engorged with blood, and accumulation of white fluid in the body cavity. Histological examination of liver revealed massive intrahepatic hemorrhage, and severe shrinkage, dissociation, and detachment of hepatocytes. Sinusoidal space was filled with blood along with mild infiltration of mononuclear cell. Nuclei of majority of the hepatocytes were condensed, fragmented, and stained dark with nuclear stain (Figure 2). Liver cells exhibited the signs of apoptosis.

The heart of exposed fish showed edema, dissociation, and congestion of blood vessels. Histological examination of myocardiocytes revealed cytoplasmic vacuolation (Figure 3) with early degenerative changes in nuclei.

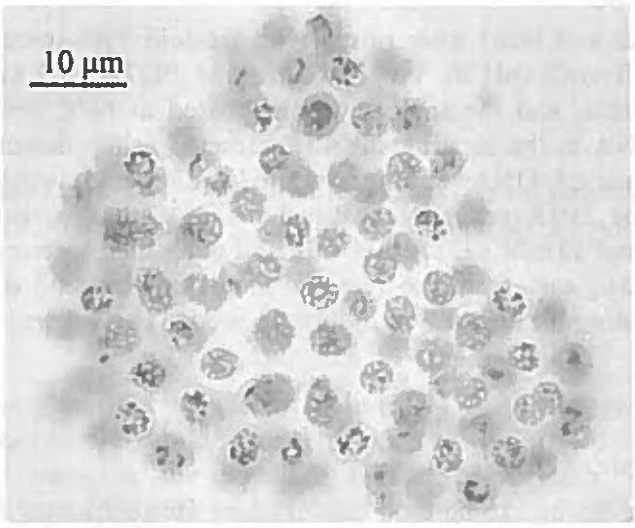

Figure 1. Live colony of *M. aeruginosa* cells packed and embedded in a gelatinous matrix. The individual cells in a colony are 3–4 µm in diameter. The dark spots in the individual cells in this picture are due to the reflection of light from the gas vesicles (magnification 1000×).

Qualitative analysis by agarose gel electrophoresis was carried out to assay the oligonucleosomal fragmentation. Agarose gel electrophoresis of DNA isolated from the liver and the heart of MCYST-treated zebrafish exhibited DNA laddering (Figure 4). However, DNA isolated from the livers and hearts of control fish did not show laddering of low molecular weight DNA, indicating that the procedure was not producing an artifactual breakdown of DNA during the isolation process. The DNA laddering was in an increment of 180–200 bp in both organs, which is consistent with fragmentation at the internucleosomal sites. Data indicate that toxicity produced by MCYST in the liver and heart is associated with features, characteristics of apoptosis.

The FADU method is based on the rate of alkaline unwinding of DNA being dependent on the length of the DNA molecule. A decrease in % DSD after treatment

Figure 2. Hematoxylin- and eosin-stained section of liver from zebrafish exposed to *M. aeruginosa* bloom extract for 36 h. The arrows point out the apoptotic cells with typical condensation and fragmentation of the chromatin (magnification 1000×).

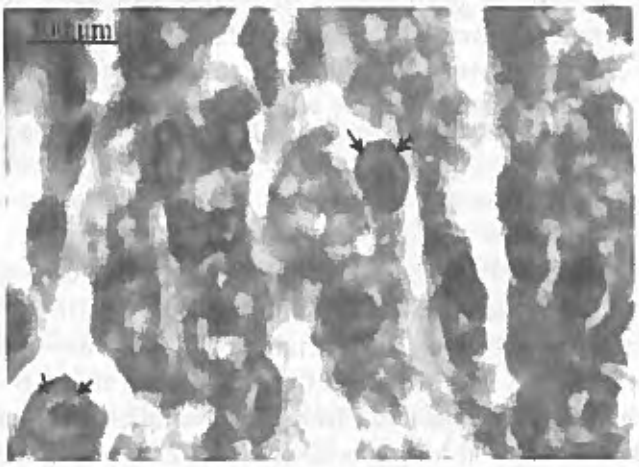

Figure 3. Section of heart stained with hematoxylin and eosin showing cytoplasmic vacuolation along with early degenerative change in nucleus. The arrows point out the vacuole in the cells (magnification 1000×).

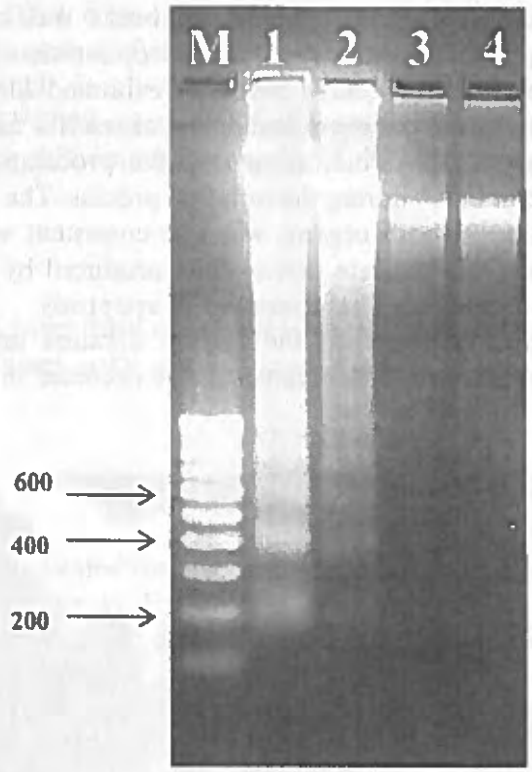

Figure 4. Agarose gel electrophoresis of DNA from the liver and the heart samples following the treatment of zebrafish with MCYST. Lane assingment; M corresponds to a 100 bp marker, Lanes 1 and 2 are DNA laddering and smearing in the livers and hearts of zebrafish, respectively. Lanes 3 and 4 are DNA from the livers and hearts of zebrafish not exposed to MCYST extract. DNA was extracted 36 h after treatment. Numbers to the left of the figure indicate DNA size in bp.

relates to the formation of strand breaks. The effect on DNA unwinding at 15°C for different durations, for both organs, is shown in Figure 5(a) and (b). The % DSD in the livers and hearts of the control fish varied from 82.6 ± 2 to 78 ± 2.08 and 80 ± 3 to 74 ± 4.9, respectively. The % DSD in both organs of treated fish were significantly lower than controls. In the livers and hearts of the exposed fish, the % DSD was 81 ± 2.82–50 ± 3.1 and 78 ± 2.82–54 ± 2, respectively. With increase in the duration of treatment, there was a linear decrease in % DSD, indicating the effect was time dependent.

Discussion

Exposure of zebrafish to *M. aeruginosa* bloom extract resulted in 100% mortality of fish within 36 h. Our study demonstrates that mortality is due to MYSCT-induced severe hepatic and cardiac DNA damage. The mortality of the fish may be correlated with higher content of MYSCT within the *M. aeruginosa* cells. Our study is consistent with some earlier studies, where MCYST isolated from field samples are found to be highly toxic (Jungmann and Benndorf 1994; Mankiewicz et al. 2002). Correspondingly, in the study by Best, Eddy, and Codd (2001), the alevins were more sensitive to cyanobacterial extracts than purified MC-LR in brown trout alevins (*Salmo trutta* L.) exposed to aqueous extracts of *Microcystis* and purified MCYST-LR.

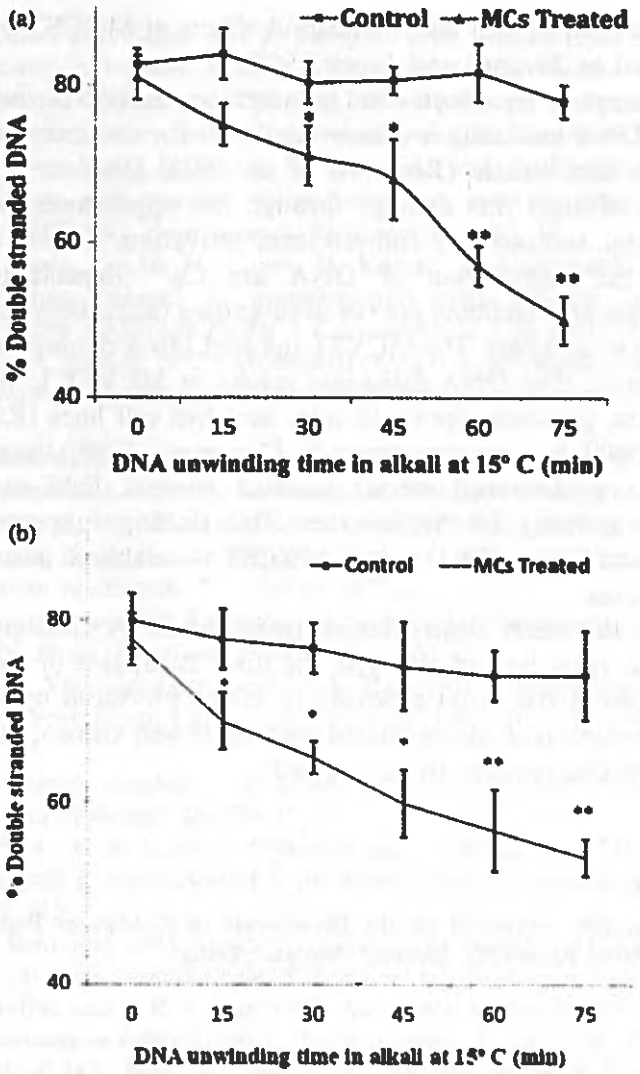

Figure 5. The kinetics of DNA unwinding of control and MC-exposed zebrafish in alkali at 15°C in (a) liver cell and (b) heart cell suspension. Percentage of DSD remaining after unwinding at different durations. Control fish are not exposed to *M. aeruginosa* extract.
Significance at $*p < 0.05$ and $**p < 0.01$ by Student *t*-test.

Apoptosis is an ultrastructural change, which is characterized by nuclear fragmentation, nuclear condensation (Kerr, Wyllie, and Currie 1972), and the presence of apoptotic bodies. Microscopic examination of the zebrafish hepatocytes revealed the nuclear fragmentation and condensation. This is in agreement with Bøe et al.'s (1991) study, who demonstrated that changes in isolated rat hepatocytes induced by MYSCT were apoptotic. In another study in carp (*Hypothalmichthys molitrix*, *Cyprinnus carpio* and *Carassius linnaeas*), Li et al. (2000) reported that toxicity produced by MYSCT in fish liver hepatocytes was also due to the induction of apoptosis. In this study, severe degenerative change in the heart in the form of vacoulation was observed.

This supports the work of Wickstrom et al. (1995) who reported cytoskeletal deformities in non-hepatocytes cell lines *in vitro*, suggesting a common mechanism of action once MYCST enter the cell. In a previous study, the heart rate, stroke volume, and cardiac output in brown trout alevins increased significantly upon exposure to *Microcystis*

extract (Best, Eddy, and Codd 2001). Harmful effects of MYCST on cardiac function in fish was also noted by Sivonen and Jones (1999).

The DNA damage in hepatocytes and myocardiocytes were further confirmed by DNA laddering assay. DNA laddering is closely related to the documented apoptotic effects of MCYST on cells and tissues (Repavich et al. 1990; Ding et al. 1998). Agarose gel electrophoresis confirmed this damage through the appearance DNA laddering in an increment of 180 bp, indicative of endonuclease activation. Several nucleases and factors responsible for the degradation of DNA are Ca^{2+} dependent. Calcium-mediated cytoskeletal changes and blebbing are two well-known characteristics of MCYST-induced toxicity (Eriksson et al. 1990). The MCYST-induced DNA damage may be attributed due to such phenomenon. The DNA damaging results of MCYST in our investigation is in agreement with the previous reports in mice and fish cell lines (Rao and Bhattacharya 1996; Rao et al. 1998). In a previous study by Ding et al. (1998), the genotoxic potential of the microcystic cyanobacterial extract isolated from a field sample induced DNA fragmentation in primary rat hepatocytes. This finding supports the conclusions of Žegura, Straser, and Filipic (2011), where MYCST were able to induce DNA laddering in mammalian cell lines.

In conclusion, this study clearly demonstrates the DNA damaging effect of MCYST. This indicates that regardless of cell type, the toxin is capable of inducing DNA damage *in vivo*. Human health risk from exposure to toxins produced by harmful algal blooms through the consumption of contaminated sea food is well known, but the risk posed from freshwater cyanotoxins remains to be assessed.

Acknowledgments

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Use of PGE for successful spawning of snow-trout, *Schizothorax richardsonii* (Gray)

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Abstract: The study highlights the results of experiment conducted on breeding operation of snow-trout, *Schizothorax richardsonii* (Gray), by dry stripping method during the period of July - November 2008 in Central Himalayas region. Healthy brooders of the age of 2-3 years were collected from river stream having the average body weight of 175 ± 5 gm & 62 ± 5 gm for female & male, respectively and successfully bred from 30th August to 3rd November at water temperature of 12.4 -18.5°C. Males were always ripe during the entire study while females were matured during 15th September to 5th October in normal condition. The general breeding season (15 September - 5 October) was extended by early maturity of female with slight dose of P.G.E. @ 1-2 mg/kg body weight and late maturity of females in poly house covered tank by raising 2°C temperatures during the breeding season. The G.S.I. was observed 1.2 - 14.3 with fecundity in the range of 10520 - 24124 eggs/kg body weight. In the present study, fertilization rate 40 - 70%, hatching rate 67- 81%, incubation period 255 - 270 hours and survival rate 55% were observed at most suitable breeding temperature of 14 - 18°C. The water quality of hatchery, nursery and brood stock pond was monitored with optimum range of temperature 8.0 - 19.3°C, pH 7.8 - 8.7, DO 7.0 - 9.4 mg/l, free CO_2 1.4 - 2.0 mg/l and total alkalinity 20-24 mg/l with water flow rate of 0.5 - 1.0 l/m in hatchery, 2-3 l/m in nursery and 10-30 l/m for brood stock tanks.

Keywords: Stripping, P.G.E., G.S.I., fecundity, survival rate.

INTRODUCTION

The major fishery in the uplands mainly consists of very popular Snow trout and Mahseer, though the introduced exotic trout are only limited to a few streams and in culture ponds. Trout, considered to be high priced and low volume fish, forms a sizable fishery in North-Western Himalayas. Snow trout is endemic to the Himalayas and, true to its name, it is found in streams and lakes which receive snow melt water from the hills. In winter months, when water in the upper reaches of the streams nearly touches 0°C, snow trout migrates downstream for a considerable distance in the Shiwalik Himalayan streams (Sehgal, 1971). Most of the snow trout species are of Central Asian origin. Nine principal species belonging to two genera viz. Schizothoraichthys and Schizothorax, inhabit the Himalayan region. Asela is considered as an economically important high value fish of the Himalayan and sub-Himalayan regions. According to the previous reports, the Schizothoracids are inhabitants of snow-melting rivers, streams and lakes usually attain sexual maturity when temperature starts warming up, but when caught in pre-spawning periods and stocked in long cemented ponds in a fish farm, they do not respond to induced spawning by stripping. According to (Joshi, 2004, 2005, 2006), the spawning period of this fish in the Kumaon region is started from 3rd September to18th September. In India, initial success in artificial breeding of wild stocks of *Schizothorax* (richardsonii), Schizothoraichthys (esocinus, niger, micropogon, longipinnis, curvifrons), was achieved by Vass *et al.*, (1978); Raina *et al.*, (1986), Raina (1992) etc. Preliminary experiments on artificial fecundation and nursery rearing of S. richardsonii under controlled conditions were conducted at Chhirapani fish farm of NRCCWF, Champawat (Kumaon Himalaya) by Joshi and Sunder (1995). Previous studies also revealed that species has 'synchronism' type of maturation as per the classification of (Yamamoto *et al.*, 1959). These observations are in agreement with the observations by other workers on the same species from Western Himalayan waters (Qadri *et al.*, 1983). Short spawning period and 'synchronism' type of maturation are the major hurdles in the seed production of this fish for stock augmentation and aquaculture purpose. The present study was aimed to try the PGE for extending the spawning period and to mature the females.

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MATERIALS AND METHODS

The experimental work was conducted at the Experimental Fish Farm, Chhirapani, Champawat (Uttarakhand), located at an altitude of 1620 m asl in the central Himalaya (Long. 80007I N, Lat. 29030I E). Healthy fish of 2-3 years age group from the Rautis gaad/stream were collected in the month of July, 2008 by cast netting during morning hours and Urli trapping at night hours. A total of 180 healthy fish of 2-3 years age group with a total biomass of 30.2 kg were stocked in the two ponds having the size of (10m x 3m x 1.25m) at stocking density of 0.5 kg/square meter. Body length and corresponding weight of the stocked females ranged between 208-315 mm and 100-240g and that of the males between 160-220 mm and 40-136 g, respectively. Continuous flow of water was maintained at the rate of 10-30 liters per minute in the brood stock pond. The stock was fed with a laboratory compounded wet diet prepared after Bhanja *et al.*, (2001), comprising 32% crude protein. Feeding was done @ 2-5% of per kg body weight.

A slight dose of Pituitary Gland Extract (PGE) @ 1-2 mg/kg body weight was given to only the female individuals of the second group during the last week of July month.

GSI were calculated by the standard formula:

$$GSI\ (\%) = \frac{\text{Weight of gonad x 100}}{\text{Weight of the fish}}$$

The stripping was conducted by 'dry method' followed by the procedure as outlined by (Greenberg 1960 and Raina 1992).

Total numbers of fertilized eggs after they were water hardened were estimated by volumetric method. The fertilization rate was calculated by the standard formula.

$$\text{GFertilization rate (\%)} = \frac{\text{Number of fertilized eggs} \times 100}{\text{Total number of eggs}}$$

The hatching rate was calculated by the formula:

$$\text{Hatching rate (\%)} = \frac{\text{Number of hatchling} \times 100}{\text{Total number of fertilized eggs kept}}$$

Survival rate was calculated by.

$$\text{Survival rate (\%)} = \frac{\text{Number of live larvae}}{\text{Total number of hatchlings kept}} \text{ x } 100$$

During the experimental period different physical and chemical parameters (flow rate, temperature, pH, dissolved oxygen, free carbon dioxide and total alkalinity) of hatchery water fry rearing tank and broodstock tank water were analyzed as per the standard methods (APHA, 1985).

RESULTS AND DISCUSSION

The previous findings reveal that the fish spawn at different elevations in different months of the year at a water temperature range 12.0-21.5°C. Jhingran and Sehgal, (1978) reported for *S. richardsonii* to breed during March, May-June and Ocober-November in Himachal Pradesh. In central Himalayan rivers (Uttarakhand), *S. richardsonii* is reported to breed twice a year i.e. July-October and Feb-May. Sharma *et al.*, (1998) have reported *S.richardsonii* and *S. progastus* to breed from March to May in north-eastern Himalaya of Assam and Arunachal Pradesh. According to Joshi (2004 & 2006), the spawning period of this fish in the Kumaon region is started from 3rd September to18th September.

It was observed that male brooder were ready to harvest the sex product (milt) in both groups of the broodstock during the last week of August. But, females of the untreated group were still immature to spawn. 'Synchronism' type of maturation in this fish was also reported by (Yamamoto *et al.*, 1959). Most of the females of the treated (PGE) group were ready to spawn during last week of the August. The first breeding operation was conducted on 30th August with PGE treated females of second group. In untreated group, most of the females respond to spawn during the period from 15th September to 5th October. Hence, the second and third breeding operations were conducted on 15th September and 5th October with females of first group. The sex of the fish was identified on the basis of distinguishing characteristics and found that the collected stock of 90 matured fish of the second group contained 35 female and 55 male with a total biomass of 15.2 kg. Hence, the frequency of the availability of the female individuals is comparatively less than the males in wild population. The Ganado-somatic indices, being the measure of sexual maturity and one of the important tool to assess the degree of ripeness of gonads, was observed as 2.1-14.3 for both

sexes of *S. richardsonii*. Raina *et al.*, (1986) reported GSI as 1.5-10.8 in *S. niger* from Dallake (Kashmir). 1.9-14.8 in *S. richardsonii* from Sind Nallah (Kashmir), 0.9-12.1 in *S. curvifrons* from river Jhelum (Kashmir). 2.2-14.2 in *S. longipinnis* from river Jhelum (Kashmir) and 2.2-14.2 in *S. richardsonii* from river Panauti (Nepal).

Table 1. Details of the stripping and incubation experiments.

Date Of stripping	Av. weight of female (g)	Av. Length of female (mm)	Av. Nos. of eggs	Av. Ova diameter (mm)	Water Temp (°C)	Fert. Rate (%)	Incu. Period (Hrs.)	Hat. Rate (%)	Surv. Rate (%)
30.8.08	170	265	2340	2.0	18.5	62	255	68	42
15.9.08	120	229	5513	2.6	17.8	70	261	74	52
5.10.08	122	232	4650	2.4	16.5	68	265	70	55
3.11.08	114	220	1245	2.5	12.4	40	270	56	48

Table 2. Physico-chemical parameters of water.

Month	Water Temp. (°C)	DO (mg/l)	Free CO_2 (mg/l)	pH	Total alkalinity (mg/l)
July -2	18.3	7.4	1.4	7.8	24
Aug-1	18.6	7.2	1.4	7.8	22
Aug-2	19.3	8.0	1.4	8.4	22
Sept-1	19.2	8.0	2.0	8.0	20
Sept-2	19.0	8.8	2.0	8.7	22
Oct-1	15.2	7.6	2.0	7.9	22
Oct-2	15.0	7.0	2.0	8.0	22
Nov-1	8.0	9.4	2.0	7.8	22

It was observed during the study that the fully ripe eggs, which were orange in colour started degeneration within 3-4 days, if not extruded in time. The over-ripe eggs turned dull orange in colour. During the study the ova diameter were observed in the range of 2.0-2.6 mm. The number of eggs released from each female varied between 1245-5513 (10520- 24124 eggs/kg body weight). In the present study fertilization rate were recorded between 40-70%, while in another experiment conducted by (Joshi, 2004) in wild stock, it was 80-94.2% at ambient water temperature range of 22.5-24°C. Theincubation period were between 255-270 hrs at 14-18 °C water temperature. The hatching rate was between 67-81% in different operation. The newly hatched sac fry are very tender, creamish-yellow in colour with a large yolk sac, which is more than half the length of the body. The length of the sac fry (alevins) varied from 7.4-8.5 mm (weight of 0.014-0.022 g). The absorption of yolk sac completes within 120-165 hrs at the temperature 16-18 °C, by that time, the fry grows to a size range of 10.0-12.5 mm in length. The sac fry swim occasionally but quite fast and rest on their lateral side due to large bulging of yolk sac. The eye spots and eye starts developing after 20-23 hours of post hatching. The melanin (black colour) appears on the body after 70 hours of hatching, which becomes darker later. Development of the dorsal and caudal fin starts after 5th day of post hatching and

transforms later to deeply emarginated shape. Development of the paired and unpaired fins is completed within 20-25th days after hatching. After complete absorption of yolk the fry starts feeding on supplementary diet with 45% protein level.

The young ones were initially reared in troughs in the hatchery and regularly fed on artificially formulated diet. The fry after attaining a size of about 20.0 mm were shifted out from hatchery to small rearing ponds having continuous water flow facilities. A total of 4773 sac fry were produced from incubating 6450 fertilized eggs, with 74% hatching. 18 days were taken from hatchling to free swimming stage with the survival rate 55% at 12-16°C.

The water quality of the brooder stock tank, hatchery and nursery trough was recorded as, water temperature 8-19.3°C, pH 7.8-8.7, DO 7-9.4 mg/l, free CO_2 1.4 - 2 mg/l and total alkalinity 20-24 mg/l which was favorable for successful breeding of this species. Findings of this study would be helpful for seed production programme of snow trout with extended breeding season and better recovery of fry at favorable water temperature.

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Recycling of piggery waste in Azolla-Pig-Fish farming system

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Abstract: An attempt was made to evaluate the integrated azolla-pig-fish farming system for the growth and production of fish, pig and water quality in fish pond under on farm trial of 12 months. Aquatic fern azolla (*Azolla pinnata*) was grown in the waste stabilization tank and used as pig fodder. Remarkable difference was observed in the values of BOD with a level of 16.85 3.85 mg/l in control pond and 6.15 3.65 in azolla integrated pond. A total of 20.06 per cent increase in total yield (5315 kg/ha) with 81.25- 98.95% survival was achieved in azolla-pig-fish integrated practice. Cost of production of pig was reduced to 14.5% through utilization of azolla in pig diet at 20% level. This is a technical and economic feasible model of integrated fish farming.

Keywords: Integrated azolla-pig-fish farming system, stabilization tank, BOD, pig diet

INTRODUCTION

Integrated livestock fish farming system is a proven environmentally sustainable and economically viable technology that encompasses rational utilization of available resources. Different forms of integrated livestock fish farming viz. pig-fish, poultry-fish, duck-fish etc. have been evolved and popularized in India (Sharma *et al.*, 1985). Cruz and Shehadeh (1980); Delmendo (1980); Sin (1980); Hopkins and Cruz (1982), Jhingran and Sharma (1986); Sharma and Olah (1986), NACA (1989); Pekar and Olah (1991); Esteky *et al.*,(1995); Zoccarato *et al.*,(1995) and Sharma *et al.*, (1998) have described culture technique and fish production in various crop-livestock integrated farming systems in countries like Hungary, Philippines, Malaysia, Germany, China, Taiwan, Madagascar and India. Many authors have emphasized the importance of fish livestock integration in recycling of waste products, income generation and diversification of products (Woyanarovich, 1979; Little and Muir, 1987; Sharma and Das, 1988; Radhey Shyam, 1995; Kaunhog, 1996; Sharma *et al.*, 1998). Singh and Das (1993) developed the model of integrated system or triple A (Aquaculture-Agriculture-Animal husbandry) system for marginal farmers. Efforts are being made to improvise the technology by way of multiplication of production potentiality and minimization of risk factor through incorporation of more components into the system.

Integrated fish farming by recycling of pig manure in fish pond have been reported by Sharma *et al.*, (1979); Cruz and Shehadeh (1980); Woynarovich (1980); Sharma *et al.*, (1985); Sharma and Olaha (1986); Sharma and Das (1988); Gavina (1994) and Borah *et al.*, (1998) in India and abroad. Pig is one of the most prolific and efficient feed converting animal. In rural India, piggery is a traditional occupation to fetch good income. Raw pig effluent is offensive in terms of its odor and also as a potential pollutant. Disposal of piggery wastes is a problem of most of the piggery farms. Pig waste is also good manure for agriculture and aquaculture practices. The application of the pig dung in fish ponds provides a nutrient base for dense bloom of phytoplankton and nanoplankton, which, in tune, form a base for intense zooplankton development. A very large proportion of the cost of pig production is invested for feeding. On an average, 5 kg. concentrate mixture is required to feed an adult pig daily. This cost can be minimized to provide feedstuff produced on the farm itself. Several aquatic plants like azolla can be grown in the associated water near the farm. These aquatic plants as well as other foliage of several terrestrial plants, such as vegetables, corn, and rice can be utilized as pig fodder.

Azolla is an aquatic fern with wide distribution all over the world having the capacity of assimilating atmospheric nitrogen through Anabaena azollae, a symbiotic blue green algae, present in the cavities of dorsal lobe of leaf. Azolla is also a bio-fertilizer for

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fish pond to enhance productivity (Ayyappan *et al.*, 1991).

Though, several workers have determined the productivity of pig dung manured fish pond. But, aerobic digestion of pig effluent before discharging in fish pond, its effect on overall productivity is quite meager. No attempt has been done to integrate azolla in pig-cum-fish farming system as manure, feed and aerobic digester in Indian conditions. Hence, an integrated farming system comprising piggery, azolla and fish rearing was designed and evaluated. Pigs were raised adjacent to ponds. Their feed was supplemented by nitrogen rich aquatic fern azolla (*Azolla pinnata*) grown in the stabilization pond. Azolla treated semi-digested manure from stabilization pond was used to fertilize the fish pond. Fish pond was managed on zero supplementary feeding cost. This farming system is expected to generate a higher income per unit area than existing systems.

MATERIALS AND METHODS

Experiment was conducted for 12 months duration at Fish Production and Pig Breeding Farm of Krishi Vigyan Kendra, Rampura, Rewari (Haryana), lies between 27.5°-28.5°N latitudes and 76°-76.5°E longitudes. Experiment was conducted in two earthen ponds of same size (0.12 ha) situated very close to piggery unit. In experimental unit, fish pond was integrated with pig sty and azolla production-cum-stabilization pond (6×4×0.6m) while control unit was designed only to integrate fish pond and pig sty. Fish ponds were stocked with advance sized fingerlings of *Catla Catla*, *Labeo rohita*, *Cirhinus mrigala* and *Cyprinus carpio* Var. *Communis* @ 8000 fingerlings/ ha in the ratio of 4:3:2:1. Weaner piglets of the variety Large White Yorkshire of 2½ months age were stocked in each pen in 6 numbers for first 6 months and 5 numbers for second six months. *Azolla pinnata* was grown in waste stabilization pond.

All sub units of each unit were connected with PVC pipeline. One group of pigs was fed with commercial diet and waste was recycled in control pond, another group was fed with 20% azolla supplemented commercial feed and waste was digested in stabilization pond, then released into experimental pond on weekly interval. Azolla was produced in stabilization pond and used for feeding of experimental pigs. Physico-chemical parameters of the pond water were analyzed followed the APHA (1985). Feed and waste was analysed by following AOAC (1980). Quantitative estimation of plankton was carried out by following Stephens and Gillespie. (1976). The primary production of phytoplankton was measured by "light and dark bottle method" (Gaarder and Gran, 1927).

RESULTS AND DISCUSSION

The average survival rate of fishes in experimental pond was 86.63 %, while it was 83.27% in control pond with highest for *Cyprinus carpio* (98.95%) and lowest for *Catla catla* (81.25%). In pig fish integrated pond, Singh *et al.*, (1972) obtained survival rate of fishes in the range of 80.0-98.9%. The present findings are comparable to these values. The highest average final weight was attained by *Cyprinus carpio* in both ponds. Average net weight of this fish was 1045.5 g in experimental pond and 878.4 g in control pond. The average net weight of *Catla catla* was 844.2 g and 726.9 g in experimental and control pond, respectively. *Labeo rohita* showed lower average net weight as 692.9 g and 576.6 g in experimental and control pond, respectively. The average net weight of *Cirrhinus mrigala* was 680.9 g in experimental and 564.4 g in control pond. Borah *et al.*, (1998) obtained an average net weight as 700g for *Catla catla*, 835g for Labeo rohita, 740g for *Cirrhinus mrigala* and 625g for *Cyprinus carpio* in a trial of 11 months under poultry-pig-fish integrated system. Abundance of benthic fauna and detritus substances has provided favorable growth conditions for *Cyprinus carpio*. Comparatively low growth of *Cirrhinus mrigala* may be due to feeding compatibility with *Cyprinus carpio* as both are bottom feeder fish. High production trend in the expermental (5315 kg/ha) and control (4427 kg/ha) pond found to be similar with the earlier reports on pig fish farming in India and abroad (Sharma *et al.*, 1979, Cruz and Shehadeh 1980, Woynarovich 1980, Sharma and Das (1988). Such high rate of fish yield was due the application of the animal excreta, recycled in the pond, which served two most important purposes for enhancing fish yield (as direct feed and pond fertilizer) and also acted as substratum for multiplication of microbial community that provide essential nutrition for fish and fish food organisms (Schroedar, 1980). A total of 20.06 per cent increase in total yield of fish reflects the superiority of pond productivity and better survival in azolla-pig-fish integrated practice over the

existing pig-fish integrated system. Out of which 4% was contributed by better survival rate which may be due to prior digestion of waste by azolla and 16.06 per cent was contributed by better pond productivity. The abundance of green and blue green algae may be the prime reason for enhanced growth of these plankton phagous fishes in experimental pond.

In present study it was also found that the nitrogen content of the pig waste of pigs fed with azolla supplemented diet is high (1.88%) than the nitrogen content in waste of pigs fed with non azolla diet (1.78%). The production of pigs was almost similar in both the sets of experiment, which revealed that substitution of 20% of standard pig feed by azolla biomass did not hamper the health and growth of pig significantly in the experimental unit. Azolla is quite efficient to fulfill the protein requirement of pig, as contain 22% crude protein. Further, in the present study the cost of production of pig was reduced to 14.5% through utilization of azolla in pig diet. In present investigation, an average gain in live weight of pigs was recorded as 63.05 kg and 64.45 kg with azolla supplemented diet and 61.95 kg and 62.30 kg with non azolla supplemented diet in first and second lot of six months rearing.

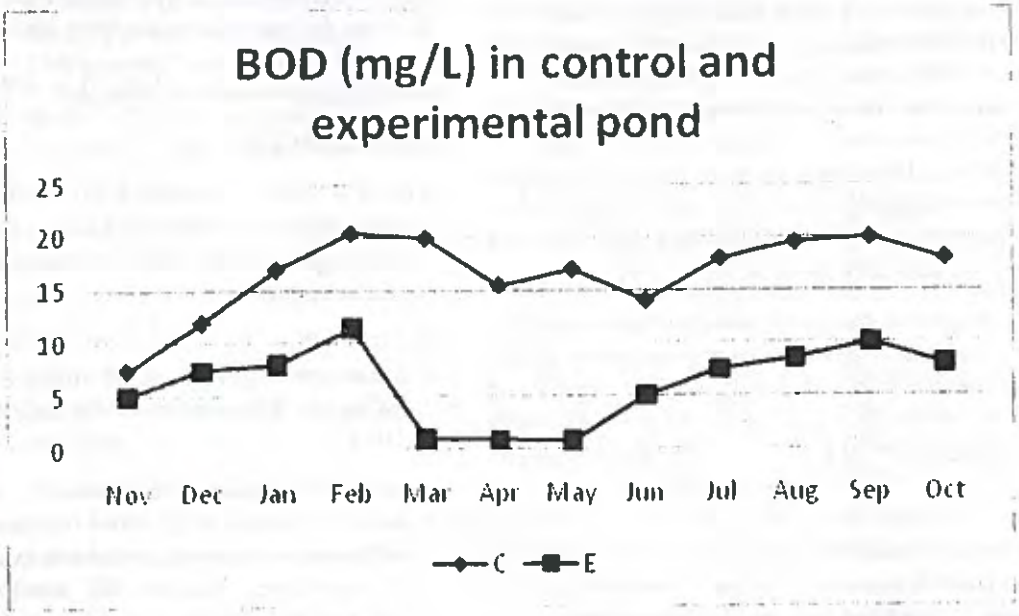

Fig. 1 BOD in control and experimental pond.

Maricel *et al*., (1990) reported 0.363 and 0.341 kg daily gain in live weight of pigs fed with 15% and 30% level protein replacement by azolla in growing phase of pig rearing. In present study 0.358 kg daily gain in live weight of pigs was observed, with 20% incorporation of azolla, which is almost similar to the above report. Lumpkin and Plucknett (1982) and Van Hove and Lopez (1983) reported that the aquatic plant species, because of their growth habit appear not to accumulate secondary plant compounds and therefore offer perhaps a greater potential than trees as a source of protein for manogastric animals. In the present study, slight difference was observed in the average feed conversion ratio between group of pigs fed on azolla supplemented diet (5.62 in first lot and 5.79 in second lot) and group of pigs fed on commercial without azolla supplemented diet (5.76 for first lot and 5.92 for feed second lot). Maricel *et al*., (1990) also reported better FCR of azolla fed pigs in finishing phase. An 1.74 kg/m^2/week net production of azolla was recorded with 3 days of doubling time except extreme cold (<20°C) and hot (>38°C) days. Gavina (1994) has also reported 3 days doubling time. Proximate composition of azolla biomass recorded as 94.2% moisture, 22% crude protein, 6.1% fat and 18.5% crude fibre, which are in conformity of Gavina, (1994). Azolla forms a permanent, heterocyst-forming cynobacterium, *Anabaena azollae*, occurs as filament

located on the plant stem apexes and inside the leaf cavities. It can develop on a medium devoid of nitrogen compounds because of the ability of reduce nitrogen to ammonia and ammonia to nitrate by bacteria, present in leaf cavities.

Raw pig effluent is offensive in term of its odor and also has high BOD, which is the amount of oxygen required for bacterial decomposition. Aquatic macrophyte-based water treatment system (AMS) offers a low energy consuming and low cost method from removing contaminants (Rengaraj *et al.*, 1999). Plants cultured over waste water perform several functions including assimilating, storing contaminants transporting oxygen and providing substrate for microbial activity which helps in the stabilization of wastes (Rengaraj *et al.*, 1999). In present study, azolla grew on the surface of waste stabilization pond. More or less facultative condition was prevailed in this pond and decomposition of organic matter was done by aerobic and facultative bacteria. One week old semi digested piggery waste was used to recycle into experimental pond, which resulted as low BOD level of pond water and readily availability of nutrients.

Wide seasonal fluctuation was observed with almost similar values of physico-chemical parameters in both ponds except for dissolved oxygen, nitrite-nitrogen and BOD. Dissolved oxygen level was observed in sub optimal level (5.41 0.77 mg/l), while nitrite-nitrogen was in the range of 0.017-0.055 mg/l in azolla integrated pond. Remarkable difference was observed in the values of BOD with a level of 16.85 - 3.85 mg/l in control pond and 6.15 - 3.65 in azolla integrated pond. In this pond, total phytoplankton density was lower during winter with mean value 1347.83 -1010.64 nos./l and it was higher in the month of April with average plankton volume (2.16 ml/50 l). Zooplankton density varied from 27 - 588 nos./l with almost equal occurrence of rotifers and Cladocerans. Gross primary productivity (GPP), Net primary productivity (NPP) and Community Respiration (CR) showed wide seasonal fluctuation with mean values 0.58 - 0.3, 0.31 -0.18 and 0.28 - 0.16 gC/m^3/hr, respectively. Period of highest GPP values (April-May) coincides with the highest NPP values. Gavina (1994) reported integration of azolla with duck-fish integrated farming system. Present study reveals the integration of azolla-pig-fish farming. Better pond productivity was experienced in this integrated system due to more input supply of nutrients into pond, capability of azolla for atmospheric N_2 fixation and semi-digestion of piggery waste by azolla. Total return of Rs. 46613/-with net profit of Rs. 23375/- was obtained from the azolla integrated unit, while it was Rs.42383/- with net profit of Rs.16101/- from control unit. Better productivity, more production of fish as well as pigs, reduction in pig production cost and better net net return reflects the technical and economic feasibility of this integrated model. The finding of the study reveals the efficacy of the system.

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Molecular cloning and expression profile of snow trout GPDH gene in response to abiotic stress

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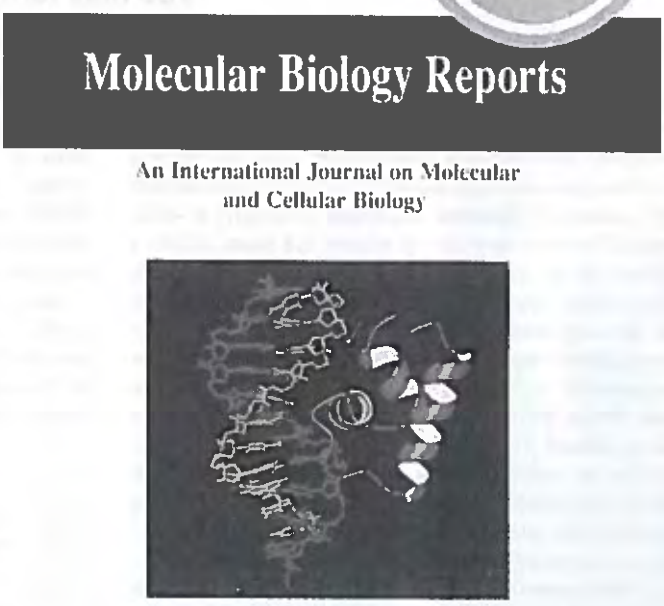

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Molecular cloning and expression profile of snow trout GPDH gene in response to abiotic stress

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Abstract Glycerol-3-phosphate dehydrogenase (GPDH) gene possibly plays a key role for cold acclimation process in snow trout during winter months when water temperature goes down to 4–5 °C. In this study, 1,012 bp nucleotide fragment of GPDH gene was obtained from two snow trout species (*Schizothorax richardsonii* and *S. niger*; family: Cyprinidae), distributed in several Himalayan rivers. The gene encoded a protein of 334 amino acids. The encoded protein sequence was very similar to GPDH of *Danio rerio* (94.36 %) using BLASTx searches. In *S. richardsonii* the qRT-PCR showed highest expression in muscle tissue followed by liver and also revealed 19 fold gene expression in liver tissue under cold (5 °C) in comparison with warm (15 °C) condition. The elevated expression levels of GPDH cDNA on cold treatment furthermore suggest that GPDH plays a role in stress related responses in *S. richardsonii*. The phylogenetic analysis showed that the two snow trout species GPDH share the same clade with characterized GPDHs from other teleost fishes suggesting a common evolutionary origin and a similar catalytic function. In addition, the *Ka/Ks* ratios of these sequences suggested that they are under purifying selection. Moreover, the expression profile of GPDH gene among co generic species of genus *Schizothorax* showed that GPDH cDNA expression was highest in *S. richardsonii* and lowest in *S. esocinus* which gives an indication of species specific adaptation in relation to different geographical areas.

Keywords Abiotic stress · Glycerol-3-phosphate dehydrogenase · Expression profile · Phylogenetic tree · Snow trout · *Schizothorax richardsonii* · *Schizothorax niger* · *Schizothorax esocinus*

Introduction

Water temperature for poikilothermic organisms including fishes is one of the most important environmental factors that affect their physiological and metabolic activities. Fishes are adapting to wide temperature variations by reorganizing their physiological process. These temperature acclimation processes include both cold stress and heat stress in relation to spatial and temporal fluctuations. For example, many fish species in cold oceans avoid freezing during the winter months by accumulating glycerol and/or antifreeze proteins. These solutes depress the freezing point effectively. Glycerol is a compatible solute that can be accumulated to higher levels that lowers the freezing point colligatively without causing physiological perturbation. Antifreeze proteins bind to ice crystals and inhibit their growth, thereby causing a non-colligative freezing point depression that is far greater than the effect on melting point [1–4]. Fish also has some warm temperature acclimation related protein *wap65* which was differentially regulated by temperature as also bacterial infections in gold fish and carps [5–7]. Rainbow smelt (*Osmerus mordax*) depresses the freezing point of their body fluids by a combination of anti freeze protein [8] and glycerol accumulation [9]. In smelt, liver glycerol-3-phosphate dehydrogenase (GPDH) gene expression was increased in

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response to low temperature [3]. This coincided with glycerol accumulation in the blood plasma, which therefore appears to be generated by activity of this enzyme. Snow trout (*Schizothorax richardsonii*) and some of its closely related species (*S. niger*, *S. esocinus* etc.) are distributed in North Himalayan rivers, snow melt springs and land locked lakes of high altitude areas in Arunachal Pradesh, Himachal Pradesh, Jammu & Kashmir, Sikkim and Uttarakhand where water temperatures decrease to 4–5 °C during winter months. But, so far there were no studies on abiotic stress the aquatic organisms face and that might alter their physiological and metabolic activities to encounter low temperature in Indian freshwater fishes. Therefore, the present study was undertaken with an attempt to clone and sequence GPDH gene possibly responsible for glycerol production in snow trout as like rainbow smelt.

We report here the partial cDNA sequences of GPDH expressed in liver of two snow trout species (*S. richardsonii* and *S. niger*) and their deduced protein sequences. The expression of *S. richardsonii* GPDH was evaluated in the different tissues and differentially regulated expression by temperature. Comparative GPDH cDNA expression analysis among 3 co generic species of genus *Schizothorax* from different locations of the Himalaya has also been carried out. It was also tried to assess the relationship between previously described fish GPDH and the mammalian GPDH based on nucleic acid and protein sequence alignments.

Materials and methods

Fish sampling and challenge experiments

Liver samples of *S. niger* and *S. esocinus* were collected from Dal lake (34°7′0″N, 74°52′0″E, altitude 1,775 m asl), Srinagar, J&K, during December 2011 (air temp. 7 °C and water temp. 5 °C). The initial identification was made on the basis of morphology [10]. Live samples of *Schizothorax richardsonii* (average weight 50–100 g.) were collected from river Kosi, near Ratighat region, Uttarakhand, (29°27.488′N, 79°28.812′E, altitude 1,033 m asl) during the month of December, 2010. Fishes were transported to wet laboratory and acclimatized at an ambient temperature (15 °C). After 15 days acclimatization, some fishes were transferred to 50 l aquarium in laboratory and the others were subjected to a controlled temperature decrease from 15 °C to 8 °C on January 15, to 8 °C on January 22 and held at 5 °C until sampled. In one aquarium water temperature was decreased by 1 °C every hour from 15 °C to 5 °C by adding ice flakes while in the other water was held at 15 °C that served as control. In each aquarium 10 fishes were held and water temperature was monitored constantly. The experiment was conducted for 96 h.

Biochemical assays

Glycerol level in the plasma of experimental *S. richardsonii* was determined directly using a glycerol assay kit (Cayman Chemical Company www.caymanchem.com). Samples were read at 520 to 540 nm after 15 min incubation at room temperature. Data were presented as mean ± SE. Comparison among groups was performed with one-way anova (ANOVA) and between groups comparisons were made using a student t test and differences were considered statistically significant at $p < 0.05$.

Tissue collection and RNA preparation

Samples of 100 mg tissue were harvested from brain, liver, kidney, spleen, heart, muscle, fin and intestine of freshly sacrificed fishes during 0 to 96 h at an interval of 24 h. Tissue samples were immediately stored in 1 ml of RNAlater (Ambion, USA) solution. Initially, tissue samples were kept at 4 °C overnight for complete infiltration of RNAlater in the tissue and then kept at −80 °C. Total RNA of all the above collected tissues were extracted using TRIZOL reagent (Ambion, USA). The concentration and quality of the RNAs were measured by UV–Vis spectrophotometer (Thermo Scientific, England) and the integrity checked by electrophoresis in 1 % agarose gel. The RNAs were then stored at -80 °C for further use. To remove endogenous DNA contamination the preparation of total RNA was digested with DNase. An aliquot of 5 µg of total RNA was digested with 5 U of DNase (Fermentas, USA) and 40 U of ribonuclease inhibitor (Fermentas, USA), and then the solution was incubated at 37 °C for 1 h. The enzyme was inactivated at 95 °C for 5 min, and RNA solution was chilled on ice. RNA quality was assessed on agarose gel showing clear 28S and 18S rRNA fragments and no smears, confirming integrities of all RNA.

Primers

In order to perform RT-PCR the primers (GPO-1R: 5′ TGGGGCTCTGCCATTGCCAAGAT 3′and GPO-1F: 5′ ACATGTGTTCAGGGTGGTTCTGCA 3′) were designed from rainbow smelt GPDH gene (GenBank accession no. AY024368) using PRIMER 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Reverse transcription and PCR

The first strand cDNA of *Schizothorax* sp. and experimental samples was synthesized from 1 µg of total RNA by

using High Capacity cDNA reverse transcription kit (Applied Biosystems, USA) as per the manufacture's instruction. Polymerase chain reaction was performed on liver (*Schizothorax* sp.) cDNA of 1 µl of first strand cDNA to the mixture containing 1 µl of 10 × Taq buffer (Invitrogen), 1 µl dNTP (100 µM), 2 µl (10 pmol) each forward and reverse primers (GPO1F/R) and 0.5 U Taq DNA polymerase (Invitrogen) in a final volume of 10 µl by adding nuclease free water. PCR was performed using Mastercycler gradient (Eppendorf) thermal cycler with the following conditions: initial denaturation step of 94 °C for 4 min, followed by 34 cycles at 94 °C for 60 s, annealing at 58 °C for 60 s and 72 °C for 90 s. The final elongation step was performed at 72 °C for 10 min. Products obtained by PCR were run on 1 % agarose gels, isolated using the Nucleospin Gel Extraction Kit (Machery Nagel) and cloned in pGMT Easy Vector (Promega). JM 109 competent cells (Fermentas) were transformed and Positive clones were selected randomly for sequencing. Sequencing was performed using Big Dye Terminator v 3.1 cycle sequencing kit with vector specific primer i.e., T7 primer in 3130 Genetic Analyzer (ABI, USA).

Multiple sequence alignment and phylogenetic analysis

Schizothorax sp. GPDH nucleotide and deduced protein sequences were compared to GPDH sequences previously described in National Centre for Biotechnology Information (NCBI) using BLASTn and BLASTx algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Predicted protein sequences were aligned using the ClustalX program version 2.0 with the default settings [11]. Identical amino acids in the alignment were shown by dots. A phylogenetic tree was constructed using the neighbor-joining (NJ) method [12] with the Poisson correction using the NJ algorithm implemented in the CLC Genomics Workbench (version 5.5). Bootstrapping (1,000 replicates) was used to evaluate the degree of support for particular grouping patterns in the phylogenetic tree. For the phylogenetic tree construction, In addition, to the GPDH protein sequences obtained in this study, we also included amino acid sequences of eleven GPDHs from yeasts and animals.

Estimation of substitution rates of amino acids

The ratios of the number of non-synonymous substitutions per non-synonymous site (*Ka*) to the number of synonymous substitutions per synonymous site (*Ks*) were calculated among GPDHs of cyprinids viz, *S. niger*, *S. richardsonii* and *Danio rerio* using the Nei and Gobojori's method implemented in the DnaSP software version 5 (http://www.ub.edu/dnasp). For *Ka* and *Ks* estimations, the alignments of the GPDH domains (http://smart.embl-heidelberg.de) were manually adjusted. *Ka*/*Ks* ratio is an indicator of evolutionary pressures acting on a class of genes. *Ka*/*Ks* greater than 1 reflects diversifying selection and a value less than 1 suggests purifying selection [13].

Quantitative real time PCR (qRT-PCR) analysis

For real time PCR, primers (F-5′ GAAAAACTCGGCAT CACCAT 3′ and R-5′ CCAGACCGTCAAAGAAACC 3′) were designed from deduced GPDH sequences using Primer 3 software for gene specific expression analysis with an expected amplicon size of 242 bp. Specificity of the primers was tested by RT-PCR and gel electrophoresis using DNA size standards, 1 kb ladder (Fermentus). Quantitative real time RT-PCR (qRT-PCR) analysis was performed for expression of genes of interest in triplicate with a BioRad CFX96™ real time PCR machine using SYBR green supermix (BioRad, USA). Reaction conditions were as follows: 95 °C for 3 min, followed by 35 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 10 s. Dissociation analysis was performed at the end of PCR reaction to confirm the amplification specificity. After the PCR program, data were analyzed with Biorad Software. Expression levels were relative to a randomly selected control sample and were calculated by delta–delta threshold cycle (Ct) method using BioRad software. The β-actin [14] (F-5′ CAGGGT/CGTG/CATGGTT/GGGT/C/GAT 3′ and R-5′ T/GGTTGGCT/CTTGGGG/ATTG/CAG 3′) was used as reference gene for normalization of expression levels. Negative controls included RNA processed for cDNA in the absence of RT (no RT) and reactions run with RNase-, DNase-free H_2O in place of cDNA template (no template). A melt curve analysis was also performed to assess PCR specificity.

Results and Discussion

Plasma glycerol levels *in S. richardsonii*

Mean initial plasma glycerol concentration was 0.54 mmol ml^{-1}. Accumulation of glycerol was increased approximately by twofold which was 1.06 mmol ml^{-1} in fishes held at lower temperature which was significantly ($p < 0.05$) higher than 0.50–0.54 mmol ml^{-1} found in the fish maintained at ambient temperature over 96 h (Fig. 1). This observation of glycerol accumulation in plasma of snow trout is strikingly higher than earlier observation in rainbow smelt where plasma glycerol level increased up to 300 µmol ml^{-1}[9]. The increase of glycerol level in plasma is possibly due to low temperature and triggered by the activity of glycerol 3 phosphate dehydrogenase.

Isolation of GPDH cDNA and sequence analysis

A partial fragment of 1012 bp were obtained from both *S. richardsonii* (accession no. JN631814) and *S. niger* (accession no. JX477099) liver cDNA by RT-PCR using the primers GPO1F/R (Fig. 2). While, a partial fragment of 200 bp was obtained from *S. esocinus* and it is excluded from the present study for its short length. The translated GPDH transcripts of two *Schizothorax* sp. showed homology with other known GPDH genes, with a maximum score of 94.36 % identity with *D. rerio* GPDH (GenBank accession number AAH55382). The sequence contained an open reading frame encoding 334 residue polypeptides (Supplementary Fig. 1). In order to determine the relationship of GPDH orthologous with other teleostean fishes and animals, some representative GPDH sequences were compared and aligned (Fig. 3) with the present *Schizothorax* GPDH sequence. All the sequences were found to be quite similar. No large sequence gap or insertions were required to align the sequences. However, ORF of GPDH sequence in the present study was smaller in length as compared to zebrafish (*D. rerio*).

Phylogenetic relationships of *Schizothorax* GPDHs

The phylogenetic analysis revealed four major clades of GPDHs from eukaryotes, mammals (clade I), animals (clade II), fungi and insecta (clade III) and teleosts (Clade IV) (Fig. 4). The GPDH homologues from *S. richardsonii* and *S. niger* along with other teleosts formed a distinct group in clade IV. High Bootstrap values indicate the statistical confidence in the present analysis. *Ka* and *Ks* values were estimated and the ratios of *Ka/Ks* were calculated for the GPDH domain of the sequences from *S. richardsonii*, *S. niger* and *D. rerio* (Table 1). Overall, the *Ka/Ks* ratios for all the sequences were <1, and ranged from 0.0106 to 0.5775 for the GPDH domain sequences. The ratios among *Schizothorax* species ratios ranged from 0.0106 to 0.0444. These results suggest that these genes have been evolving under purifying selection [13].

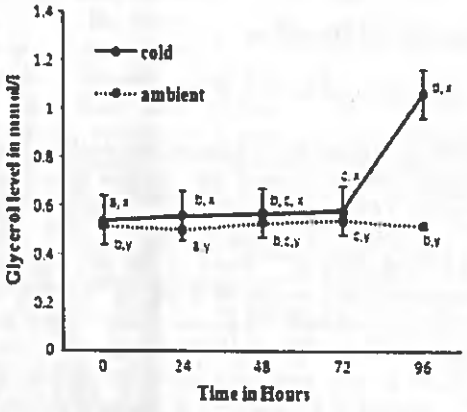

Fig. 1 Different superscripts (*a*, *b*, *c*, *d*) in the same row and (*x*, *y*) in between different temperature (5 and 15° C) were significantly ($p < 0.05$) different. Data expressed as mean ± SE, $n = 3$

Analysis of GPDH expression in *S. richardsonii*

In order to gain insight into gene expression in relation to function, RT-PCR analysis was conducted using RNA isolated from various tissues of *S. richardsonii*. The GPDH gene expressed essentially in all the tissues analyzed including muscle, brain, kidney, heart, fin, intestine, liver and spleen. However, it is expressed highly in muscle followed by liver (Fig. 2b). β-Actin was used as control (Fig. 2c). The same expression profile was observed using real time PCR analysis. The highest mRNA expression level of GPDH was detected in muscles which was 52.70 fold ($p < 0.05$) than that of fin which was lowest among all the tissues. The expression level in intestine, kidney and spleen were approximately, 1.40, 1.55, and 2.94 folds respectively. The dissociation curve of amplification products showed a single peak in all cases, indicating that the amplifications were specific. Though in the present findings revealed a highest expression site in muscle tissue, we have opted liver mRNA for studying in comparative expression analysis in temperature treatment because a highest level of glycogen occurs in liver followed by heart, with lower levels in a number of other tissues [9]. Glycogen is the major carbon source of glycerol accumulation which was observed by Driedzic et al. [2].

It was also observed differential gene expression in relation to downshift of temperature using real time PCR analysis. A significant up regulation of GPDH transcripts in liver was observed (Fig. 5) when the fishes were exposed to 4–5 °C in comparison to 15 °C control group. Low temperature (4–5 °C) led to approximately 19 fold ($p < 0.001$) elevated expression of GPDH during 96 h. There was no significant increase during the initial day (24 h), though there was little elevation (5 fold) during 48 h and a fall (2.5 fold) during 72 h in comparison to control. The transcriptional response of GPDH gene in this species appeared to be quite slow and irregular, days after cold treatment. It appears that slow response is likely a consequence of many other upstream regulatory gene actions. The similar activity was also observed in glycerol accumulation (Fig 1) where it was very slow up to day 3 (72 h).

Expression analysis of GPDH gene among co generic species of genus *Schizothorax*

To examine the expression profile of GPDH gene among co generic species of genus *Schizothorax* total RNA was

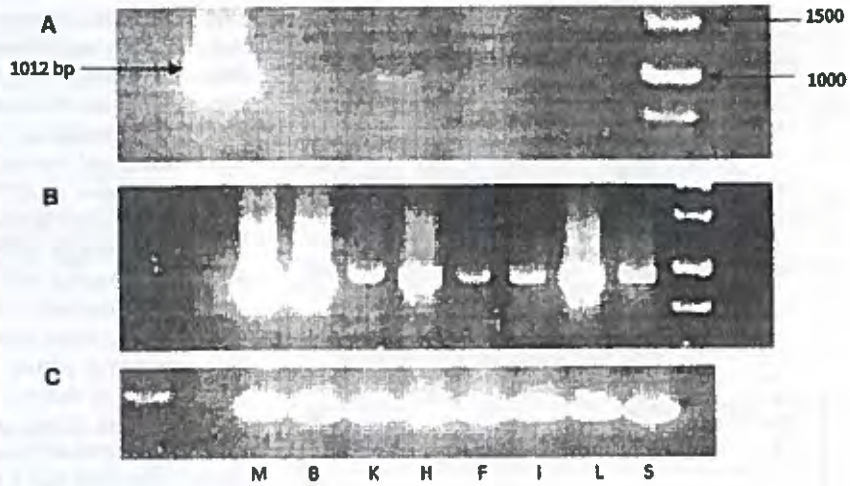

Fig. 2 a RT-PCR of GPDH gene in *S. richardsonii*, b Tissue specific GPDH expression, c Amplification of β-actin for normalization of cDNA of different tissues (*M* Muscle, *B* Brain, *K* Kidney, *H* Heart, *F* Fin, *I* Intestine, *L* Liver, *S* Spleen)

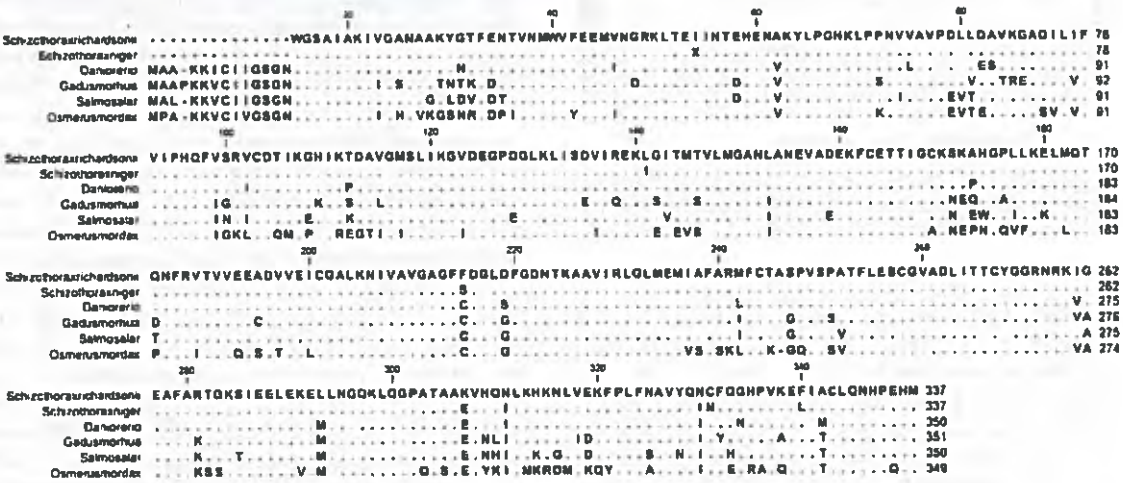

Fig. 3 Multiple alignment of the deduced amino acid sequence of *S. richardsonii* (accession no. JN631814), *S. niger* (accession no. JX477099) GPDH with GPDH orthologous of other teleostean fishes *Danio rerio* (NP956000), *Salmo salar* (ACH70721), *Gadus morhua* (AAT47549) and *Osmerus mordax* (ACO10070). Identical amino acid residues between each other are shown by *dots* respectively

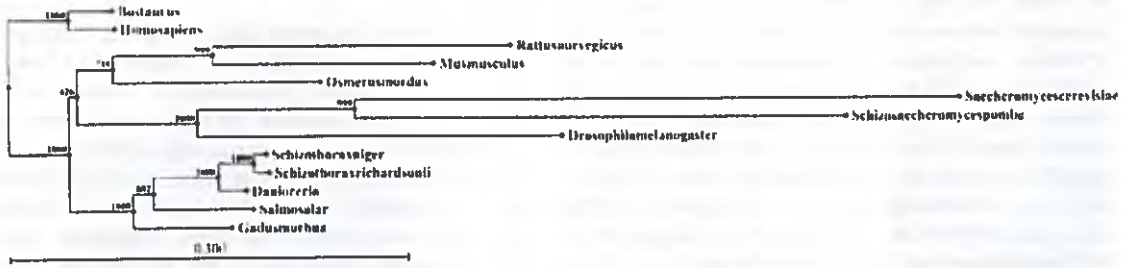

Fig. 4 A phylogenetic tree showing the relationship between GPDH cDNA from *Schizothorax richardsonii*, *Schizothorax niger* and other organisms (GenBank accession no. *Homo sapiens* P21695; *Bos taurus* Q5EA88; *Danio rerio* NP956000; *Schizothorax richardsonii* JN631814; *Salmo salar* ACH70721; *Gadus morhua* AAT47549; *Mus musculus* BAC34327; *Ratus norvegicus* XP001060681; *Osmerus mordax* ACO10070; *Drosophila melanogaster* P13706; *Schizosaccharomyces pombe* P21696; *Sacchcromyces cerevisiae* Q00055). The tree was built using neighbor-joining method

Table 1 The number of nonsynonymous substitutions per site (*Ka*), the number of synonymous substitutions per site (*Ks*), and their ratio (*Ka/Ks*) in the GPDH domain between GPDH sequences from *S. richardsonii*, *S. niger* and *Danio rerio*

Sequence pair	GPDH domain		
	Ka	*Ks*	*Ka/Ks*
Danio rerio vs *S. richardsonii*	0.5775	0.0348	0.06
Danio rerio vs *S. niger*	0.5738	0.0365	0.06
S. richardsonii vs *S. niger*	0.0444	0.0106	0.23

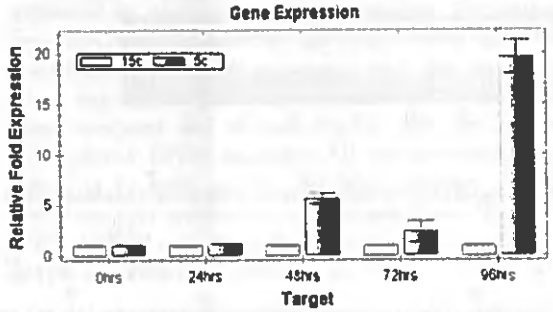

Fig. 5 Real time PCR analysis of *S. richardsonii* GPDH gene expression after cold temperature (4–5 °C) treatment with 15 °C (ambient temperature) as control

extracted from liver of *S. niger*, *S. richardsonii* and *S. esosinus*. The cDNA synthesized as described above was used as a template for qRT-PCR analysis. The results showed that GPDH cDNA expression was highest in *S. richardsonii* and lowest in *S. esocinus*. The expression level in *S. richardsonii* was about 10.18 and 8.45 fold ($p < 0.05$) than that of *S. esocinus* and *S. niger* respectively.

In the present study we isolated and characterized partial cDNA sequences of GPDH gene and also predicted the protein sequences from two snow trout species (*S. richardsonii* and *S. niger*) (family: Cyprinidae). Both nucleotide and protein sequences were very similar to that of rainbow smelt and *Danio*. In addition, both *Schizothorax* GPDHs shared the same phylogenetic cluster with other teleosts suggesting a common evolutionary origin and similar catalytic function (Fig. 4). The GPDH domain is present in all teleosts examined so far, taken together all the above evidence suggest that the *Schizothorax* GPDH sequences analyzed in this study are homologues to the GPDHs characterized from *O. mordax* and *D. rerioi* and could have a role in the synthesis of G3P, and probably also have a role in glycerol accumulation. Furthermore, our results suggest that these genes are under purifying selection, which means that they are subject to evolutionary constraints that maintain the catalytic function of their protein products, since any mutation that compromises the function of the enzyme could be detrimental for the cell. This is in agreement with the essential role that the GPDH enzyme plays for the survival of the cell under cold stress [15]. We also analyzed *S. richardsonii* expression in different tissues and regulation under cold temperature. The tissue distribution of GPDH mRNA was determined by both RT-PCR and real time PCR. In both analyses GPDH mRNA was detected more or less in all tissues but highly expressed in muscle and Liver. This implies that GPDH may play important role in cold acclimation process through all the major organs. During winter months fishes were observed to sustain a temperature as low as 4–5 °C and summer they thrive at 18–20 °C. Usually, they migrate to warmer area during the winter months. But there are some areas in high altitude Himalayas where they are found to survive in land locked lakes during winter months. Like rainbow smelt, *O. mordax* [3] this snow trout was also observed to produce glycerol through DHAP to acclimatize extreme cold temperature and accumulation of glycerol was triggered with the elevated activity of GPDH enzyme. This result was supplemented with 19 fold elevated expression of GPDH gene under cold stress using real time PCR analysis. It was also observed a seasonal variation of GPDH expression in rainbow smelt with higher levels during winter using qPCR analysis [15]. GPDH was also found to be regulated in yeast [16, 17], insects [18], fish [3] and mammals [19] under stress condition. The species distribution of GPDH gene expression reveals that *S. richardsonii* liver is unique in having very high levels of this enzyme than the other *Schizothorax* species. The low levels of GPDH transcripts in *S. niger* and *S. esocinus* may be due to they are non-glycerol accumulators. The similar phenomenon was observed in Atlantic Salmon, capelin, tomcod and flounder liver [20, 21]. The different levels of gene expression in different species of *Schizothorax* are indicating different levels of cold adaptation in respect to geographical locations and also may be species specific nature.

In summary, the partial cDNA and protein sequences of GPDH gene were identified and characterized from two snow trout species (*Schizothorax richardsonii* and *S. niger*). In *S. richardsonii* the GPDH mRNA was found constitutively in muscle, brain as well as in liver and some other tissues. The upregulation expression of GPDH cDNA on cold treatment as compared to the control condition, suggested that GPDH was stress responsive gene in *S. richardsonii*. Furthermore, the phylogenetic analysis showed that the two snow trout species GPDH share the same clade with characterized GPDHs from other teleost fishes suggesting a common evolutionary origin and a similar catalytic function. In addition, the *Ka/Ks* ratios of these sequences suggested that they are under purifying

selection. Moreover, the expression profile of GPDH gene among co generic species of genus *Schizothorax* showed that GPDH cDNA expression was highest in *S. richardsonii* and lowest in *S. esocinus* which gives an indication of species specific adaptation in relation to different geographical areas.

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OBSERVATION ON THE USE OF ARTIFICIAL SUBSTRATES FOR PERIPHYTON BASED CULTURE OF *SCHIZOTHORAX RICHARDSONII* (GRAY) IN RACEWAYS OF MOUNTANEOUS REGION OF KUMAON, UTTARAKHAND

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Field experiments were conducted in three raceways of running water system with a view to find out the suitable substrate for periphyton based culture of *Schizothorax richardsonii* (Gray). Different artificial substrates; sugarcane bagasse, paddy straw, tree branches, bamboo poles (dead organic materials), plastic sheet, plastic pipes (non bio degradable) and other materials like stones and pebbles were used for the production of periphyton community. Bamboo poles and other non-biodegradable substrates were found suitable substrates having without any adverse effect on the water quality of the pond. Growth, survival and production performance of snow trout was evaluated in the 12 months field experiment using bamboo poles and plastic sheet. Periphyton based culture of *Schizothorax richardsonii* is profitable eco-friendly aquaculture practice for hills.

Aquaculture is not always a truly sustainable practice, so far the supply of external feeds, chemicals and energy inputs are concerned[1]. Therefore, the trophic status of periphyton led researchers to realize it as a future potential of sustainable system hiding under the water Horne and Golderman[2] reported that the herbivorous fish in nature feeds largely on benthic, epilithic or epiphytic algae rather than on phytoplankton. Dempster *et al*[3], also obtained same result when experimented on *Oreochromis niloticus* in a glass fiber tank. The possibility of consuming periphyton by fish is more due to several reasons. Wetzel[4] reported that the production of periphytic algae per unit water surface area is higher than phytoplankton. Westlake *et al*[5] explained that the periphytic algae are generally more stable than phytoplankton and the risk of collapse is much lower. Horne and Golderman[2] stated that it is mechanically more efficient to scrap or graze a two dimensional layer of periphyton than a filter algae from three dimensional planktonic environment. Dempster *et al*[3], also showed that biomass ingestion rates of filter feeding fish on planktonic cyanobacteria were significantly lower than those grazed on periphyton. In recent years, extensive researches are going on the traditional periphyton based aquaculture practices as fisheries enhancement technique throughout the world.

The periphyton community is an important component of aquatic eco-system[4]. In natural communities, periphyton contributes significantly to primary production[6,7] and represents readily available food for many vertebrates[8] including fish[9].

The pioneering work in the Indian sub-continent on substrate based aquaculture was carried out at the North-west Fisheries Extension Project (NFEP), Parbatipur aquaculture complex in Bangladesh. Enhancement of fish pond through provision of substrate for periphyton growth has been demonstrated for carp species[10-13]. The major fishery in the uplands mainly consists of very popular Snow trout (*Schizothorax richardsonii*) and Mahseer. Snow trout is endemic to the Himalayas and, true to its name, it is found in streams and lakes which receive snow melt water from the hills. Most of the snow trout species are of Central Asian origin. Nine principal species belonging to two genera viz *Schizothoraichthys* and *Schizothorax*, inhabit the Himalayan region. From the foregoing account, it will be apparent that there is not much information available on the fishery position of snow-trout. The growth of this fish is not very encouraging, so that its culture in captivity has not attracted the attention of the aquaculturists. As this is an important fish for hill biodiversity and is a preferable fish of the people, it is desirable to develop a culture technique of this fish. Attempt has been made by DCFR (erstwhile NRCCWF), Bhimtal to evaluate the growth of this fish in pond condition. *Asela* is a phytophagous fish and has a special mouth adapted to scraping attached algae from the surfaces of stones. It feeds on attached algae including *Spirogyra*, *Ulothrix*, *Oedogonium*, as well as on the benthic insect larvae. Fry feed on larvae of chironomids and caddis flies, but also on microscopic algae.

In the present study, different types of substrates were used to evaluate the performance of each substrate for

the production of an important coldwater indigenous food fish, *Schizothorax richardsonii* in pond condition and its impact on the water quality of the pond.

MATERIALS AND METHODS

Experiment was carried out in the raceways (Size- 8.73m x 1.63m x 0.84m) at Department of Zoology, D.S.B Campus, Kumaun University, Nainital (1938 msl, Long. 29° 23 'N, lat. 79° 30' E) and at experimental field Centre, DCFR, Champawat(1670 msl, Long. 80° 07' N, lat. 29° 30 'E)during the period April, 2008 to Aug, 2009 (5 months for periphyton production and 12 months for fish rearing). Raceways were cleaned and filled with tube well water. The flow rate of water was maintained 5 liter/minute in all the raceways. In the first phase of experiment, for substrates study, sugarcane bagasse, paddy straw, tree branches, bamboo poles (dead organic materials) plastic sheet, plastic pipes (non- bio degradable) and other material like stones and pebbles were used. Sugarcane bagasse, procured locally was sun dried and added at the rate of 10 kg in first raceway (R1). The dried sugarcane bagasse was spread uniformly in the bottom of the pond with approximately 3 m^2 surface area. In the second raceway (R2), paddy straw, tree branches, and bamboo poles were inoculated at the same rate in the pond water. All non-degradable organic material and solid material i.e plastic sheet, plastic pipes and stones and pebbles were inoculated in the water of the third raceway (R3). Sampling was done from the all treated raceways for the qualitative and quantitative study of the penphyton. Performance of the tested substrates was evaluated on the basis of quality and quantity of grown periphyton and their impact on pond water quality. The similar experiment was repeated at field centre, DCFR Champawat during the period Sept.2009-Dec 2009.

In second phase of the experiment, selected substrates, bamboo splits (dead organic materials) and plastic sheet (non - degradable organic material) were inoculated in the water of raceway prior to one month of stocking having total surface area of 3m^2 in the raceways (R2). The raceway (R1) was without the substrate as control having exclusively artificial feeding. Healthy fry of *Schizothorax richardsonii* (Stocking density 8-10 fish/m^2) of average weight 1.11 0.04 to 1.85 0.07g were stocked in each raceway. The pelleted artificial feed (25% crude protein) was prepared by using mixture of rice bran (40%), mustard oil cake 25%, soyabean oil cake 25% and fish meal 10% and provided @3% of the total fish biomass to the control group.

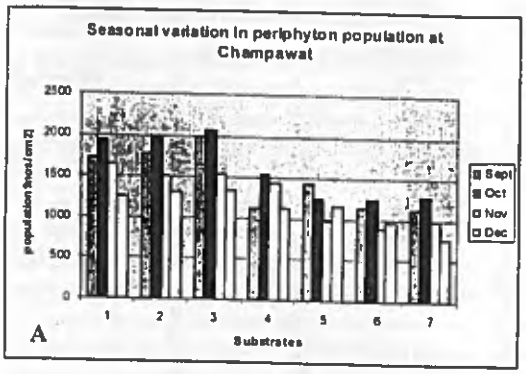
Fortnightly analysis of water samples was carried out following the standard methods of APHA[14]. Artificial diet and periphyton diet were analysed for proximate composition (crude protein, crude fat, moisture and ash content) by the AOAC method[15]. For the numerical estimation of periphyton, sample from 1cm^2 area was used. Depending on the density of organisms the scraping was dispersed in 10 to 100ml water in a beaker. One ml of the dispersed material was placed in the sedgewick rafter counting cell and the counting was done. The counts were expressed as cells or filaments per square centimeter of substrate area.

Cells/cm^2 area = cells/ml suspended scrapings

For wet biomass analysis, 1cm^2 of scraping was taken from natural substrate and then by using pre-weighted filter paper, extra moisture was removed by putting scrapping material on this filter paper and there after weight of filter paper with periphyton sample was taken using Digital balance and obtained biomass was expressed in mg/cm^2. Randomly[2] fishes from each raceway were selected for taking length weight data for computing C.F. and S.G.R. Each harvested fish was measured and weighed to quantify the production. Weight gain, condition factor (CF), specific growth rate (SGR), feed conversion ratio (FCR), survival and yield were calculated according to Castell and Tiews[16] and Devendra[17]. The mean values were compared and data were analysed using analysis of variance.

RESULTS AND DISCUSSION

Production of periphyton biomass: Observed data on seasonal variation in periphyton population (No./cm^2) at Nainital on different substrates (Fig. 1) reveal that highest population was produced with bamboo poles (1608 nos /cm^2) followed by sugarcane bagasse (1508 nos /cm^2) and plastic sheet (1433 nos./cm^2). The average of the periphyton population on all tested substrate was 1291 nos./cm^2 on all substrates. Population on the bamboo pole was 24.6% higher, on sugarcane bagasse 16.8% higher and on the plastic sheet 11% higher than the average. Minimum population was recorded with stones/pebbles (943nos /cm^2), which is 27% less than the overall average. Seasonally, population on all substrates was higher in the month of May followed by April and June. Almost similar pattern of seasonal variation in periphyton population at Champawat was observed on different substrates (Fig. 1).



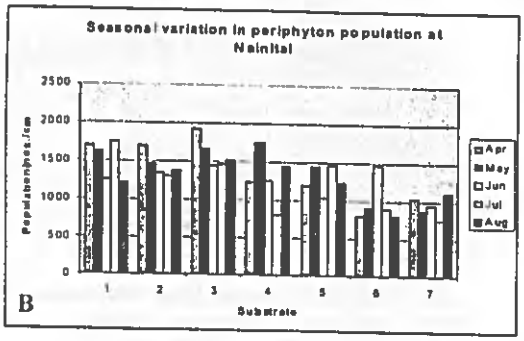


Fig.1. A&B Seasonal variation in periphyton population with different substrates.

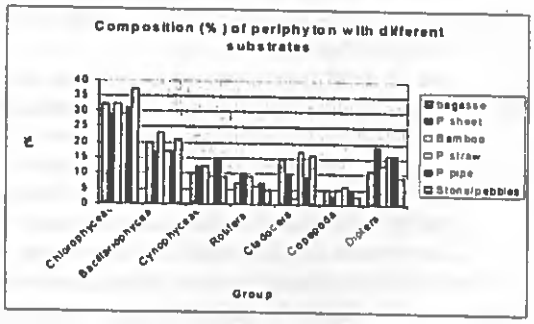


Fig. 2. Percentage of periphyton with different substrates.

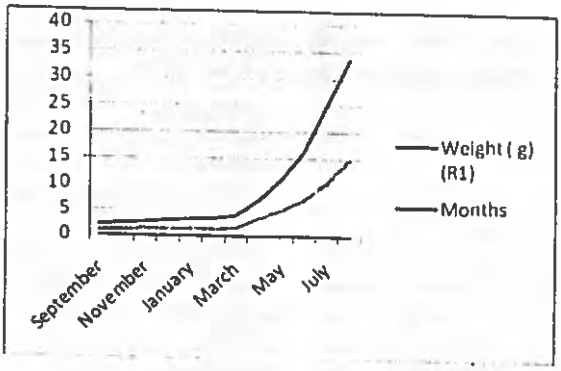


Fig. 3. Growth performance of snow trout in control/ experimental raceways.

Table 1. Seasonal variation in periphyton biomass on dry matter base (mg/cm2) with different substrates at Nainital.

Month	Sugar cane bagasse	Plastic sheet	Bamboo poles	Paddy straw	Plastic pipe	Tree branches	Stones/ pebbles
Apr	1.42	1.40	1.42	1.22	0.98	1.24	1.20
May	1.28	1.28	1.64	1.24	1.26	0.86	0.84
Jun	0.98	0.87	0.93	0.80	0.64	0.78	0.64
Jul	1.26	0.96	0.88	0.66	0.64	0.58	0.56
Aug	0.86	0.94	1.14	1.04	0.68	0.42	0.86

Table 2. Seasonal variation in periphyton biomass on dry matter base (mg/cm²) with different substrates at Champawat.

Month	Sugar cane bagasse	Plastic sheet	Bamboo poles	Paddy straw	Plastic pipe	Tree branches	Stones/ pebbles
Sept	1.45	1.52	1.56	1.24	1.28	1.40	0.98
Oct	1.42	1.28	1.64	1.46	1.45	1.34	1.20
Nov	1.20	1.46	1.24	1.20	1.12	0.46	0.48
Dec	0.64	0.88	0.86	0.82	0.40	0.44	0.26

Table 3. Mean values (± SD) of physico-chemical characteristics water having different substrates (R1, R2, R3).

Parameters	Raceways		
	R1	R2	R3
Water temperature (°C)	15.98 ±2.709	15.96±2.667	15.8±2.670
pH	7.28±0.370	7.49±0.407	7.44±0.434
Dissolved oxygen (mg/l)	6.70±0.581	6.68±0.530	6.40±0.466
Free carbon dioxide (mg/l)	2.22±0.147	2.36±0.209	2.2±0.135
Total alkalinity (mg/l)	168.6±0.013	168.41±1.176	168.3±1.468
Ammonia-nitrogen (mg/l)	0.11±0.066	0.11±0.067	0.102±0.060
Nitrite-nitrogen (mg/l)	0.011±0.002	0.11±0.001	0.11±0.001
Nitrate-nitrogen (mg/l)	0.129±0.025	0.12±0.022	0.114±0.019
Phosphate-phosphorus (mg/l)	0.223±0.065	0.24±0.069	0.19±0.055

Seasonally, population on all substrates was higher in the month of Oct. followed by Sept. and Nov. These results in pond condition regarding the periphyton population are in higher side than the previous reports for natural water bodies. Sukumaran *et al.*[18] reported 14-66 nos. /cm² periphyton population in Lalbagh tank of Karnataka and also observed 18-127 nos. /cm² periphyton population in a pond at Cuttak (Orissa).The maximum periphyton biomass at Nainital was observed in bamboo poles (6.01 mg/cm²) followed by sugarcane bagasse (5.8 mg/cm²) and plastic sheet (5.45 mg/cm²) on dry matter base. The similar pattern was observed at Champawat with maximum biomass as (5.3 mg/cm²) on bamboo poles (Tables 1&2). In general, periphyton population and biomass was comparatively higher on biodegradable substrates than the non- biodegradable substrates.

About 26 phytoplankton and 20 species of zooplankton were recorded from the collected biomass of the periphyton on all substrates. Out of 26 species, 11 species belonging to

Table 4 . Growth performance of fish in control/ experimental raceways (R1&R2)

Parameter/ Raceway	R_1 (Artificial, control)	R_2 Natural	Difference from control
Pond size (m²)	1442	1442	-
Stocking no	120	120	-
Initial Av. Weight (gm.)	1.1	1.1	-
Final Av. Weight (gm.)	14.9	18.8	+3.9 (26.2%)
Net gain in Av. Weight (gm.)	13.8	17.7	-
Initial Av. Length (mm.)	13.8	17.7	-
Final Av. Length (mm.)	18	19	-
Net gain in Av. Length (mm.)	132	142	-
Total production (Kg.)	114	133	-
Survival (%)	1649	2143	+0.534 (33.2%)
SGR	90	95	
FCR	2.67	2.90	+0.23 (8.6%)
Condition factor(k)	6.85	-	
Protein content (%)	0.931	0.951	

chlorophyceae, 10 species belonging to bacillariophyceae and 5 species belonging to cynophyceae were observed. Zooplankton species were recorded as 7 species of minor phyla rotifera, 6 species of cladocera, 4 species of diptera, 3 species of copepoda and 1 species of oligochaeta. In species composition, maximum percentage was contributed by chlorophyceae followed by bacillariophyceae, cladoceran and dipteran (Fig 2).

Azim *et al.*[19] reported, rich periphyton biomass with 32 genera of algae and 10 genera of plankton by placing bamboo poles in carp rearing ponds. On analysis of proximate composition, the natural food, periphyton contains the crude protein as 61.2%, while it was 25.55 in the artificial feed. The fat content was lower in natural diet i.e. 4.8%, while it was 6.2 % in the case of artificial diet.

Impact of different substrates on water quality of the pond: Water qualities must be within the tolerant limit for fish throughout the rearing period. Temperature was in lower side during the months of April and June (15.0-19.2) in all raceways. Little difference was observed in water temperature in the pond having biodegradable substrates and non-biodegradable substrates. But, significant differences were observed in dissolved oxygen content. In the case of sugar cane bagasse the dissolved oxygen content was in decreasing trends after one months of the substrate placing and continued up to next two month, similar trends of the dissolved oxygen contents was also observed in raceway R2, it might be due to the presence of paddy straw, got rotten after 15 days. In case of sugar cane bagasse and paddy straw the dissolved oxygen

concentration went to down up to critical level (4.7 ppm.). Favorable dissolved oxygen range (6.0-6.5 ppm) was observed in the pond water having non-biodegradable substrates. Water pH and free carbon dioxide was more stable in the pond having non- biodegradable substrates. Similar trend of the alkalinity was observed in all raceways having lower concentration in June. Concentration of free ammonia fluctuated between 1.0-1.7 and did not show clear trends in time. It showed downfall towards the end of experiment. Pond of non- biodegradable substrates contains comparatively low concentration on ammonia. The concentration of ammonical nitrogen (NH_4-N) and NO_3-N fluctuated around 0.20 and 0.05 ppm, respectively. NH_4-N was comparatively low in the pond having bamboo poles and plastic sheets. Concentration of NO_2-N was observed in the range of 0.01-.015 ppm with higher concentration in the pond having sugarcane bagasse. NO_3-N was in lower concentration in the pond of non- biodegradable substrates. Mean PO_4-P concentration decreased from 0.28-0.04 ppm during the experiment with a peak during June, then showed continues downfall towards the end of the experiment (Table 3). The results of the present study were in the conformity[20]. Keshavanath[21] also resulted that bamboo poles and sugar cane bagasse yields greater periphyton production but sugar cane bagasse created serious water quality problems in terms of low dissolved oxygen concentration. The present results reflected that bamboo poles and other non- biodegradable substrates are suitable for aquaculture points of view having without any adverse effect on the water quality of the pond. Figures having same superscript in each row are significantly different ($P < 0.05$)

Fish growth and production: In periphyton fed fish, the average final weight was found as 18.8 gm. with 2.143 kg (15.31 kg/100m²/yr) fish production. It was 26.2% higher in average growth and 33.2% higher in total production over the control. 7 % increase was due to the better survival (95%) of this group. In the control, raceway (R1), the average weight gain was 14.9g with total production of 1.609kg. (11.50 kg/ 100m²/yr) and survival of 90%. SGR in R2 was 2.90, which was 8.6 % more than the control. FCR of the fish was 3.74 while it was 6.85 in the fish fed with artificial diet in control without periphyton. Condition factor was also better for periphyton fed group (Table 4).

The results of weight gain in the present study are superior to the previous reports. Joshi *et al.*[22] reported that fish merely attained an average length of 8.4 cm, 12.1 cm, 15.6 cm, 18.2 cm, 20.4 cm, with corresponding weight of 5.2 g, 10.6 g, 25.8 g, 44.6 g and 72.0 g. during 1st to 5th year life span, respectively. Azim *et al.*[20] studied growth and production of Indian major carps, rohu (*Labeo rohita* and *Labeo gonius*) using bamboo substrate in ponds and recorded a 77% higher production of rohu with bamboo substrates than the ponds without substrates.

It was concluded that the growth, survival, production and nutritive value is better in natural periphyton fed snow trout. Net yield of snow trout may be increased by providing bamboo poles and other non- biodegradable substrates like plastic sheet for periphyton production despite the fact that there was positive impact on growth and production without deteriorating water quality.

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PERMANENT GENETIC RESOURCES NOTE

Permanent Genetic Resources added to Molecular Ecology Resources Database 1 June 2012–31 July 2012

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Abstract

This article documents the addition of 96 microsatellite marker loci to the Molecular Ecology Resources Database. Loci were developed for the following species: *Clarias batrachus, Marmota himalayana, Schizothorax richardsonii, Sitophilus zeamais* and *Syagrus romanzoffiana*. These loci were cross-tested on the following species: *Clarias dussumeri, Clarias gariepinus, Heteropneustus fossilis, Sitophilus granarius* and *Sitophilus oryzae*.

This article documents the addition of 96 microsatellite marker loci to the Molecular Ecology Resources Database. Table 1 contains information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column. The MER database and GenBank accession numbers and the authors responsible are also listed. A full description of the development protocol for the loci presented here can be found on the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/).

Correspondence: Molecular Ecology Resources Primer Development Consortium, E-mail: editorial.office@molecol.com

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Table 1 Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. primers developed	Other species tested	MER database no.	GenBank accession no.	Authors
Clarias batrachus	27	*C. dussumeri*, *C. gariepinus*, *Heteropneustus fossilis*	49729–49755	GR955281, GR955282, GR955285, GR955306, GR955308, GR955313, GR955315, GR955321, GR955322, GR955324, GR955333, GR955341, GR955342, GR955346, GR955349, GW397086, GW397095, GW397120, GW397131, GW397132, GW397160, GW397163, GW397164, GW397171, GW787427, GW840539, GW840553, GW840578, GW840586	Mohindra, Vindhya; Singh, Akanksha; Patangia, Ruchi; Tripathi, Ratnesh K.; Singh, Rajeev K.; Sah, Rama Shankar; Lal, Kuldeep K.
Marmota himalayana	13	n/a	49766–49778	JQ317689–JQ317692, JQ317694–JQ317697, JQ317700, JQ317704–JQ317706, JQ317709	Huailei, Ma; Liang, Lu; Qiyong, Liu
Schizothorax richardsonii	27	n/a	49803–49829	JN128128–JN128134, JN128136–JN128139, JN128141–JN128147, JN128150–JN128152	Chandra, Suresh; Matura, Rakesh; Barat, Ashoktaru; Singh, Birender Kumar
Sitophilus zeamais	9	*S. granarius*, *S. oryzae*	49756, 49757, 49759–49765	JX303735–JX303743	Corrêa, A. S.; Vinson, C. C.; Guedes, R. N. C.; Oliveira, L. O.
Syagrus romanzoffiana	20	n/a	49504–49523	JX075189–JX075198, JQ890315–JQ890322, JQ087381, JQ087385	Giombini, M. I.; Tosto, D. S.; Bravo, S. P.

AQUACULTURE ASIA

CONTENTS

Management in seed production of golden mahseer, *Tor putitora* in hatchery conditions

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Angling - a good catch.

Golden mahseer, *Tor putitora* are large cyprinids, inhabiting the clear, pristine and fast flowing waters of Asia, from the cool waters of Himalayan streams to the tropical rivers of South East Asian jungles. Mahseer in the Indian sub-continent are described as the 'King of Indian Aquatic Systems'. Encounters occur in the Tor zone (600-1,200 m) of the glacier-fed Himalayan rivers with much more extended distribution to the lower reaches in the peninsular Indian rivers.

Mahseer as a sport fish provides unparalleled recreation to anglers from all over the world, better than salmon. For the fishermen, mahseer is of considerable importance because of its large size. As a food fish, it is highly esteemed and fetches the highest market price in North and North East of India. Despite their abundance at one time, the mahseer population has been declining in number and size in natural waters and is in serious danger of extinction. Its population is declining in the natural water bodies because of degradation of the aquatic environment and biological changes in the ecosystem due to urbanisation as well as over fishing especially with pressure from illegal catching methods such as electro fishing, poisoning, and dynamiting. As a result, the population has become unsustainable with fish catch from fisher communities being low in most parts of the country, and the fish likely be declared endangered in the near future. Developments of captive breeding and culture techniques are the means for conservation and promotion of a sustainable fish population[1].

Almost 20 different species of Tor have been reported from many rivers, streams and lakes of all along the mid-Himalayan belt from Assam and Sikkim, and more broadly Afghanistan, Bangladesh, China, Myanmar, Thailand, Cambodia, Laos, Nepal, Pakistan, Vietnam, Indonesia and Malaysia. In natural systems the fish has been known to reach 2.75m in length and 54 kg in weight, although specimens of this size are rarely seen nowadays.

Food and feeding

Golden mahseer is known to be an omnivorous fish in its adult stage. In earlier days considering the mouth opening and massive size, the fish was thought to be a carnivore. Mahseer have been found to also feed on green filamentous algae, insect larvae, small molluscs and algal coatings on rocks[2]. In natural habitats the food of mahseer fingerlings has been reported to consist of insect matter (81.4 percent) plant matter (15.9 percent) and other items including fish (1.6 percent). Scientists have noted that mahseer is an intermittent feeder[3].

Green filamentous algae and other water plants, biofilms and insect larvae have been recorded from the stomach contents of the Putitora mahseer. Diatoms formed the most preferred food component supported by green algae, blue green algae and both micro and macro-benthic animals. Various species present in the gut included *Navicula*, *Amphora*, *Cymbella*, *Synedra*, *Fragillaria*, *Oscillatoria*, *Zygnema*, *Spirogyra*, *Tribonema*, *Areella*, *Keratella* and *Chironomus*[4].

Site selection of mahseer farm

In successful aquaculture programs the selection of site for a farm is most important. The available quantity of water must be taken into consideration for the capacity and type of farm to be developed.

The water source to hatchery should be of good quality and adequate in quantity. Water from a spring sources is the most ideal for mahseer cultivation, as temperature regime does not fluctuate much. The oxygen content of water is of paramount significance and should be in the range of 7.0-9.0 mg/l at all times in all seasons. The water temperature between 20.0-25.0°C during breeding and marginally higher during rearing phase is desirable[3].

For water supply to a mahseer farm direct access to the spring or stream through a feeding channel should be preferred. It is always better that the water is passed though a de-silting device or a deep storage chamber before it is fed to the farm. The source of a stream, a brook or a spring should have enough water to compensate for losses through seepage, infiltration and evaporation. The distribution of water in the farm should be so regulated that each unit of the farm should have separate inlets to receive the required quantity of fresh oxygenated water in various components such as the hatchery, nursery and raceways or growing ponds with proper outlets also. Overhead tanks with pumping facilities can be a suitable alternative[4,5].

Hatchery unit of golden mahseer

Hatcheries need appropriate facilities for incubating the eggs as well as for their development up to the stage suitable for shifting into nursery tanks. Low cost hatchery structures

Fertilisation

can be built from tin or plastic sheets supported on frames For long term prospects, the structure can be a room made of concrete bricks etc. or erections on the wood logs if it is considerably cheaper at the site. The floor should be cemented with a gradient to facilitate cleaning and removal of water. The hatchery should be protected from direct sunlight and should have adequate neat and clean working space.

Troughs

Hatchery troughs and trays are basic requirements of a hatchery for incubating the fertilised eggs and raising fry up to first feeding stage. The hatching troughs may be of various shapes and sizes, but should have the capacity to hold sufficient water for rearing the eggs, larvae and early fry. We used rectangular troughs (220 x 50 x 40 cm or 220 x 60 x 50 cm) to rear mahseer eggs. The depth of these troughs may be increased by 10-25 cm, to facilitate the rearing of spawn and fry of mahseer. These troughs can be made from cement concrete, aluminium, flat galvanised iron sheets but preferably of fiberglass. The arrangement of hatching troughs may be in a series so that, water from source flows into the first or head trough to subsequent troughs. Additional water supply to augment the dissolved oxygen content can be provided for each trough. Each trough should have separate inlet and outlet mechanism for water. A trough with at least five hatching trays can hold 20 000-25,000 fertilised eggs

Trays

The shape and size of the hatchery trays are in accordance with troughs, so as to fit about 4-5 trays in each trough. The trays are made of fiberglass / wooden frame and may be rectangular or square in shape. The bottom of each hatching tray is fitted with the synthetic netting cloth (mesh size 2 mm) to ensure regular water movement and the height of each tray ranges from 7.5-10 cm. The outside dimensions of each tray are such that they can be accommodated in series along the length of a trough. Water enters at one end of the trough and leaves from the other after passing in each of the serially arranged hatchery trays. Each tray (50 x 30 x 10 cm) has a capacity to hold 4 000-5,000 fertilised eggs

Nursery ponds

The nursery ponds are the other important component of a hatchery, which are used for rearing the early fry of mahseer during their initial feeding stage. These tanks may vary in shape and size but should not be very deep. Efficient nursing of tiny mahseer fry can be possible in shallow tanks. The suggested size of the rectangular nursery tanks can be 2.0 x 0.5 x 0.6 m or 2.0 x 0.75 x 0.60 m and circular tanks (diameter 2.2 m x 0.75 m or 0.60 m) preferably of cement / fibreglass with suitable water inlet and outlet facilities can also be used. A water flow rate 3-4 litres/minute for raising 0-3 months old fry should be maintained in the nursery ponds

Rearing ponds

To provide sufficient space for rearing mahseer fingerlings two types of earthen ponds with proper water supply system can be prepared in the farm. First, the smaller ponds (5.0 x 1.5 x 0.75 m) for immediate stocking of advanced fry from the nursery tanks and secondly, the larger size of rearing tanks (10.0 x 4.0 x 1.0 m) to grow one year old fish. These fry ponds/tanks can also be constructed using stone pitching, cement or made of fiberglass with continuous water renewal facilities (flow rate 4-6 liter/min.). In these ponds fry can be stocked at a density of 1,000 m^2.

Preparing mahseer seed for transport

Water reservoir (outlet channel)

The outlet channel of a mahseer farm should be wide enough and preferably extending from above to the end of the farm to hold the outgoing water from nurseries, rearing and stocking ponds. A direct inlet can also be provided from main feeding channel, so that the flow rate could be raised as and when desired for stocking the brood fish. The outlet channel can also function as spawning channel during the spawning season when brood fish need running water environment.

Water supply

The available quantity of water is also to be taken into consideration for the capacity and type of farm to be developed. The ideal requirement of water in term of quantity at various stages of mahseer rearing is as below

Water flow	Rearing capacity
1 litre/minute	Incubation and rearing 2,000 eggs at 20-28°C
3-4 litres/minute	Rearing 2,000 fry (0-3 months) at 20-27°C.
4-6 litres/minute	Rearing 1,500 fingerlings (4-9 months old)
Flow through hatchery	
Overhead tank	1,000 litres capacity installed at a height of 5 m above
Hatchery tanks	Galvanised iron sheets or fiber glass of 200 x 60 x 30cm size.
Hatching trays	50 x 30 x 10 cm. with synthetic netting cloth 1 mm mesh size 5,000-6,000 eggs can be stocked.

Spawning

Golden mahseer are an intermittent breeder and fish lay eggs at intervals through out the year, but peak spawning occurs in the monsoon. The mahseer prefers clean water for breeding and has migratory habits. During the floods, the mahseer ascends to upper reaches of the river, traversing long distances to find fresh breeding grounds for spawning. They lay their eggs in sheltered rock pools, a batch of eggs at a time, repeating the process several times in a season. Mahseer most certainly breed at the commencement of the rains. The breeding season as well as spawning in many hill-stream fishes, including golden mahseer, are initiated by a specific combination of temperature, pH, velocity, turbidity and rains, which collectively induce the fish to spawn. We observed was identified five distinct stages in breeding females, which we categorised as stage I (immature), stage II (maturing), stage III (ripening), stage IV (ripe) and stage V (Fully ripe)[4].

Artificial propagation

Supportive breeding programmes are one possible option for increasing the population of mahseer in natural water bodies. This involves culturing them and propagating their seed on a large scale, with due consideration of genetic issues of both broodstock and wild populations, and transporting them to streams, lakes and reservoirs for release. Seed of mahseer was earlier collected from natural sources but recently it is produced through artificial propagation.

Breeding and rearing

Induced spawning can be carried out in an ordinary manner. Selected broodstock are stripped of their eggs and milt by exerting pressure on the caudal portion of the fish. The stripped eggs are collected in the plastic trays and the milt is spread over the eggs and then mixed with a feather and allowed to stand for five minutes. After that, the eggs are washed thoroughly with clean oxygenated water three to four times to remove the excess milt. Then the trays containing eggs are filled with fresh water and allowed to stand for 15-20 minutes in shade to allow the eggs to swell and harden before releasing them in hatching trays. The fertilised eggs are demersal, lemon yellow or brownish golden in colour. We observed a fertilisation rate of 90-100%. Hatching period of *Tor putitora* is 80-96 hours in water temperature 22-24 0°C. Once the yolk-sac is completely absorbed and swim up fry start moving freely, the stock is shifted to nursery tanks and stocked @ 8 000-10 000/tank with water flow of 2-3 litres per minute. The young ones are fed with artificial feed. With a view to develop table size fish or brood stock, the natural seed or hatchery reared seed can be stocked in the earthen ponds, cement ponds, running water ponds or cages[5,7].

Air transport of eggs

Fish with empty gut contents consume less oxygen. Moreover, faces, urea and ammonia produced during digestion deteriorate water used for transport. So, fish should be prepared for stress before transport. For fish transport different sizes of plastic bags or containers of different size and shape manufactured from PVC, fiberglass, iron or aluminium are used. Fish are frequently injured during conditioning and transportation. Use of 0.05–0.3% kitchen salt during transportation decreases activity and stress-sensitivity of fish. Overloading of transport facilities must be avoided. After transportation, gradual equalisation of temperature and water quality is essential during release of the fish[6].

Seed ranching

The lack of a well established hatchery technology for mahseer and for rearing of its seed was one of the major obstacles in introducing the mahseer ranching. The Directorate of Coldwater Fisheries Research, Bhimtal, ICAR has taken a very bold step for seed production of golden mahseer in the hatchery complex of the Directorate and releasing the seed in the different streams/rivers/lakes in all over India to increase the population of this fish in the natural habitat and also to conserve the germplasm from extinction. The hatchery-produced seed has been transported to Department of Fisheries, West Bengal, Department of Fisheries, Sikkim as well as other Institutions. The Directorate also stocked golden mahseer in Shyamlatal lake of Kumaun region, India during 2001 where in it has survived very well, grown to mature sizes and now turning out to be an attraction for tourists. It can be expected that stock so introduced may continue for generations and may be served as natural sanctuaries. These kinds of efforts can be suggested in all the regions wherever mahseer exists.

Conclusion

To conclude, mahseer, which enjoyed the status of a fighting opponent to the fishers for a quite long time, is presently struggling for its mere existence. Sincere input from the scientific community as well as the planners in the right direction is the need of the hour to save these creatures from extermination. The potential of the species as a cultivable and sport fish has to be exploited with further research and planning, keeping an eye on their conservation. The efforts taken by the early workers especially the anglers and fishery biologists to study this group in a comprehensive manner have to be gratefully acknowledged. It was their studies, which have laid the strong foundation of mahseer research in India.

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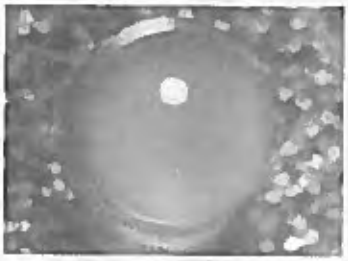
Fertilised egg.



Early morula stage

Blastopore

Formation of somites.

Appearance of eye.

Pigmented and circulated eye

Three quarters of egg surface covered

Appearance of pectoral fin

Hatching.

Hatchling

Early stage hatchling

One day old hatchling

Swim up fry

30 day old fry

90 day old fry

Subject	Publications' Number
Fish Biology and aquaculture	1, 10, 11, 12, 13, 16, 22, 24, 26, 28, 29, 31, 33,
Molecular phylogeny, diversity and species characterization	2, 3, 5, 7, 8, 9, 13, 17, 19, 20, 25,
Fish Nutrition	4, 24, 26
Fish Disease	5, 14, 27,
Molecular Biology	6, 7, 8, 9, 16, 17, 20, 21a, 21b, 23, 25, 27, 30,
Statistics	15, 18,
Water quality	15, 22,
Morphometric and Meristic	18, 24, 26,
Taxonomy	19,

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