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Proteins Diversity in Various Tissue of the Body of Carps

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Abstract: The production of culture and capture fishery of Indian major carps and Exotic CARPS, biochemical analysis has been conducted in farmed reared composite species of Indian major carps and Exotic carps to study its biodiversity. General protein from gill, muscle, heart and kidney were separated in SDS-PAGE (10% gradient) in which no polymorphism was observed. The Rm values were critically analysed using Jaccard's coefficient and the dendogram generated on UPGMA basis by computer software gives the closeness among the species, but the overall observation found more or less to be tissue specific.

Keywords: Fish, Carps, Protein, Rm value

1. Introduction

Indian major carps; Catla (*Catla catla*), Rohu (*Labeo rohita*), Mrigal (*Cirrhinus mrigala*) are the fastest growing fish available for freshwater aquaculture in the country. A fish that grow relatively in short period of time using cheap feed sources is desirable for fish farmer. Exotic species such as common Carp (*Cyprinus carpio*), Grass carp (*Ctenopharyngodon idella*) and Silver carp (*Hypophthalmichthyes molitrix*), which are native to China and also well established in India are also used in polyculture along with Indian Major Carps.Polyculture or composite culture of carps **involving three** Indian major carps and three species of exotic carps was developed by research institutes in 1970's has undergone refinement and modification over the years.,to get better yield.

Genetic modification occurs inadvertently in a cultured population. Since there is no competition for food and fear for predators, a farmed fish population experiences different kinds of selection regimes unprecedented in natural waters, . The composite or multi-species culture technologies so far developed are based on species manipulation and application of certain management practices. These technology no doubt have boosted the fish culture in several folds. However, at present it is felt that any further improvement in production may not be possible and the researcher gradually realizing the importance of other aspect such as genetic quality and improvement of the candidate species by fully exploiting their hitherto untapped genetic potentials. Molecular genetic markers have been applied to three fisheries areas in particular-stock structure analysis, aquaculture and taxonomy/systematic (Ward and Grewe, 1994) with varying degree of success (Carvalho and Hauser, 1994). All these techniques have their advantages and disadvantages in analysis of genetic variability in fishes (FAO, 1981, Bhatia et al; (1997a, 1997b; Ferguson and Danzmann, 1998;). The various methods available earlier, much before the advent of biochemical and molecular (DNA) techniques for stock identification or to study the existence of different population in a given species were only the

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morphometric measurement and meristic counts. These methods however, do not provide the degree of polymorphism distinguishable by modern methods especially within species (Reddy, 1999, Ward, 2000). Technological advancement in molecular biology and biochemistry has led to the identification of a variety of discrete, inheritable and stable genetic markers. These markers can be used for addressing problems of relevance to the management and conservation of various fishery stocks Bhatia et al;(2003, 2005).

Some advantages of protein electrophoretic methods include their speed and simplicity; disadvantages include a requirement for fresh or frozen tissue, relatively low level of variation and uncertainty over whether the variability recorded is natural or subject to selective pressure. Keeping in view, the foregoing points, in this study analysis were carried out on samples of three Indian major carps (viz. Rohu, Catla and Mrigal) and exotic/Chinese carps (viz. Grass carp, Silver carp and Common carp). However, in the absence of proper breeding plans, this has led to a gradual decline in the genetic quality of the seed. Consequently the negative effect on inbreeding started appearing with the characteristic poor survival and slow growth, besides disease susceptibility of the hatchery produced seed (Ibrahim *et al.,* 1982, Smith and Conroy 1992). All this suggest that it is an appropriate time to act seriously about genetic improvement of these carps. Thus genetic characterization of this fish species is the appropriate step in this direction.

Considering the above aspects, the present study has been undertaken with following objectives on six species of Catla (*Catla catla*), Rohu (*Labeo rohita*), Mrigal (*Cirrhinus mrigala*), Common carp (*Cyprinus carpio*), Grass carp (*Ctenopharyngodon idella*) and Silver carp (*Hypophthalmichthyes molitrix*).

2. Materials and Methods

Analysis of tissue specific protein polymorphism of the six species had been done and presented here.

Protein Extraction

Frozen tissue samples were thawed at 4^0 C by keeping in a refrigerator and 1g of different tissues was cut into small pieces with scissors. Weighing was done in digital balance using Aluminium foils. Homogenization was carried out under chilled conditions in 0.01 M Tris-HCl buffers of pH 6.8 having 1 m EDTA. Ground glass homogenizer was used for this purpose. Homogenized samples were centrifuged at 10,000 rpm for 30 minutes at 4^0C to remove the debris. Supernatant was further used for protein profiling by SDS-PAGE in muscle, gill, heart and kidney.

Protein Estimation

The method described by as of Bradford (1976) was followed, slight modifications.

Reagents

A. Preparation of Dye

Ten milligrams of dye (Co omassie brilliant blue, G-250) was dissolved in 5 ml of absolute alcohol. To this 10 ml of Orthophosphoric acid (85%) was added and the volume was made upto 100 ml with distilled water. Then it was filtered and kept in dark bottle and used within 2 weeks.

B. Standard Protein

Standard protein solution (BSA) was prepared by dissolving 50 mg BSA in 100ml of distilled water.

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Procedure

(i). 0.02, 0.04, 0.06, 0.08 and 0.10 ml aliquots of the standard protein solution were pipetted out in 5 cleanly washed dried test tubes.

(ii). Volumes of 5 tubes were made upto 0.3 ml with distilled water. 0.3 ml of distilled water was poured in a test tube for blank.

(iii). 3.0 ml of dye was added in each tube and colour intensity was measured at 595 nm. A standard curve of protein concentration vs O.D. was drawn.

(iv). Tissue extraction fraction was treated in the same way using water blank for measuring the content of protein.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The procedure of Laemmli (1970) (with certain modifications) was used to check the protein banding in gill, muscle, heart and kidney in order to determine polymorphism.

Reagents

Monomer Solution (30.8%T 2.7%C_{bis})

60g acrylamide with 1.6g N, N' methylene bis-acrylamide was prepared in 200 ml distilled water. The solution was filtered through.

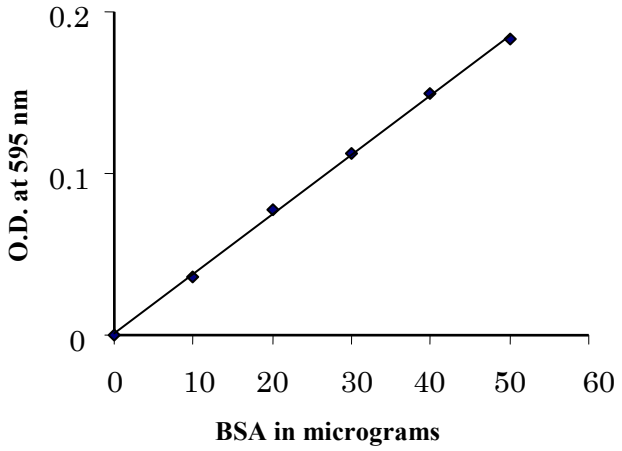

Fig 2.1 Standard curve for protein (Bradford dye binding method)

Whatman No. 1 and stored in dark bottle at 4^0 C and used within 2 months.

4X Running Gel Buffer (1.5 M Tris-Cl, pH 8.8)

36.3g Tris hydroxymethyl aminomethane (Tris) was prepared in 150 ml distilled water and the pH was adjusted to 8.8 with HCl. Final volume was made upto 200 ml. The solution was stored at 4^0C in dark bottle and used within 2 months.

4X Stacking Gel Buffer (0.5 M Tris-Cl, pH 6.8)

3.0 g Tris was prepared in 40 ml distilled water and the pH was adjusted to 6.8 with HCl. The final volume was made upto 50 ml. The solution was stored at 4^0C in dark bottle and used within 2 months.

10% Ammonium Persulphate (initiator)

0.1g Ammonium persulphate was prepared in 1 ml distilled water just before use.

10% SDS

10g sodium dodecyl sulphate was prepared in 100 ml distilled water and stored up to 6 month in room temperature.

TEMED

N, N, N', N', tetramethyl ethylene diamine (TEMED) was used as such (commercial preparation).

2X Sample Buffer (0.125 M Tris-Cl, 4% SDS, 40% v/v Gylcerol, 0.2 M DTT/ 8% β Mecaptoethanol, 0.02% Bromophenol Blue, pH 6.8)

0.7 ml of β Mecaptoethanol, 4 ml of 10% SDS, 2 ml of 40% glycerol and 2 mg Bromophenol blue dye were added in 2.5 ml stacking gel buffer of pH 6.8 to a final volume of 10 ml.

Tank/Electrode Buffer (0.025 M tris, 0.192 M glycine, 0.1% SDS, pH 8.3)

3.028 g Tris base, 14.413 g glycine and 1g SDS dissolved in 1000 ml of distilled water and pH was adjusted to 8.3 with concentration HCl if needed.

Water-Saturated n-Butanol

50 ml of n-butanol was mixed with 5 ml of distilled water in a bottle and shaked. The top phase was used to overlay gels and stored at room temperature indefinitely.

Staining Solution

Solution of 1.0 g coomasie brilliant blue R-250 in the 1 litre of water: methanol: acetic acid (1:5:4) was prepared.

Destaining Solution

Destaining was done by using 4% NaCl solution.

Procedure

(i). Electrophoretic glass plates (16 x 19 cm) were washed with soap water, rinsed thoroughly in distilled water and dried. Then concentrated alcohol was used to further clean if any grease left over.

(ii). Two glass plates were placed together with high vacuum-greased spacer between them, along the two sides and along the bottom edge. The thickness of the spacer determined the thickness of the gel (1.5 mm).

(iii). The plates were clamped with clamps and using base clamps it was kept in vertical position.

(iv). Approximately 60 ml of the separation gel of 10% concentration were prepared; 30ml/plate as given in table (3.2) and poured in between the plates to a level of 5 cm below the notch. For the purpose of casting gradient gel (higher concentration gel solution layered towards bottom of the plate followed by lower concentration gels at the top). Water saturated n-butanol was used @ 0.3 ml per gel to get a smooth surface of the separating gel and allowed to polymerized atleast for one hour. Thus, after polymerization overlayed water along with saturated n-butanol was washed away thoroughly using distilled water atleast three times.

Table 2.1 Running gel solution (10%) (60 ml; 1.5 mm thick two gels)

Monomer solution	20 ml
4X Running gel buffer	15 ml
10% SDS	0.6 ml
ddH_2O	24.1 ml
10% Ammonium persulphate	300 µl
TEMED	20 µl

(v). Stacking gel solution was prepared as given in table 3.2 and poured over the separating gel. Simultaneously 13 fingered combs were inserted in between the plates from the notch to leave a place of about 1 cm in between the surface of the separating gel and finger of the comb. It was also kept for one hour for polymerization.

(vi). After the stacking gel had polymerized, the bottom spacer was removed and the whole unit was attached to the electrophoresis apparatus.

(vii). Lower and upper chamber were filled with electrode/tank buffer and trapped air bubbles near the lower surface of the separating gel were removed by inclining the assembly.

Table 2.2 Stacking gel solution (4% acrylamide; for two gels)

Monomer solution	2.66 ml
4X Running gel buffer	5.0 ml
10% SDS	0.2 ml
ddH_2O	120 ml
10% Ammonium persulphate	100 µl
TEMED	10 l

(viii). 10-20 µl of samples containing 30 µg of protein were dissolved in sample buffer. Final concentration of SDS and β-mercaptoethanol were adjusted to 2 & 4 % respectively in these samples.

(ix). The loading samples were kept in water bath of 100^0 C for 90 seconds.

(x). Then samples were applied in each slot using micro pipette.

(xi). Power pack (MAC) was connected with the electrophoretic chamber with cathode in the upper chamber and anode in the lower chamber.

(xii). Electrophoresis was performed at 80 volts constant voltage for 20 minutes and then 110 volts till indicator dye (bromophenol blue) approached the bottom of the gel.

(xiii). Spacers were removed and the plates were gently separated with a spatula and gel was transferred to a dish containing staining solution for one hour.

(xiv). Dye was replaced with destaining solution till the background was clear.

Determination of Rm of SDS-PAGE bands

Relative mobility (Rm) values were calculated from the following formula

$$\text{Rm} = \frac{\text{Migration distance of polypeptides}}{\text{Migration distance of tracking dye}}$$

Analysis of Gel by Jaccard's Co-Efficient

Since genetic expression can vary due to many factors, analysis of similarity co-efficient like Jaccard's co-efficient can be very helpful (Jaccard, 1908). The co-efficient of Jaccard's omits consideration of negative matches. The Jaccard's co-efficient is expressed as follows

$$\text{J. C.} = \frac{\sum \text{Common bands}}{\sum \text{Common bands} + \sum \text{Different bands}}$$

Reagents

30% Acrylamide:Bisacrylamide Solution (37.5:1)

75g acrylamide with 2g NN' methylene bisacrylamide was prepared in 250 ml distilled water and stored in brown glass bottle for 3 months at 4^0C.

4X Separating Gel Buffer

36.3g Tris Hydroxymethylamine methane (Tris) of pH 8.8 was prepared in 150 ml distilled water. pH was adjusted to 8.8 with HCl and the final volume was made up to 200 ml and stored in brown bottle for 3 months at 4^0 C.

4X Stacking Gel Buffer

15.1g Tris-HCl was prepared in 40 ml distilled water. pH was adjusted to 6.8 with HCl and the final volume was made up to 50 ml and stored in brown bottle for 3 months at 4^0 C.

10% Ammonium Persulphate

1g ammonium persulphate was prepared freshly in 10 ml distilled water.

TEMED

N, N, N', N', tetramethyl ethylene diamine (TEMED) was used as supplied, stored in cold dry place.

H_2O-Satureted n-butanol

50 ml of n-butanol was mixed with 5 ml distilled water and stored at room temperature.

Electrophoresis Buffer

6.0 g of 0.025M Tris was dissolved in 1 litre of water containing 28.8g of 0.192M glycine. If weighed accurately pH would come 8.3 otherwise adjust with 1 M HCl.

2X sample Buffer

Sample preparation was carried out by mixing about 25 µl tissue extracted protein solution with 1 µl of bromophenol blue (0.5%) and 40 % glycerol. The amount of protein taken depended upon the type of enzyme to be stained. Same concentration of protein was loaded in each slot/well for staining one type of enzyme.

Staining and destaining solutions for visualizing total protein bands were same as for SDS-PAGE.

Table. Recipe for gel preparation for nondissociating discontinuous buffer system

Stock solutions	Stacking gel 4%	Separating gel mixture 7.5%
30% Acrylamide: Bis solution	2.66 ml	15 ml
4 x Separating gel buffer	—	15 ml
4 x Stacking gel buffer	5.0 ml	—
Distilled water	12.2 ml	29.7 ml
10% Ammonium per sulphate	100 µl	300 µl
TEMED	10 µl	20 µl

3. Results and Discussion

Protein Based Markers

Non-specific staining of soluble muscle, gill, heart and kidney proteins and specific staining of enzymes as in the above tissues (muscle, gill, heart and kidney) were carried out to visualize genetic polymorphism in these six composite species of Indian major; *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* and Exotic carps; *Ctenopharyngodon idella*, *Hypophthalmichthys molitrix*, *Cyprinus carpio* respectively.

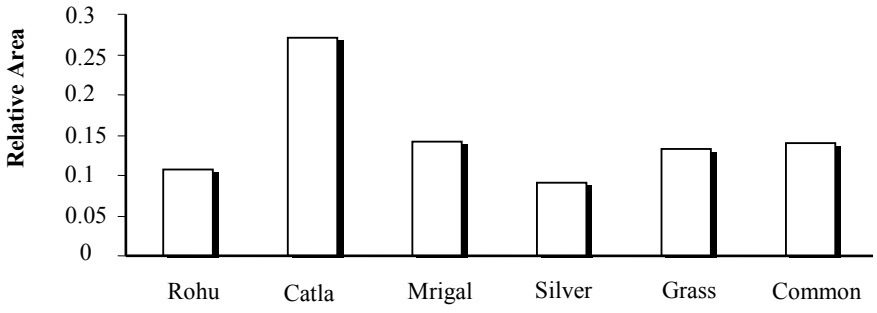

Figure 3.1. Relative gill area

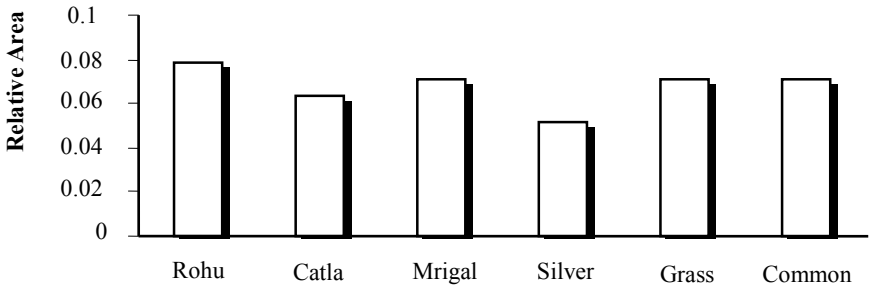

Figure 3.2. Relative heart area

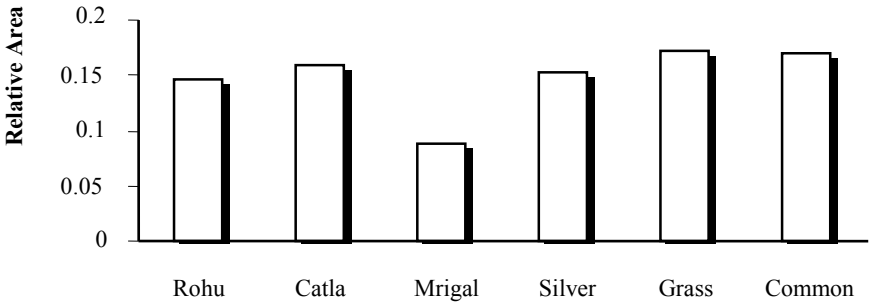

Figure 3.3. Relative kidney area

Interpretation of Gels

After staining an enzyme varying number of bands appeared in the tissues extracts from each species. These bands corresponded to:

(a) The products of the expression of several genes at several loci,

(b) The products of the expression of several alleles of a given gene at a given locus,

(c) Conformational changes of a given protein molecule,

(d) Molecule synthesized by a given gene or group of genes, which have undergone various post-translational modification.

Based upon the above facts the gels of proteins under present study were visualized as described below.

General Proteins

Soluble proteins pattern was recorded for four tissues (gill, muscle, heart and kidney). A gradient gel of (10% polyacrylamide) using SDS (10%) along with β Mecaptoethanol (8%) found suitable to resolve denatured proteins optimally.

Table 4.6 Total score between gills (G) of six species

Comparisons	Similar Bands	Total Bands	Jaccard'S Co-Efficient
G1-G2	6	26	0.23
G1-G3	4	23	0.17
G1-G4	2	21	0.09
G1-G5	3	23	0.13
G1-G6	5	25	0.2
G2-G3	5	27	0.18
G2-G4	4	25	0.16
G2-G5	4	27	0.14
G2-G6	4	29	0.13
G3-G4	4	22	0.18
G3-G5	6	24	0.25
G3-G6	5	26	0.19
G4-G5	3	22	0.13
G4-G6	4	24	0.16
G5-G6	6	24	0.25

Table 4.7 Total score between muscles (M) of six species

Comparisons	Similar Bands	Total Bands	Jaccard'S Co-Efficient
M1-M2	5	30	0.16
M1-M3	5	28	0.17
M1-M4	5	28	0.17
M1-M5	1	28	0.03
M1-M6	0	34	0.0
M2-M3	7	34	0.2
M2-M4	5	34	0.14
M2-M5	5	34	0.14
M2-M6	4	40	0.1
M3-M4	4	32	0.12
M3-M5	2	32	0.16
M3-M6	4	38	0.1
M4-M5	4	32	0.12
M4-M6	4	38	0.1
M5-M6	7	38	0.18

Table 4.8 Total score between hearts (H) of six species

Comparisons	Similar Bands	Total Bands	Jaccard'S Co-Efficient
H1-H2	1	28	0.33
H1-H3	0	28	0.0
H1-H4	2	32	0.06
H1-H5	6	32	0.18
H1-H6	2	31	0.06
H2-H3	4	30	0.13
H2-H4	2	34	0.05
H2-H5	3	34	0.08
H2-H6	5	33	0.15
H3-H4	4	34	0.11
H3-H5	2	34	0.05
H3-H6	4	33	0.12
H4-H5	7	38	0.18
H4-H6	4	37	0.1
H5-H6	2	37	0.05

Table 4.9 Total score between kidneys (K) of six species

Comparisons	Similar Bands	Total Bands	Jaccard'S Co-Efficient
K1-K2	6	22	0.27
K1-K3	3	20	0.15
K1-K4	2	25	0.08
K1-K5	1	21	0.04
K1-K6	0	23	0.0
K2-K3	4	22	0.18
K2-K4	3	27	0.11
K2-K5	3	23	0.13
K2-K6	0	25	0.0
K3-K4	3	25	0.12
K3-K5	1	21	0.04
K3-K6	2	23	0.08
K4-K5	1	26	0.03
K4-K6	2	28	0.07
K5-K6	0	24	0.0

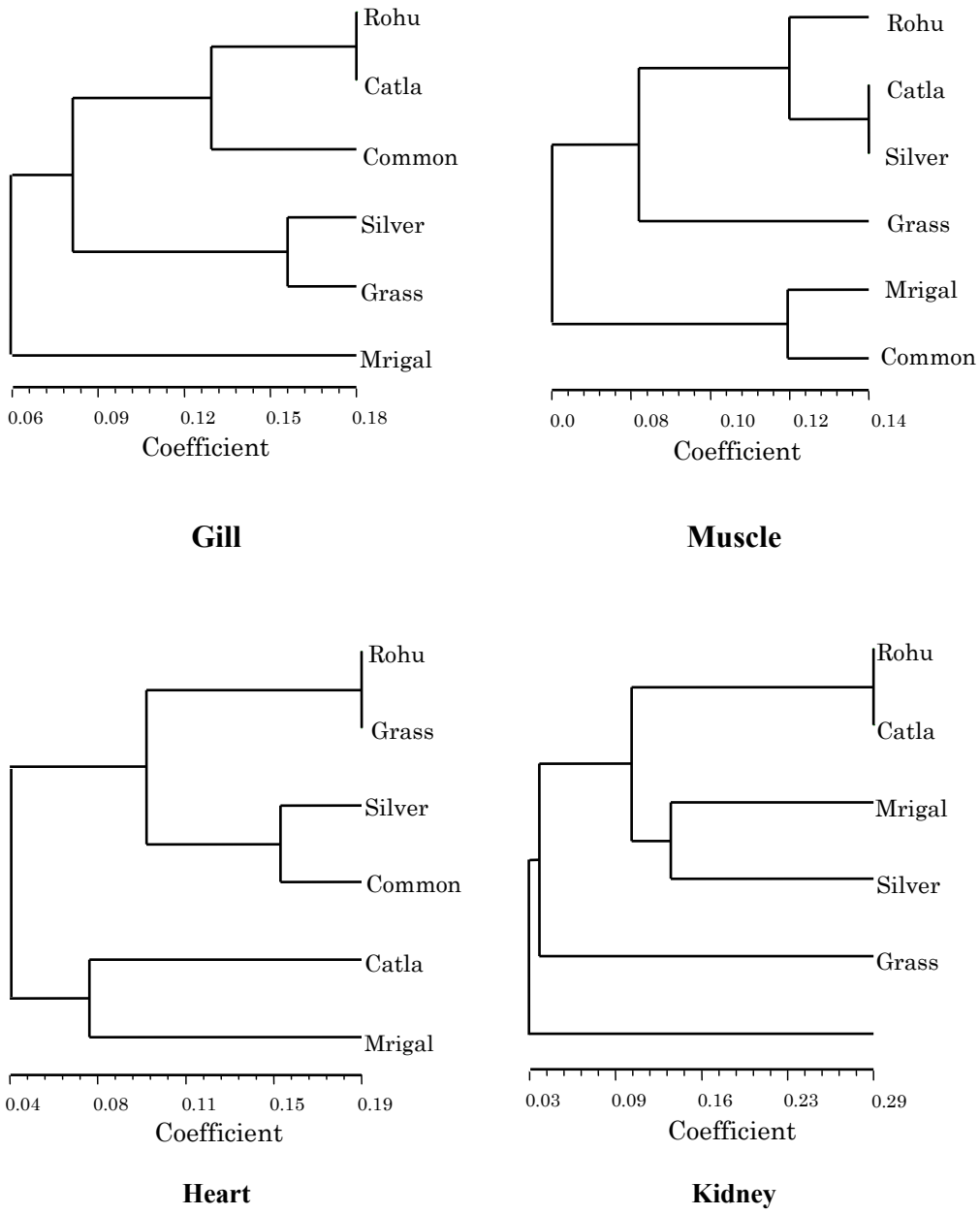

Figure 3.4. Dendogram representation of all the six species with respect to their Rm values.

Jaccard's co-efficients are shown in Table 4.6, 4.7, 4.8 and 4.9 for gill, muscle, heart and kidney for all the six species respectively. Dendogramic representation of all the above observation was shown is figure 4.5 clearly depicts the closeness between the species. In the case of gill tissue *L. rohita* & *C. catla* were closely related followed by *H. molitrix* & *C. idella*. *C. carpio* was closely related with *L. rohita* & *C. catla* then *H. molitrix* & *C. idella*. *C. mrigala* was observed to have the farthest relationship with remaining species.

In the case of muscle tissue *C. catla* & *H. molitrix* were observed to be closely related with much closer link with *L. rohita* followed by *C. mrigala* & *C. carpio*. *C. idella* has closer relation with the above three species then the later two.

In the case of heart tissue *L. rohita* & *C. idella* were observed to be closely related with closer link with *H. molitrix* & *C. carpio*. *C. catla* & *C. mrigala* follows next which were farthest from the above four species.

In the case of kidney tissue *L. rohita* & *C. catla* were observed to be closely related with closer link with *C. mrigala* & *H. molitrix*. *C. idella* was closely related with the above four followed by *C.mrigala*.

Gene structure determines protein structure and genetic variation at protein coding loci results in changes in the amino acid sequences of proteins. At the protein level, electrophoresis is one technique which can be utilized to detect such differences. In numerous early electrophoretic studies of fish stock variation of general proteins in muscles as revealed by non-specific protein stain such as amino black or commassie blue, have been employed for analysis of genetic variability (Choudhry,et.al' 1975 ,Shaklee and Salini, 1985; Chakraborty, 1990; Smith 1990 ,King and Pate, 1992 and Pandey and Hasnain, 1994). However under present investigation no polymorphic loci were detected in all the tissues (gill, muscle, heart and kidney) of all the six species of Indian major carp and Exotic carp using SDS-PAGE. Similar type of observation was also recorded earlier in muscle extract of three species of farmed reared Indian major carp using SDS-PAGE (Abella1982 ,Kothari, 2002 ,Bhatia et al;(, 2003, 2005 ,Saxena,2006)). Jaccard's co-efficient observed in the present study was found be tissue specific. Bhatia, (1998) claimed that *C. catla, L. rohita, C. carpio* and *C. mrigala* to be closely related based on protein profile observed through Jaccard's co-efficient. Problem were faced by many earlier workers which can only be resolved either by modifying present electrophoretic method using other denaturing agents such as Urea/CTAB instead of SDS (Pandey and Hasnain, 1994) or by isoelectric focusing (Whitmore, 1990,Ward 2000)) or two dimensional electrophoresis (Hames and Rickwood, 1981). Thus, different denaturing agents under different gradient can be tried to resolve any possible results. Interpretation of the banding pattern by Jaccard's co-efficient using Rm values can give a preliminary picture of the investigation.

The composite species of Indian major carp and Exotic carp forms the backbone of Indian aquaculture, even though the later was brought to India which now has been well adapted to the local conditions and forms an integral part of the freshwater genetic diversity. But it has been seen recently that the aquaculture production come to a standstill and simultaneously capture fishery of these species are also declining at a faster rate. This may be due to the fact that the breeding programme in most of the hatchery uses limited stock and there is little or no precaution for the genetic variability of the brooders. The wanton destruction of both adult and juvenile fish, ecological degradation, impact of river valley projects, pollution, introduction of competitive and fast growing exotics are some of the causes in natural fisheries.

Knowledge of genetic variability in past have been proven fruitful to fishery managers in identifying discrete breeding populations. Besides, estimation of stock mixture, indicating problems in fish culture, recognizing and quantifying hybrid populations as well as providing insights into conservation problems may be tackled by molecular technique using protein(s). Keeping in view of the above aspects an attempt was made to employ protein analysis as effective means of studying genetic diversity in this composite fish species. Highest production per unit area has been obtained in the polyculture of carps in India Murthy, (2002).

The salient observations of the study are summarized.

1. The relative anatomy of the target organs except muscle was also studied in which some species has better result than the others due to their difference in physiological behaviour.
2. Non-specific staining of denatured proteins of tissues viz., gill, muscle, heart and kidney on 10% gradient SDS-PAGE revealed similar banding pattern in all the species and observed to be tissue specific.

Thus, it is concluded that the species investigated being of the same family possess very close relationship with respect to the morphology and protein.

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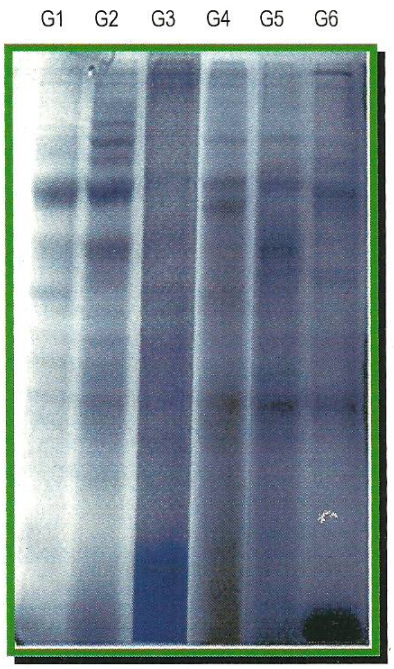
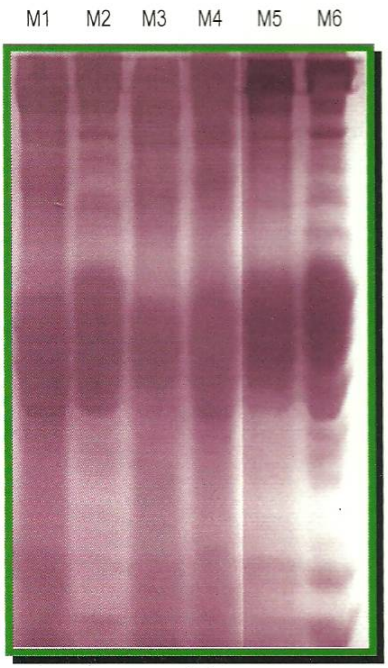
Appendix



(a)



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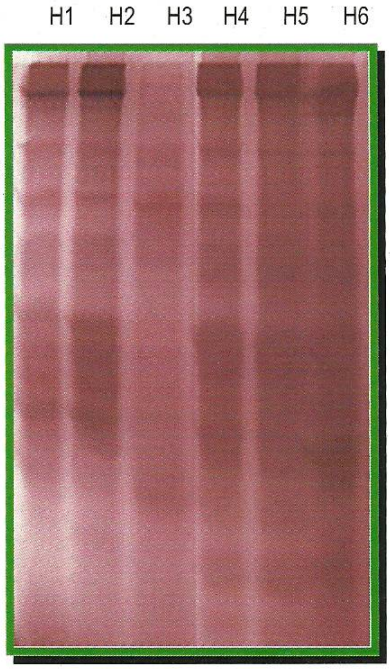

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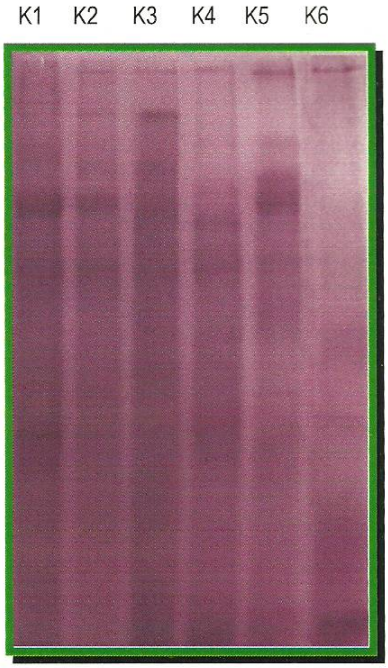

(d)