FREQUENCY DISTRIBUTION, BIOCHEMICAL CHARACTERIZATION AND INTRAGENERIC RELATIONSHIP OF TRANSFERRIN PHENOTYPES OF *Channa punctatus*

THESIS SUBMITTED FOR THE DEGREE OF Doctor of Philosophy IN ZOOLOGY

BY NIKHAT NABI

SECTION OF GENETICS DEPARTMENT OF ZOOLOGY ALIGARH MUSLIM UNIVERSITY ALIGARH (INDIA) 1999
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ABSTRACT

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ABSTRACT

Transferrin (Tf) is a monomeric iron binding protein which plays a central role of physiological importance in transporting iron to the bone marrow and to storage organs such as liver. In addition, it maintains the optimum concentration of iron in plasma which in turn prevents iron intoxication. The high degree of polymorphism displayed by transferrins is extremely useful in working out biochemical genetics of the concerned species. In fishes, it carries additional importance due to its possible application in identification of population units, breeding and maintenance of stocks. Besides, certain pathogenic bacteria which are associated with clinical conditions of fish species have been shown to use transferrins as iron source.

Channa punctatus Bloch, the fish species whose Tf's have been investigated here is amongst important food fishes of India and other Southeast Asian countries and makes significant contribution to fresh or brackish water capture as well as culture fisheries of these countries. The investigations included in this study deal with: (i) the identification of Tf isoforms of C. punctatus Bl. by PAGE and using obtained data on the polymorphism in discerning genetics of its populations; (ii) biochemical characterization of Tf isoforms and iii) lastly, the intrageneric relationships among Tf's of four available species of genus Channa viz. C. punctatus Bl., C. marulius Ham., C. gachua Ham. and C. striatus Bl. by microcomplement fixation analysis (MC'F).

Presuming polymorphism in Tf of C. punctatus under the control of codominant alleles as in other fishes, the single band isoform A, B and C in electropherogram would actually represent super-imposed identical polypeptides encoded by homologous pair of alleles, TfAcp, TfBcp or TfCcp, whereas their random combinations would give rise to two banded heterozygotes. Thus, in all six Tf phenotypes, AA, BB and
CC (homozygotes), plus AB, BC and AC (heterozygotes) should be formed which have in fact been discovered in different populations of *C. punctatus* inhabiting an area of about 73,815 sq. kms. Though in different proportions, virtually all phenotypes except AA are present within the above area. Among them phenotypes, BB and BC are quite in abundance whereas AA is found only in samples of Aligarh district. Contrary to other reports, frequency distribution pattern indicate that homozygote BB (frequency, 31%), are not very much at disadvantage as compared to heterozygote BC (frequency, 44%).

At 5% level of significance, no inter area heterogeneity is observed among samples procured from Aligarh, Bulandshahr, Badaun and Bareilly districts as the calculated value of $\chi^2$ (13.983) is less than the tabulated value ($\chi^2_{15,0.05}=25$). A comparison of the frequency of Tf alleles i.e. TfAcp, TfBcp and TfCcp between Aligarh and other 3 districts using Student's ‘t’-test reveals no significant differences except for the frequency of TfCcp in Badaun and Bareilly districts which differs significantly from that of Aligarh. The observed discrepancy for TfCcp may be attributed to random sampling or small number of samples examined from these districts. Since homozygote BB and heterozygote BC exist in differing proportions in one or the other habitats, differences in fertility as the cause of limiting population of these phenotypes may be ruled out. Observed differences may, therefore, depend more on post-hatching survival rather than the former factor. The statistical analysis of the electrophoretic data also supports the presumption that Tf polymorphism in randomly mating population of *C. punctatus* is controlled by 3 co-dominant alleles as pointed out above and the existence of a particular phenotype is independent of sex, size as well as the distribution of this fish species in four districts covered in the present study.
The three transferrin bands designated as A,B and C isoforms of *C. punctatus* were purified to homogeneity by a combination of rivanol precipitation and preparative polyacrylamide gel electrophoresis (PAGE). Commercial preparation of human Tf was used as the control. Biochemical analyses of Tf isoforms demonstrates that pI values of A,B and C isoforms are 5.3, 4.9 and 4.0 respectively, whereas the molecular weights of each type is ≈ 75 kD. Variations in pI of Tfs suggest differences in interactions which determine the charged state of these molecules. Spectral features of iron binding properties confirms the transferrin nature of the above isoforms beyond doubt. Studies on their iron binding capacities further shows that the number of atoms of iron bound per molecule of Tf is invariably ≈ 2 in all cases, though slight variations in the rate of binding can not be ruled out. The evidence thus favours that inspite of *C. punctatus* being a highly specialized fish, its Tfs are functionally and structurally similar to those of other vertebrates. In other words, it is a bilobar molecule with one site each for iron binding per lobe and one or more for binding membrane receptors at the tissue where it unloads iron. It is also proposed that the later site(s) appear to have a role in determining differential survivability of various phenotypes.

The quantitative estimation of reactivity between rabbit anti-TfBB of *C. punctatus* and Tfs of 4 species of genus *Channa* was performed by MC'F. The antiserum concentration which gives 75% complement (C') fixed at peak is 3,200; 2,900; 2,800; and 2,500 for *C.punctatus*, *C.gachua*, *C.striatus* and *C. marulius*, respectively. A linear correlation can be observed when antiserum concentration is plotted against % C' fixed at the peak. The immunological distance (ID) calculated on the basis of MC'F data indicates that among four species of genus *Channa*, antigenic determinants of Tfs of *C. punctatus* and *C. marulius* are most distantly related whereas those of Tfs of *C. striatus* and *C. gachua*
appears to be the closest. Out of them *C. punctatus* may be the species of most recent origin. The affinity of Tfs within genus *Channa* according to MC'F analysis may be of the following order: *C. punctatus* > *C. gachua* > *C. striatus* > *C. marulius* which is in accordance with the evolutionary relationship described on the basis of morphometric data.
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1999
This is to certify that the thesis entitled "Frequency distribution, biochemical characterization and intrageneric relationship of transferrin phenotypes of Channa punctatus" has been completed under my supervision by Ms. NIKHAT NABI. This work is original and has been independently pursued by the candidate.

I permit the candidate to submit the thesis for the award of Doctor of Philosophy in Zoology of the Aligarh Muslim University, Aligarh.

Dr. Absarul Hasnain  
(Supervisor)
Dedicated to
my father

(Bate Dr. Mohd. Noor Kabi)

Who provided me virtuous and
spiritual guidance during the
formative years of my life.
ACKNOWLEDGEMENTS

It is insurmountable to acknowledge all that one owes to ones benefactors, however, few words would be in order.

I wish to express my sentiments and immeasurable debt to my revered supervisor Dr. Absarul Hasnain, Section of Genetics, Dept. of Zoology, A.M.U. Aligarh. His resolute guidance and perseverance through out the progress of this work was the veritable stimulus. His scientific knowledge and methodological perspectivism have crucially contributed to the formulation of this thesis. I obediently offer my sincere gratitude to him.

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I deem it my immense pleasure in expressing my profound acknowledgement to my husband Abdul Hai Shah whose unwavering encouragement and cooperation at every stage made it possible to finish this thesis despite my various responsibilities.

(NIKHAT NABI)
## CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CHAPTER -1 (INTRODUCTION)</td>
<td>1-6</td>
</tr>
<tr>
<td>2. CHAPTER -2 (MATERIALS AND METHODS)</td>
<td>7-27</td>
</tr>
<tr>
<td>3. CHAPTER- 3 (RESULTS)</td>
<td>28-40</td>
</tr>
<tr>
<td>4. CHAPTER- 4 (DISCUSSION)</td>
<td>41-51</td>
</tr>
<tr>
<td>5. CONCLUSIONS</td>
<td>52-53</td>
</tr>
<tr>
<td>6. REFERENCES</td>
<td>54-63</td>
</tr>
</tbody>
</table>
CHAPTER -1
INTRODUCTION

Transferrin (Tf) is a monomeric protein characterized by the function of binding specifically and reversibly most of the acid soluble iron in plasma (Davis et al., 1962). Two other synonyms of it, siderophilin and β1 metal-binding protein are no more in use. Whereas it unloads bound iron in liver, it is unrelated to ferritin, which is an iron storage protein of this organ (Fletcher and Huehns, 1968).

The following vital functions of iron regulation are attributed to Tf's:

i) The iron derived from catabolism of hemoglobin and other proteins is conserved by its quantitative return through Tf to hematopoietic tissues (Fletcher and Huehns, 1968).

ii) The buffering action of Tf on the free iron concentration of plasma prevents the ferric concentration from elevating to toxic levels and it also impedes the possible oxidative or inhibitory effects of ferric ions (Laurell, 1960).

iii) Lastly, by holding the concentration of free iron in plasma it checks the loss of iron by urinary excretion. Experiments further indicate that during cyclic processing of iron by Tf, it binds ≥2 iron atoms per molecule (Palmour and Sutton, 1971; Welch, 1990).

Apparently, each human Tf monomer has two homologous iron binding sites incorporated in each of the two domains (MacGillivray et al., 1983) specified as N (residues 1-336) and C (residues 337-679). In addition, for the binding of ferric ion at each of the two iron binding sites of Tf, a concomitant binding of a bicarbonate ion is also essential (Aisen et al., 1973). The complex that is formed between purified...
Tf and ferrous ion is not chemically or spectrometrically different from that prepared using ferric salts. Loci for Tfs map on positions 21-25 on short arm of chromosome 3 of human karyotypes (Yang et al., 1984).

It has also been suggested that by competing for iron and keeping the plasma concentration low Tf protects against infections. This suggestion is supported by the fact that patients with atransferrinemia (the congenital absence of Tf) suffer from recurrent infections (Heilmeyer, 1966). Furthermore, patients with hypotransferrinemic conditions are more predisposed to infections than those with normal Tf levels (Bernstein, 1987). There are some evidences which suggest a correlation between Tf levels in sera of fish species and certain clinical conditions (Pratschner, 1978; Winter et al., 1980; Hirono and Aoki, 1995).

Though Tf can also combine loosely with the metals of transition and lanthanide series including manganese, copper, cobalt, chromium, scandium, terbium and zinc (Charlwood 1963; Aisen et al., 1969; Gafni and Steinberg, 1974) only the transport of iron is known to have a physiological significance.

Earlier estimates of molecular weights of vertebrate transferrins had settled to a value of ≈90 kD (Koechlin, 1952; Bezkorovainy et al., 1963 Aisen et al., 1966). Charlwood (1963), first disputed this figure and instead determined a value of 68 kD for human, monkey and rat Tfs. Later on, further low values for human Tfs close to 76.5 kD were reported by several workers (Roberts et al., 1966; Mann et al., 1970, Palmour and Sutton, 1971; Welch, 1990). No differences in molecular weights of apo-Tf or those binding either one or two iron atoms has ever been detected.

Values (68-85 kD) similar to those agreed upon for human Tfs have been
recorded for Tfs of other vertebrates (Hara 1984, Welch, 1990). Fish Tfs are also known to follow this pattern with a value of 77 kD reported for hagfish (Aisen et al., 1972) and Hershberger (1970) reported a value of 78 kD for brook trout Tfs. In contrast, Stratil et al. (1985) calculated rather a low value of 68 kD for catfish against 86.8 kD for pike Tf. This range of molecular weights in vertebrate stand confirmed by SDS-polyacrylamide gel electrophoresis (Welch 1990).

For the purification of Tf numerous strategies making use of ammonium sulfate, rivanol (2-ethoxy-6,9-diaminoacridine lactate) or ethanol as precipitants, and purification by column chromatography or gel filtration were attempted. Out of them, the use of rivanol (originally proposed by Boettcher et al., 1958) has proved most effective and rapid. The ratio of rivanol to serum, however, may vary from case to case. The documented ratios for different vertebrates are: 1:3 for cattle (Patras and Stone, 1961) 1:3.5 for human (Sutton and Karp, 1965), and 1:2 for brook trout (Hershberger, 1970) Tfs.

Another important characteristic of Tfs which has drawn considerable attention is the high degree of polymorphism it displays in most of the vertebrate species examined so far. Such data is extremely useful in working out the biochemical genetics of the concerned species. Whereas for human beings due to a correlation with clinical aspects Tf data on polymorphism carries additional importance, as well as in case of other vertebrates it may find applications in identification, breeding and maintenance of animal stocks. The discovery of first Tf polymorph was made by Smithies (1957) who identified a heritable variant Tf-D in the sera from Negroes and Australian natives. However, the most common of them is Tf-C which was originally detected as the
third component in the β-globulin region in starch gel electropherogram of human serum (Smithies, 1957). Further studies demonstrated that genetic polymorphism at Tf loci of vertebrates may be a rule rather than an exception. The cited examples include, zebras (Stratil et al., 1992); rat (Nagabuchi et al., 1993); brown hare (Hartle and Ferrand, 1993); hounds (Sherer and Kluge, 1993); donkey (Bell, 1994) and cattle (Blott et al., 1998).

So far as fishes are concerned, a high degree of Tf polymorphism has been reported in several species such as tunas (Barrett and Tsuyuki, 1967; Fujino and Kang, 1968); brook trouts (Hershberger 1970); carps (Valenta et al, 1976). Some species of the family cyprinidae (Valenta and Stratil, 1977); European hake (Mangaly and Jamieson, 1979); sturgeons (Keyvanfar, 1986); Channa sp. (Hasnain et al., 1981; Sahoo and Khuda-Bukhsh, 1989) and coho salmon (Utter et al., 1970; Hirono and Aoki, 1995; Van-Doornik and Milner, 1996).

Phylogenetic relationships of a number of organisms have been worked out by making immunological investigations on several proteins. There exists published evidence which deals with glycerol 3-phosphate dehydrogenase (Fink et al., 1970); lysozymes (Arnheim and Wilson, 1967; Prager and Wilson, 1971); albumins (Prager et al., 1974); histones (Bustin and Stollar, 1973); azurins (Champion et al. 1975). The reports dealing with phylogenetic relationship of Tfs where immunological approach was applied, are scarce. Among them is the report of Prager et al. (1976) on phylogenetic correlation of flightless land birds. As for fish species, only one report was found where phylogenetic interrelationships between Tfs of a number of elasmobranchs were determined by immunological methods (Lawson et al.; 1995).
Thus, there exists a paucity of information on immunological interrelationships of proteins of vertebrates in general and on Tf in particular.

**STATEMENT OF THE PROBLEM**

*Channa punctatus* Bloch is among important food fishes of India as well as most of South Asian countries. It makes a significant contribution to fresh and brackish water capture fisheries of these countries beside being harvested as a component of paddy-cum-fish culture. The information about population structure, in general, is important from the viewpoint of better management of natural resources as well as aquaculture practices. In biochemical genetics, polymorphic protein and isozyme markers have been extensively employed to achieve the above objectives. Unfortunately, very little information is available on these aspects of transferrin (Tf) isoforms of *Channa punctatus* Bl. and their biochemical characterization remains virtually unattended.

An interesting aspect of the fish species belonging to genus *Channa* is the adaptation to accessory airbreathing habit for which, specialized organs exist in them. This divergence justifies additional interest in these species because fishes already occupy a central position in the evolution of vertebrates.

Taking the above considerations into account, the present investigations on Tf isoforms of *Channa punctatus* Bl. were envisaged so as to include the following aspects:

1) To determine the extent of polymorphism at transferrin locus by electrophoretic typing of transferrin isoforms and assess the significance of obtained data in
discerning population genetics of *C. punctatus* Bl.

2) To purify its Tf isoforms to homogeneity and biochemical characterization of them by monitoring functional property of iron binding and estimation of molecular weights.

3) To trace evolutionary relationships among Tfs of four available species of genus *Channa* by microcomplement fixation (MC'F) analysis using purified Tf isoform of *C. punctatus* as reference immunogen.
CHAPTER - 2
MATERIALS AND METHODS

This chapter comprises details of methods and protocols followed during the present investigations, which are split up into following sections and sub sections.

1. SOURCE OF TRANSFERRINS 9

2. KEY TO INDIAN SPECIES OF GENUS CHANNA 9

3. AREA OF SAMPLING 10

4. COLLECTION OF SERA 11

5. GENERAL METHODOLOGY 12
   (A) Source of chemicals 12
   (B) Protein estimation 13
   (C) Polyacrylamide gel electrophoresis (PAGE) 13
      a) Non-denaturing polyacrylamide gel electrophoresis (PAGE) 13
      b) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) 14
   (D) Staining 16
      a) with coomassie brilliant blue. 16
      b) with silver stain 16
      c) with nitroso-R stain (specific staining) 18
   (E) Photography 18

6. PURIFICATION OF TRANSFERRINS 18
<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Partial purification by rivanol</td>
<td>18</td>
</tr>
<tr>
<td>(B) Purification of individual Tf isoforms by preparative polyacrylamide gel electrophoresis (PAGE)</td>
<td>19</td>
</tr>
<tr>
<td>7. ISOELECTRIC FOCUSING (IEF)</td>
<td>20</td>
</tr>
<tr>
<td>8. MOLECULAR WEIGHT ESTIMATION</td>
<td>20</td>
</tr>
<tr>
<td>9. ABSORPTION SPECTROSCOPY OF TRANSFERRINS</td>
<td>20</td>
</tr>
<tr>
<td>10. PREPARATION OF APOTRANSFERRINS</td>
<td>21</td>
</tr>
<tr>
<td>11. IRON BINDING ANALYSIS</td>
<td>21</td>
</tr>
<tr>
<td>12. STATISTICAL ANALYSIS OF ELECTROPHORETIC DATA</td>
<td>22</td>
</tr>
<tr>
<td>(A) Chi-square ($\chi^2$) contingency test</td>
<td>22</td>
</tr>
<tr>
<td>(B) Calculation of allelic frequencies</td>
<td>22</td>
</tr>
<tr>
<td>(C) Standard deviation ($\sigma$) and Student’s 't' test</td>
<td>22</td>
</tr>
<tr>
<td>13. IMMUNOLOGICAL METHODS:</td>
<td>23</td>
</tr>
<tr>
<td>(A) Diluent</td>
<td>23</td>
</tr>
<tr>
<td>(B) Antigen</td>
<td>23</td>
</tr>
<tr>
<td>(C) Raising antiserum</td>
<td>23</td>
</tr>
<tr>
<td>(D) Ouchterlony's immuno-diffusion method</td>
<td>24</td>
</tr>
<tr>
<td>(E) Source of complement</td>
<td>24</td>
</tr>
<tr>
<td>(F) Source of red blood cells (RBCs)</td>
<td>24</td>
</tr>
<tr>
<td>(G) Hemolysin titration</td>
<td>25</td>
</tr>
<tr>
<td>(H) Preparation of sensitized cells</td>
<td>25</td>
</tr>
<tr>
<td>(I) Protocol for microcomplement fixation experiments</td>
<td>26</td>
</tr>
</tbody>
</table>
1. SOURCE OF TRANSFERRINS:

As explained under the Statement of the Problem, the present investigations envisaged the distribution of phenotypes and characterization of transferrin isoforms in *C. punctatus* Bl. (fig. 1) only. The transferrins of the other three species i.e. *C. marulius*, *C. gachua* and *C. striatus* have been included for the sake of determining phylogenetic interrelationship by complement fixation. These fishes are commonly known as murrels or snakeheads as their body is subcylindrical and anteriorly covered with plate-like scales. They have a wide distribution not only in India but also in several other countries of Southeast Asia (Day, 1958) and make a significant contribution to their fishery resources, including paddy-cum-fish culture.

2. KEY TO INDIAN SPECIES OF GENUS CHANNA:

During the present investigations identification was performed on the basis of the key proposed by Srivastava (1980). It is being reproduced below:

(i) 15-20 scales between the snout and origin of dorsal fin; 9-10 scales between the orbit and the angle of preopercle.

(ii) 12-13 scales between the snout and origin of the dorsal fin; 4-5 scales between the orbit and the opercle.

(iii) 18-20 scales between the snout and the origin of the dorsal fin; 9 scales between the orbit and the angle of preopercle. Grey and black band descending from the lateral side to the abdomen, without an ocellus at the tail.

* C. striatus (Bl.)

(iv) 15-16 scales between the snout and the origin of the dorsal fin; 9-10
scales between the orbit and the angle of the preopercle. No band like *C. striatus*.

A large black ocellus at the upper part of the base of the caudal fin.

*C. marulius* (Ham.)

(v) Ventral 2/5 length of pectoral. Pectorals with alternating orange and blue transverse bands. No bands on the body.

*C. gachua* (Ham.)

(vi) Ventral 2/3 to 3/4 length of pectoral. Pectoral without transverse bands and with uniform colouration. Several bands or patches from back pass down the abdomen.

*C. punctatus* (Bl.) (Fig. 1).

3. **AREA OF SAMPLING:**

Samples of *C. punctatus*, *C. striatus* and *C. marulius* were obtained from local fish markets of the districts of Uttar Pradesh namely, Aligarh, Badaun, Bulandshahr and Bareilly with a coverage of about 73,815 sq. kms. area (Fig. 2). It is a routine that mixed catches from freshwater ditches, ponds, small streams and rivers are collected and transported via important towns (localities) listed in Table-1 to the disposal outlets which in most of the cases are townships or the main district/cities. Because of the presence of accessory airbreathing organ these species, most of the times are brought alive to markets in aerated hard-knits stuffed with wet grates. The transportation of *C. marulius* from distant localities however, requires special care which in the present case was taken by carrying them in oxygen filled water
care which in the present case was taken by carrying them in oxygen filled water bags.

Live specimens of *C. punctatus* are available in the market throughout the year, while *C. striatus* and *C. marulius* of sizes (>32 cm) appropriate for good yield of blood are available from September to December. *C. gachua* is the least abundant of all being very rare in Aligarh, specifically.

4. COLLECTION OF SERA:

Blood was taken from live fish specimen by cardiac puncture using sterilized plastic syringe equipped with the needle # 23. While being withdrawn from the heart, blood starts clotting in case of *C. marulius* which chokes the needle of syringe. Rinsing the syringe with the anticoagulant (trisodium citrate - 0.8 gm %; D-glucose - 2.05 gm %; sodium chloride - 0.72 gm %) helped to overcome this problem. Sterile glasswares and vials were preferred for keeping blood as well as for subsequent storage of sera, since contamination was noticed to cause hemolysis.

To obtain sera, blood was allowed to clot at room temperature (25-30°C) for 2-3 hrs and as soon as the clot started to shrink the containers were shifted to lower chamber of the refrigerator (10 °C) for another 2-3 hrs. The serum was then drawn by a 2 ml sterilized plastic syringe and centrifuged at 2,000 rpm for 10 minutes to sediment out contaminating blood corpuscles. Clear sera were stored in microfuge tubes at -20°C till further analysis.
5. GENERAL METHODOLOGY:

(A) Source of Chemicals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Acrylamide</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Alcohols</td>
<td>Riedel-de Haën (Germany)</td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Coomassie brilliant blue G250</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Coomassie brilliant blue R250</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Freunds complete adjuvant</td>
<td>GIBCO BRL (USA)</td>
</tr>
<tr>
<td>Human transferrin</td>
<td>Fluka biochemica (Switzerland)</td>
</tr>
<tr>
<td>IEF Marker</td>
<td>Pharmacia Biotech (Sweden)</td>
</tr>
<tr>
<td>Nitroso-R</td>
<td>Sigma Chemicals (USA)</td>
</tr>
<tr>
<td>Pharmalyte (pH 3-10)</td>
<td>Pharmacia Biotech (Sweden)</td>
</tr>
<tr>
<td>Polyethylene glycol (PEG)</td>
<td>CDH-India (AR Grade)</td>
</tr>
<tr>
<td>Potato starch</td>
<td>CDH-India (AR Grade)</td>
</tr>
<tr>
<td>Rivanol</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Sigma chemicals (USA)</td>
</tr>
<tr>
<td>Trizma base</td>
<td>Sigma chemicals (USA)</td>
</tr>
</tbody>
</table>

Other chemicals and acids were of AR Grade.
(B) Protein estimation

It was performed according to Bradford (1976). The protocol given below was followed:

In a final volume of 0.1 ml, serial dilutions (1 μg - 9 μg) of BSA (bovine serum albumin) were made in 0.01 M phosphate buffer (pH 7.2). Each tube received 2 ml of coomassie brilliant blue (CBB G250, 10 mg dissolved in 4.7 ml of ethanol and 10 ml of orthophosphoric acid which was made up to 100 ml with distilled water). The absorbance was read at 595 nm. Concentration of unknown samples of protein was determined against this standard which was linear up to 7 μg/tube.

(C) Polyacrylamide gel electrophoresis (PAGE):

(a) Non-denaturing polyacrylamide gel electrophoresis (PAGE). It was performed in the discontinuous buffer system of Laemmli (1970) with the modification that no SDS was added to any of the solutions. Gels were cast using following stock solutions:

i) 30% Acrylamide + 0.8% Bisacrylamide

ii) Lower Tris x 4 (pH - 8.6)

Tris Base 1.5 M

pH adjusted to 8.6 with HCl

iii) Upper Tris x 4 (pH - 6.8)

Tris Base 0.5 M

pH adjusted to 6.8 with HCl

iv) Tris Glycine - Running Buffer x 4 (pH - 8.3)

TRIS BASE 100 mM

GLYCINE 1 M
v) Ammonium per sulphate 10%

**Electrophoretic runs:**

Routine screening of sera was done on 7.5% gel with a final composition
acrylamide =7.5%, 0.2% bisacrylamide in 0.375 M Tris-HCl (pH - 8.6). The
polymerization was achieved by adding ammonium per sulfate 0.06% and 0.04%
TEMED (N, N, N', N' tetra ethyl methylene diamine). Stacking gel was 3% with a
final composition of 3% acrylamide, 0.08% bisacrylamide in 0.125 M Tris-HCl (pH
6.8) and polymerized by adding 0.03% Ammonium per sulphate and 0.1% TEMED.
The electrophoretic runs were made in slab gels of 10x15x1mm at a current of 8
mA/ gel until the tracking dye (bromophenol blue) entered the resolving gel.
Subsequently, the current was raised to 12 mA/gel until the dye migrated 1 cm short
of anodic end.

(b) Polyacrylamide gel electrophoresis in the presence of sodium dodecyl
sulfate (SDS-PAGE):

It was performed in a discontinuous but highly porous system of Doucet
and Trifaro (1988), essentially as described by them. This protocol which is specifically
suitable for glycoprotein requires the following stock solutions:

i) 40% Acrylamide + 0.4% Bisacrylamide

ii) Tris (x5) 1 M

iii) Glycine (x5) 0.5 M

iv) EDTA (x5) 20 mM

(v) Tris-HCl Buffer (x5) (pH - 6.7)

Tris 350 mM

pH adjusted to 6.7 with HCl
vi) SDS (x5) 2%

vii) Upper tank buffer (x 4) (pH - 8.5)
- SDS 0.4%
- Tris 400 mM
- Glycine 600 mM

viii) Lower tank buffer: 1 volume of upper tank buffer diluted with
1 volume of distilled water.

ix) Sample buffer (x 2)
- Urea 16 M
- SDS 6%
- Dithiothreitol 200 mM
- Tris-HCl buffer of pH - 6.7, 70 mM

**Electrophoretic runs:**

The separating gel had the following final composition: 10% acrylamide and 0.1% bisacrylamide in 0.4% SDS, 5% glycerol, 200 mM Tris, and 100 mM glycine (final pH 9.0); 0.1% ammonium persulfate and 0.05% TEMED was added for polymerization. The stacking gel contained 4% acrylamide, 0.04% bisacrylamide, 0.4% SDS, 4 mM EDTA, 5% glycerol and 70 mM Tris-HCl (pH 6.7). The mix had to be essentially degas for 15 minutes before adding ammonium persulfate and TEMED in the final concentrations of 0.1% and 0.05% respectively. Gels were cast in slabs of 10 x 15 x 1 mm.
The protein samples containing sample buffer in equal volumes were denatured by heating at 100°C for 2 minutes and before loading the wells were washed twice with the upper tank buffer. Electrophoresis was performed at a constant voltage of 60 V (about 10 mA/gel) till the BPB-buffer line was 1 cm short of anodic end.

(D) Staining:

a) With coomassie brilliant blue:

To stain the gels for general protein patterns, routinely, coomassie brilliant blue R-250 was employed.

The stock solution (1 x) had the following ingredients:

- Coomassie Brilliant Blue (R250) 2.5 gm
- Methanol 450 ml
- Acetic acid 90 ml
- distilled H₂O To 1L

Destaining was carried out in 7% acetic acid.

(b) with silver stain:

Silver staining of the gel was performed by the method of Oakley et al. (1980). The following solutions were required for this purpose.

i) Fixative 1: 50% methanol in 7.5% acetic acid

ii) Fixative 2: 5% methanol in 7.5% acetic acid

iii) Fixative 3: 10% glutaraldehyde

iv) Developer: 0.05% citric acid

For each ml of 0.05% citric acid, 5 ml of 37% formaldehyde solution was also added.
v) Stain:

NaOH  0.09M; 53 ml
NH₄OH (concentrated)  3.5 ml
AgNO₃ solution (20 g%)  8 ml

Final volume made up to 250 ml with distilled water. This stain had to be made just before use.

The gel was incubated for 20 minutes in 300 ml of each fixative serially followed by thorough rinsing in large volumes of distilled water. At least four such changes were made before the final overnight rinse. Gel was kept for 20 minutes in the stain and the browning of gel margins was taken as the signal of optimal staining. It was then washed in distilled water for 5 minutes and allowed to develop till the bands of desired intensity appeared. Finally, it was placed in 7% acetic acid.

vi) Destaining:

The following destaining solutions were used:

Solution A:

NaCl  11.1 g
CuSO₄  11.1 g

Final volume made up to 300 ml with distilled water.

Just before use ammonia solution (25%) was added until the precipitate cleared to give a neat blue colour.

Solution B:

44 g of sodium thiosulfate pentahydrate was dissolved in 85 ml of distilled water and made up to 100 ml.
Solution C:

Kodak hypo cleaning agent, 20 g dissolved in 800 ml of distilled water.

Solution A and B (3:1) were mixed and diluted with an equal volume of distilled water. Gel was incubated in 100 ml of this solution until the background cleared. The gel was immediately rinsed with distilled water and incubated in solution C for 30 minutes followed by washing with large volume of distilled water.

(c) with nitroso-R stain (specific staining):

It was performed according to Mollar and Naevdal (1966). The gel was incubated for 15 minutes in hydroxylamine hydrochloride solution (100 mg/50 ml acetic acid). Subsequently, nitroso-R (25 mg/ml of distilled water) was added and incubation was continued in dark until the maximum development of green bands. The gel was then washed twice with distilled water and stored in 7% acetic acid containing 5% methanol.

(D) Photography:

Fresh specimens of all four channid species were used to study morphological features and photographed on Kodak Gold colour print film. Electrophoretic patterns of gels were documented on 125 ASA black and white negative films after placing them on an ordinary transilluminator.

6. PURIFICATION OF TRANSFERRINS:

(A) Partial purification by rivanol:

It was accomplished by precipitating out non-transferrin proteins of fish sera by rivanol (2, ethoxy-6,9 diaminoacridine lactate). Essentially, the methodology of Sutton and Karp (1965) and Hershberger (1970) was followed.
Typically, to 1 ml of sera 15 mg of ferrous ammonium citrate was added to saturate the transferrins with iron which is considered to increase its stability. One part of this serum was then diluted with 0.5, 1, 1.5, 2 and 2.5 parts of 0.4% rivanol in 0.005M Tris-HCl of pH 8.8. The supernatant was passed through a pad of 1g of dry potato starch that was previously washed with the same buffer. This amount of starch is known to adsorb about 14 mg of rivanol (Sutton and Karp, 1965). The filtrates were run on non-denaturing PAGE as described above. Samples were dialysed against 3 changes of distilled water and were concentrated with polyethylene glycol-600 to a desired volume.

**(B) Purification of individual Tf isoforms by preparative polyacrylamide gel electrophoresis (PAGE):**

Sera of the same phenotype were pooled to give sufficient volume of sample for partial purification with rivanol. For this purpose, non-denaturing PAGE as described under 5C(a) was employed with the modification that larger gels 100 x 180 x 4 mm containing only a single slot were used. The slot was 9 cm long with at least 0.5 cm flanking arms to prevent seepage accommodating upto 2 ml of partially purified and concentrated transferrin sample. After the run was over, 1 cm slice of gel along the vertical axis was cut off and stained with CBB-R250. The portion of unstained gel corresponding to the bands of transferrins in the stained slice were cut out and frozen at -20°C overnight. The frozen bands were homogenized by a mechanical blender at 3,000 rpm in 3 volumes of Tris-HCl buffer (25 mM, pH 7.5) and centrifuged in microfuge tubes with a built-in glass filter. The eluted samples were dialyzed against 3 changes of distilled water and freeze dried.
(7) ISOELECTRIC FOCUSING (IEF):

It was essentially performed according to the Calibration Kits for pI determinations using isoelectric focusing provided by the supplier Pharmacia Biotech, (Sweden). Polyacrylamide gels were T=5% and C=3% containing pharmalyte pH 3-10. Focusing was achieved with in 2 hours at 1,500 V and 50 mA. Broad range pI markers were also from Pharmacia Biotech.

(8) MOLECULAR WEIGHT ESTIMATION:

For this purpose the purified isoforms were run in the system of Doucet and Trifaro (1988). Molecular weight of pure transferrin phenotypes was calculated using marker. Relative mobilities were calculated from the standard formula of Weber and Osborn (1969).

\[
\text{Relative mobility} = \frac{\text{distance migrated by protein}}{\text{distance migrated by tracking dye}} \times \frac{\text{length of gel before stain}}{\text{length of gel after staining}}
\]

(9) ABSORPTION SPECTROSCOPY OF TRANSFERRINS:

Absorption spectra of iron saturated transferrin was determined in the visible range (Palmour and Sutton, 1971) using spectrophotometer (Spectronic-1001). Samples were dissolved in 25 mM Tris-HCl, pH 7.5 containing NaHCO3 at a final concentration of 30 mM. The samples were dialyzed extensively against several changes of the same buffer to remove any excession. An aliquot of the final dialysis fluid was used as a blank. Human transferrin was used as a reference protein.
(10) PREPARATION OF APOTRANSFERRINS:

The transferrins were deprived of iron by dialyzing the samples against at least 4 changes of 1.0 M citric acid (Palmour and Sutton 1971). The contents were centrifuged at 10,000 rpm and the supernatant was extensively dialyzed against distilled water with 5-6 hourly change for 48 hrs.

(11) IRON BINDING ANALYSIS:

Iron binding studies were carried out on apotransferrin of individual isoforms of *C. punctatus* according to Bates and Schlabach (1973) and Welch (1990). Human transferrin of commercial origin (Fluka, Switzerland) was used as the control. The system makes use of FENTA or ferric nitrilotriacetic acid (composition: nitrilotriacetic acid, 0.19 g/3 ml of D. H₂O; NaOH 1 M, (2ml); 0.5 M ferric chloride, 2.0 ml; final volume made up to 10 ml with distilled water) containing 0.1 μg of Fe/μl of FENTA. Titrations were carried out in the presence of sodium bicarbonate at a final concentration of 30 mM. The binding of iron to transferrin was monitored by following the increase in absorbance at 460 nm. Transferrin was supposed to be fully saturated with iron when addition of FENTA did not result in an increase in absorbance any more. Atoms of iron (Fe) bound per molecule of Tf was calculated by the following formula:

\[
\text{atoms of Fe bound/Tf molecule} = \frac{\text{mole of iron}}{\text{mole of Tf}}
\]

Where, 1 mole = \[
\frac{\text{Weight of the substance used}}{\text{Molecular weight of that substance}}
\]
(12) STATISTICAL ANALYSIS OF ELECTROPHORETIC DATA:

On the basis of recorded data on transferrin phenotypes, the following statistical calculations were made to check the magnitude of equilibrium among subpopulations according to Hardy-Weinberg equation.

(A) Chi-square ($\chi^2$) contingency test:

It was made using the formula:

$$\chi^2 = \frac{\sum (O-E)^2}{E}$$

Where, $O =$ observed frequency

$E =$ expected frequency

(B) Calculation of allelic frequencies:

It was calculated according to Ferguson (1980b) using the formula:

$$\frac{2H_o + H_e}{2N}$$

where, $H_o =$ number of homozygotes for that allele

$H_e =$ sum of number of heterozygotes for that allele and

$N =$ number of individuals examined.

(C) Standard deviation ($\sigma$) and Student's $t$ test:

$$\sigma = \frac{\sqrt{\sum d^2}}{n-1}$$

Where, $d =$ deviation from the mean.

$n =$ number of observations
\[ t = \frac{\bar{x}_1 - \bar{x}_2}{S} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \]

Where,
\( \bar{x}_1 \) = mean of the first sample
\( \bar{x}_2 \) = mean of the second sample
\( n_1 \) = number of observations in the first sample
\( n_2 \) = number of observations in the second sample
\( S \) = combined standard deviation

13) IMMUNOLOGICAL METHODS:

(A) Diluent:

Isotris was used as a diluent for all the reagents given below. It was prepared by adding 400 ml of stock solution (NaCl - 81.6 g; Tris base - 12.1 g, pH 7.45 with HCl, final volume raised to 2L with distilled water); 6.66 ml of MgSO\(_4\) (0.15 M); 3.0 ml of CaCl\(_2\) (0.1M) and final volume was raised to 2L with distilled water.

(B) Antigen:

PAGE purified homozygous transferrin electromorph BB of \textit{C. punctatus} were used as an antigen. The method of purification has been described under 6 A and B.

(C) Raising antiserum:

Antiserum was raised in rabbits by injecting purified antigen for a period of 4 months. The initial injection consisted of 2 mg of protein in Freund's complete adjuvant that was given intradermally. The booster doses of protein were given
subcutaneously at an interval of 3 weeks. Bleeding by marginal vein of the ear was performed one week after each injection to check the antibody titre by Ouchterlony's double diffusion technique (described below). The resulting antisera was heated at 60°C for 20 minutes to inactivate rabbit complement. The heat-treated sera was centrifuged at 20,000 rpm for 15 minutes (Champion et al. 1974). The supernatant was saved and stored at -20°C. For convenience 1/100 dilution of antiserum was made and used as a stock solution.

(D) Ouchterlony's immunodiffusion method:

Double diffusion was performed at room temperature according to Ouchterlony's (1962) which facilitates the monitoring of antigen-antibody cross reaction by diffusion across agarose gels. The gel medium was made as follows:

A solution of 0.9% agarose was prepared in 0.07 M phosphate buffer (pH 7.2) Antigen and antiserum wells were made 5mm apart and each of them was 3 mm in diameter. 10 ml of solution containing 0.05 mg of protein per ml was added to antigen well while antiserum well received 10 ml of undiluted serum.

(E) Source of complement:

Fresh chicken serum was used as a source of complement which was stored at -20°C, in case it was not used immediately. A dilution of 1/5 times gave satisfactory results during microcomplement fixation (MC'F) (described below). Sera older than one week were discarded.

(F) Source of red blood cells (RBCs):

Freshly drawn sheep red blood cells were mixed with an equal volume of Alsever's solution (glucose, 24.6g; sodium citrate dihydrate, 9.6g; NaCl, 5.0 mg; final volume made up to 1200 ml with D.H₂O and pH adjusted to 6.1 with citric
To raise antibodies (hemolysin) against sheep RBCs. This suspension was centrifuged at 3,000 rpm for 5 minutes. The pellet was washed thrice with normal saline (0.9% NaCl) and finally 0.1 ml of packed cells was suspended in 0.5 ml normal saline. This suspension of RBCs was injected in rabbits until satisfactory titre of hemolysin was obtained. This required injection of booster doses at intervals of 15 days. The rabbit was bled after 4th day of 3rd, 4th and 5th booster. The antiserum of each bled was stored in equal volume of glycerol at -20°C. Each batch of antiserum was monitored for hemolysin titre.

(G) Hemolysin titration:

The titration of hemolysin was performed by setting a series of test tubes containing 5 ml of diluent and 1 ml of complement dilution (1/5). Several preparations of sensitized cells were made (described below) using different concentration of hemolysin. To each tube 1 ml of sensitized red cells were added and incubated at 35°C. The hemolysin concentration that produced 85% of lysis with in 35 minutes was used for subsequent MC'F experiments.

(H) Preparation of sensitized cells:

5 ml of sheep blood in Alsever's solution was centrifuged at 3,000 rpm for 3 minutes. The pellet was washed thrice with 5 ml of diluent (isotris). Finally, 1 ml of cell pellet was diluted (1/18) with diluent and 1 ml of this dilute suspension was lysed by diluting it with 25 ml of distilled water. The $A_{541}$ of this lysed suspension was kept between 0.47 to 0.50 as per recommendation (Champion et al., 1974). Accordingly, the concentration of 1/8 dilution was adjusted by adding more washed cells or isotris. The sensitized RBCs were prepared by mixing the dilute suspension of RBCs with an equal volume of 1/80 dilution of hemolysin in isotris with slow but
continuous swirling. The mixture was incubated at 35°C for 15 minutes and further
diluted 1/10 times with diluent. The sensitized RBCs were kept at 4°C and used
within 10 hrs.

(I) Protocol for microcomplement fixation (MC'F) experiments:

Essentially the protocol described by Champion et al. (1974) was followed. Accordingly, 4 concentrations of antiserum (rabbit anti *C. punctatus*
transferrin) was tested against 6 concentrations of antigen. This experiment involved
40 reaction tubes each of which received 7 ml of reagent i.e. diluent (3 ml) diluted
antiserum (1 ml), diluted complement (1 ml), diluted antigen (1 ml) and sensitized
cells (1 ml). Control tubes lacked antiserum, complement or antigen. The deficit
being made up by additional diluent.

Procedure:

The tubes were placed in ice water bath. The diluent was added in each
tube and 1 ml of desired dilution of antiserum was also added to appropriate tube #
1-36 and 2 ml to tube # 37 and 38. No complement was added to tube # 39 & 40.
The tubes were swirled and then 1 ml of each dilution of antigen was added to the
appropriate tube (Table-10) with continuous swirling. After sealing tubes with
aluminium foil, they were incubated for 20 hrs at 4°C. Subsequently, the tubes were
placed in ice water bath and 1.0 ml of freshly prepared sensitized RBCs were added
and placed in 35°C water bath for 35 minutes with swirling after every 5 minutes.
During this incubation lysis could be seen in tubes # 37-38 within 18-20 minutes by
clearing of cloudy red suspension. After 35 minutes of incubation the tubes were
again placed in ice water bath following cooling, the contents were then poured into
pre-chilled numbered centrifuge tubes and spun at 6,000 rpm for 15 minutes to
sediment unlysed RBCs. The supernatant was carefully decanted and the absorbance measured at 413 nm. The percentage complement fixed (Y) in each tube was calculated by the following equation.

\[
Y = \frac{A_{413}^{(As \ control)} - A_{413}^{(experimental \ tube)}}{A_{413}^{(As \ control)} - A_{413}^{(cell \ control)}} \times 100
\]

**Formulae used in MC'F experiment:**

(A) % lysis in As control = \[
\frac{A_{413}^{(As \ control)} - A_{413}^{(cell \ control)}}{A_{413}^{(double \ C' \ control)} - A_{413}^{(cell \ control)}} \times 100
\]

(B) % sequence difference (P) = \[
\frac{\text{Immunological distance (ID)}}{5}
\]
CHAPTER -3
RESULTS

This chapter comprises the data collected as per envisaged programme which
has been divided into following sections :

1) STATISTICAL ANALYSIS OF ELECTROPHORETIC DATA OF
TRANSFERRIN PHENOTYPES IN Channa punctatus BLOCH

   A. Inter-area heterogeneity test
   B. Inter-area analysis of allele frequencies
   C. Intra-area analysis of allele frequencies
      a. Aligarh
      b. Bulandshahr
      c. Badaun
      d. Bareilly

(2) BIOCHEMICAL CHARACTERIZATION OF TRANSFERRIN (TF)
ISOFORMS OF Channa punctatus BL.

   A. Initial identification of Tf isoforms by specific staining with nitroso-R
   B. Partial purification of Tf isoforms by rivanol
   C. Purification of Tf isoforms by preparative PAGE
   D. Determination of isoelectric point (pI)
   E. Molecular weight estimation
   F. Iron binding analysis
(3) IMMUNOLOGICAL STUDIES:

A. Antiserum production

B. Titration with homologous antigen

C. Titration with heterologous antigen(s)

D. The relationship between immunological distance (ID) and percentage sequence differences (P) among Tf's of four species of genus *Channa*

E. Intrageneric relationships of Tf's within genus Channa

Out of the four species of genus *Channa* included in the present study, the major part of this work comprising subpopulation structure and biochemical characterization, deals with the Tf isoforms of *C. punctatus* Bloch. Fig. 1a shows the photograph of a live specimen of *C. punctatus* whereas in Fig. 1b diagnostic characters of the same have been displayed diagrammatically. *C. punctatus* is a small sized (>23 cm) fish making a significant proportion of mixed catches from ditches, ponds, canals and rivers of the area covered during the present study. Detailed information about the catches is provided in Table-1.

1. STATISTICAL ANALYSIS OF ELECTROPHORETIC DATA OF TRANSFERRIN PHENOTYPES IN *Channa punctatus* BLOCH

Polyacrylamide gel electrophoresis (PAGE) of sera from 978 specimens of *C. punctatus* Bl. procured from Aligarh, Bulandshahr, Badaun and Bareilly districts (located in western belt of the state, Uttar Pradesh), covering an area of about 7,3,815 sq. Kms. (Fig. 2), reveal the presence of six electrophoretic variants in β-globulin region of electropherograms (Fig. 3a). Line diagram of the detected
phenotypes is given in Fig. 3b and their relative mobilities summarized in Table-2. As demonstrated under the section on "Biochemical Characterization", specific staining, rivanol precipitation, iron binding analysis, pI values and molecular weight values confirm these variants as Tfs. As shown in Fig. 3a, three of these variants (lanes 6, 8 and 9) comprising single bands, on the basis of their mobility are designated as 'A' the slowest, 'B' the intermediate and 'C' the fastest moving isoforms (Table-2). The remaining 3 variants are two banded (lanes 1, 3 and 5). For the reason explained under "Discussion", the single band transferrin variants are considered homozygotes and two band variants heterozygotes. Thus, the six phenotypes (variants) recorded here would genotypically be AA, BB, CC (homozygotes) and AB, AC and BC (heterozygotes).

(A) Inter-area heterogeneity test:

The statistical analysis was made with the assumption that the observed polymorphism in Tfs of *C. punctatus* is controlled by 3 codominant alleles A, B and C and its existence is independent of geographical distribution. This is consistent with the published evidence on the genetic expression of Tf genes in vertebrates including fishes. To test it, pooled data of observed phenotypes from different areas were subjected to inter-area heterogeneity test. The expected frequencies of the observed phenotypes were calculated according to Hardy-Weinberg principle using chi-square test for contingency table (Table-3). The calculated values of chi-square statistic was found to be 13.983 which is less than its tabulated value $\chi^2_{15,0.05} = 25$, thereby indicating that there is no significant difference between the expected and observed values at 5% level of significance. Therefore, the phenotype frequency
analysis of all specimen collected from different localities suggest that the observed six phenotypes of *C. punctatus* Bl. form a system that hereafter will be specified as Tfcp. This would, in turn imply that the polymorphism is determined by three codominant alleles TfAcp, TfBcp and TfCcp. During typing of Tfs, it was also observed that the frequency of a particular phenotype has no relation with sizes and sexes of fish samples (Table-1). Moreover, a comparison of the observed and expected incidences of phenotypes indicates that phenotype AA is rare as only 5.0 specimens of this phenotype are found in Aligarh. The existence of this group is not detected among 328 specimens collected from other three districts. Usually there is an excess of phenotype BB (31%) and BC (44%), and a deficiency of CC (7.0%) and AC (5.0%) within the samples examined. Therefore, the percentage of AB (13%) as compared to CC and AC is high (Table-3).

**B) Inter-area analysis of allele frequencies**

Frequencies of three codominant alleles TfAcp, TfBcp and TfCcp were also analyzed by Hardy-Weinberg equation including both the homozygotes and heterozygotes of the particular allele in total number of specimens examined. As shown in Table 4, the frequency of TfAcp is maximum (0.11) in Bulandshahr and minimum in Badaun (0.07) whereas in Aligarh and Bareilly the values are 0.10 and 0.08, respectively.

The frequency of TfBcp is the same (0.58) for samples of Badaun and Bareilly, but the value increases to 0.60 for samples of Aligarh and an intermediate value of 0.59 is recorded for those of Bulandshahr.

Whereas the frequency of TfCcp remains the same (0.30) in Aligarh and
Bulandshahr, a relatively high value of 0.34 and 0.35 has been recorded for Bareilly and Badaun, respectively. On the basis of calculated frequencies the relative abundance of different alleles is as follows: TfBcp with the frequency range of 0.58-0.60 is the most abundant allele followed by TfCcp with a range of 0.30 to 0.35 and TfAcp allele shows minimum occurrence with the frequency range of 0.07-0.10. The same trend is observed for the relative abundance of these alleles in all the four districts.

(C) Intra-area analysis of allele frequencies:

A total of 978 specimens of *C. punctatus* Bl. were obtained from four districts during 1997-1999. The mean range and standard deviation of TfAcp, TfBcp and TfCcp were calculated for samples of each district. A comparative account of these analyses based on Student's 't'-test at 5% level of significance is given below:

a) Aligarh: In all, 650 specimens were procured from this district reaching a total of 30 lots with each lot comprising 20-25 fishes. As shown in Table-5, though the allele frequency of TfAcp ranges from 0.06 - 0.16 the mean value is 0.10 and S.D. ±0.040.

For TfBcp the frequency range is 0.52-0.66 which carries a mean value of 0.6 and S.D. of ± 0.051, while the frequency of allele TfCcp has a range of 0.27-0.33 carrying a mean value of 0.30 and S.D. of ±0.024.

b) Bulandshahr: A total of 100 specimens were collected in 6 lots comprising 15-19 fish / lot. In this district, as compared to Aligarh, the mean value of TfAcp increases by 0.01 (t=0.41*) and that of TfBcp decreases by 0.01 (t=0.41*), while the mean value of TfCcp remains the same (0.30). The mean range and S.D. of these
3 alleles in Bulandshahr are shown in Table-6.

c) Badaun: A total of 78 specimens were collected in 7 lots of 10-15 fishes each. In comparison to Aligarh, in Badaun the mean frequency of allele TfAcp decreases by 0.03 (t=2.25*) while that of TfBcp decreases by 0.02 (t=1.1*); however, there is a significant increase in the frequency of TfCcp by 0.05 (t=3.75**). Table-7 shows the mean, range and S.D. of these 3 alleles in Badaun.

d) Bareilly: In Bareilly in all 150 specimens were collected in 10 lots comprising 12-18 fishes/lot. As compared to Aligarh the mean frequency of TfAcp and TfBcp decreases by 0.02 (tA = 1.387*, tB = 1.057*) while the mean frequency of TfCcp increases significantly by 0.04 (t = 4.62**). The mean, range and S.D. of TfAcp, TfBcp and TfCcp in Bareilly are shown in Table-8.

Frequency (mean, range and S.D.) of TfAcp, TfBcp and TfCcp in different districts is graphically presented in Fig. 4.

2. BIOCHEMICAL CHARACTERIZATION OF TRANSFERRIN (TF)

ISOFORMS IN Channa punctatus BL.

(A) Initial identification of Tf isoforms by specific staining with nitroso-R:

Blood volume depending on the size of fish (15-22 cm) varies between 1.5 to 2.5 ml. The yield goes up to 5 ml in case of C. marulius and C. striatus of moderate sizes (28-32 cm). Individual sera samples of each lot were screened by gel electrophoresis and Tf bands identified following specific staining. Fortunately, during subsequent biochemical characterization, all of those bands which were initially identified as Tfs (after specific staining), could be confirmed as transferrins.

* Statistically non-significant
** Statistically significant at 5% level of significance
During electrophoretic runs, iron saturated Tf s move as yellowish brown bands which after staining with nitroso-R turn dark green. Though a few other non-transferrin protein bands also take up this stain, they are visibly faint as compared to those of Tf s (Fig. 5). Routinely, line diagrams and direct counting of phenotypes in the PAGE patterns were employed as the method of screening the samples of each lot and data thus collected was used for statistical analyses (Section-1 of this chapter). Only the representative patterns from selected lots were photographed on negative black and white films.

As shown in Fig. 3, six electrophoretic variants or phenotypes were observed; 3 of them comprising single bands (lanes 6, 8 and 9) and an equal number of phenotypes representing two-band system (lanes 1, 3 and 5) generated by random combinations of the 3 single band genotypes (some explanation of phenotyping has already been given above under Section-1 and further details are available under Chapter-4). In order to raise the samples in amounts sufficient to carry out biochemical and immunological investigations, sera with identical phenotypes were pooled.

(B) Partial purification of Tf isoforms by rivanol:

As revealed by PAGE patterns (Fig. 6), addition of serum to 0.4% rivanol in a ratio of 1:0.5, 1:1 and 1:1.5 (v/v) does not precipitate non-transferrin proteins as effectively as the ratio of 1:2. A comparison with PAGE patterns of serum control (lane 1) of the same figure shows Tf band making the most distinct appearance at this ratio (1:2). Upon further raising the proportion of rivanol, the yield of Tf fraction declines (Fig. 6, lane-2). During further studies, partial purification of Tf was therefore accomplished at a ratio of 1:2 serum to rivanol that gives maximum purification without
affecting the yield of Tf. A passage of supernatant containing transferrin through potato starch adsorbs rivanol completely and the sparkling clear filtrate is obtained having only faint reddish brown color typical of iron saturated Tf. Apparently there is no negative effect on either the yield or properties of Tfs as originally reported by Sutton and Karp (1965). The detail of this pretreatment which facilitates rivanol fractionation are given in 6A of Materials and Methods.

(C) Purification of Tf isoforms by preparative PAGE:

Tf phenotypes obtained by partial purification with rivanol were further purified by preparative gel electrophoresis. Because of the relative abundance of phenotype BB and CC the bands B and C were obtained by purification of rivanol treated homozygous serum BB and CC through preparative gel electrophoresis. Since phenotype AA is rare TfA band has been purified from rivanol treated heterozygous AC phenotype where due to higher electrophoretic mobility of TfC, elution of TfA is convenient (Fig. 7). Heterozygote AB resolves in narrow zone preventing the purification of TfA by preparative gel electrophoresis. Samples purified in this way were used for isoelectric focusing, molecular weight estimation, apotransferrin preparation and subsequent iron binding analysis by absorption spectroscopy as well as immunological studies.

(D) Determination of isoelectric point (pI):

Fig. 8 shows the results obtained by focusing commercial preparation of human Tf along with selected isoforms of C. punctatus. No extra bands appear in case of fish isoforms, thereby indicating that the preparations were of the highest purity. The calculated pI values for isoform A, B and C were 5.30, 4.90 and 4.0 respectively.
(E) Molecular weight estimation:

The discontinuous system of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of Laemmli (1970) proved inadequate for estimating the molecular weight of Tf isoforms of *C. punctatus*. It is already known that the presence of carbohydrate moieties affects the electrophoretic migration in this system. Instead, a highly porous gel electrophoresis system recommended by Doucet and Trifaro (1988) has been found suitable for estimating the molecular weights of Tf isoforms of this fish. Fig. 9 demonstrates that satisfactory resolutions of individual Tf band could be obtained in this system. Tfs of *C. punctatus* migrated with a relative mobility which differs slightly from that of human serotransferrin.

A linear correlation is apparent upon plotting the log molecular weight of marker polypeptides versus corresponding relative electrophoretic mobilities of individual bands (Fig. 10). The relative locations of Tf of *C. punctatus* and human Tf give approximate values of molecular weights as 75 kD for fish and 80 kD for human Tfs.

(F) Iron binding analysis:

Absorption spectra of complex of iron with individual pure transferrin phenotype in the visible range is shown in Fig. 11. Tf isoforms A, B, and C of *C. punctatus* show maximum absorbance at a wavelength of 460 nm (curve 1, 2 and 3 of Fig. 11, respectively). This is in accordance with the spectral behaviour of human Tf (curve 0 of the same figure).

The peak value of iron-transferrin complexes at 460 nm (Fig. 11) was used to determine iron binding capacity of fish and human Tfs. Each experiment was continued till consecutive 5-6 tubes showed no changes in optical density after adding iron
solution. The standard iron solution was ferric nitrilotriacetic acid (FENTA) 2 mM which contains 0.1 μg of Fe/μl of FENTA. Whereas the concentration of Tf was kept constant, increasing quantities of FENTA were added till A_{460} reached the maximum value. These results are shown in Fig. 12.

The calculated value of atoms of iron bound per molecule of Tf is approximately 2. The similar value is obtained for human Tf (Table-9).

(3) IMMUNOLOGICAL STUDIES:

(A) Antiserum production:

To elicit antibody production in rabbit the most abundant homozygous transferrin phenotype BB of \textit{C. punctatus} was used as an immunogen. As shown in Fig. 13, antibody production was qualitatively monitored by Ouchterlony's immunodiffusion technique. A prominent precipitin arc can be seen between purified homozygous transferrin phenotype BB and antisera of maximum titre raised against it. Quantitatively, the antibody titre was determined by microcomplement fixation (MCF), typical results of which are presented in Fig. 14. The titre in these cases has been taken as the factor by which the antiserum has to be diluted to give 75% C' fixation with the homologous antigen at the peak of MCF curve. Immunodiffusion experiments indicated that antibody level increases after the first booster and the maximum value reaches after the 3rd immunization that was maintained up to 4th booster, whereafter it gradually declines. The antiserum titre obtained for Tf (BB) of \textit{C. punctatus} is 3,200 (Fig. 14). Limited experiments made with other phenotypes AA and CC did not reveal any remarkable quantitative difference between their antigenicity.
To obtain satisfactory results of MC'F analyses, the protocol was standardized which under our experimental set up gives best values when:

(a) Antigen, antibody and complement are allowed to react for 21 hours at 4°C, (b) incubation time to achieve 85% lysis in antiserum control is 35 minutes at 35°C, (c) $A_{414}$ of diluted SRBC reagent is 0.48-0.5.

(B) Titration with homologous antigen:

As shown in Fig. 14, bell shaped curves are obtained when percentage of the complement fixed is plotted against different antigen concentrations. The amount of antigen that produces maximum complement fixation at different dilution of antisera is $\approx 95$ ng. There is little complement fixation at the extremes of antigen excess (tubes, 1, 7, 13 and 19) i.e. 3-13% or at the extremes of antibody excess (tubes, 6, 12, 18 and 24) i.e. 6-14% (Table-10). The plot of the peak heights of C' fixation curve versus the logarithm of the antiserum concentration gives a straight line with a slope of 142.48 (Fig. 15).

(C) Titration with heterologous antigen(s):

At the antiserum concentration 3,200 i.e. the titre which gives 75% C'fixation with Tf phenotype BB of $C. punctatus$, comparatively lesser cross reactivity is observed with Tfs of other 3 species of genus $Channa$. The order and magnitudes of cross reactivity with other species are, however, not the same.

For instance, at this titre percent C' fixed in case of Tf of $C. striatus$ is only 62%. Therefore, the antiserum concentration has to be raised to 2,800 to obtain a peak of 76%. For Tf of $C. gachua$, 75% peak of C' fixed is obtained when antiserum concentration is raised to 2,900 otherwise at the concentration of 3,200 it remains
at 65%. Similarly, in *C. marulius* due to the lowest value of 48% C' fixed at the concentration taken as titre for *C. punctatus*, the antiserum concentration has to be raised to 2,500 to obtain 74.8% C' fixed at the peak (Table 11). The relationship between peak value of percentage C' fixed and the log of antiserum concentration is linear for all the other three species with the slope values of 158.13 for *C. striatus*, 152.38 for *C. gachua* and 173.70 for *C. marulius* (Fig. 16).

**D. The relationship between immunological distance (ID) and percentage sequence differences (P) among Tfs of four species of genus *Channa*:**

To determine the degree of immunological correlation between Tfs of all four channid species, six possible combinations of them were made (Table 12). The immunological correlation expressed by the notion ID or immunological distance has been defined, 100 times the Briggsian logarithm of the factor by which the antiserum titre has to be raised to give a cross reaction with the heterologous protein equivalent to that of the homologous protein.

As shown in Table-12, the highest value of 10.720 denoting maximum immunological distance (ID) is observed between transferrins of *C. punctatus* and *C. marulius*. ID values of 5.799 and 4.275 are typical of correlation between Tfs of *C. punctatus* and *C. striatus* and between those of *C. punctatus* and *C. gachua*, respectively. The least of all, an ID value of 1.523 is obtained between transferrins of *C. gachua* and that of *C striatus*. As shown in Table 12, this amounts to only 0.30% P or percentage sequence differences between transferrins of *C. gachua* and *C. striatus*. Whereas a higher value of P(2.14%) is obtained for transferrin of *C. punctatus* and *C. marulius*, the value for transferrins of *C. punctatus* and
C. striatus is 1.16% whereas for transferrins of C. punctatus and C. gachua it is 0.85%.

(E) Intrageneric relationships of Tfs within genus Channa:

To facilitate the display of phylogenetic relationships of transferrins of C. punctatus to C. striatus, C. marulius and C. gachua based on MC'F analysis, a dendrogram was constructed using unweighted pair-group arithmetic average (UPGMA) clustering method (Ferguson, 1980c). For this calculation, immunological distance observed between transferrins of six groups of channids were employed as operational taxonomic unit (OTUs). As shown in Table-13, the two OTUs to be clustered are those with the lowest immunological distances, i.e. OTU2 and OTU3 (1.523). The maximum distance is 10.72 i.e. between OTU1 and OTU4. The scale to cover this range (1.523-10.72) is drawn and OTU2 and OTU3 are joined by a vertical line at the value of 1.523 (Fig. 17). Similarly, OTU 2/3 and OTU1 (Table 13b) are joined at a distance of 5.037. The similarity value of OTU 2/3 to OTU1 is calculated by taking the mean of OTU2 to OTU1 and the mean of OTU3 to OTU1. The joining distance between OTU 1,2 and 3 to OTU4 was 7.362. The similarity value of a grouped OTU is the mean of the similarities of its constituent member to that OTU. The dendrogram so obtained (Fig. 17) demonstrates that the affinity of Tf of C. punctatus to other 3 species of Channa may be of the following order: C. gachua > C. striatus > C. marulius.
<table>
<thead>
<tr>
<th>DISTRICT</th>
<th>MALE</th>
<th>FEMALE</th>
<th>SIZE RANGE (cm)</th>
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<td></td>
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</tr>
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</tr>
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<tr>
<td>Ujhani</td>
<td>21</td>
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<td>15 – 22</td>
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<tr>
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<tr>
<td>Gunnor</td>
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<td>Nawabganj</td>
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TABLE 2: Relative mobilities of *Channa punctatus* transferrins (Tfs) as compared with human transferrin (used as a marker) on non-SDS PAGE

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<tr>
<th>PROTEINS</th>
<th>RELATIVE MOBILITIES</th>
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<td>0.477</td>
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<td>TfBcp</td>
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<td>TfCcp</td>
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<td>Bulandshahr</td>
<td>100 Observed</td>
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<td>0.51 Expected</td>
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<tr>
<td>Badaun</td>
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<td>0.39 Expected</td>
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<tr>
<td>Bareilly</td>
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<tr>
<td></td>
<td>0.76 Expected</td>
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</table>
TABLE 4: Allele frequencies of TfAcp, TfBcp and TfCcp of *Channa punctatus* in different districts

<table>
<thead>
<tr>
<th>DISTRICT</th>
<th>NUMBER OF SAMPLES EXAMINED</th>
<th>ALLELE FREQUENCIES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TfAcp</td>
</tr>
<tr>
<td>Aligarh</td>
<td>650</td>
<td>0.10</td>
</tr>
<tr>
<td>Bulandshahr</td>
<td>100</td>
<td>0.11</td>
</tr>
<tr>
<td>Badaun</td>
<td>78</td>
<td>0.07</td>
</tr>
<tr>
<td>Bareilly</td>
<td>150</td>
<td>0.08</td>
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</tbody>
</table>
TABLE 5: Frequency of alleles TfAcp, TfBcp and TfCcp of *Channa punctatus* in Aligarh

<table>
<thead>
<tr>
<th>ALLELE</th>
<th>MEAN</th>
<th>RANGE</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfAcp</td>
<td>0.1</td>
<td>0.06 - 0.16</td>
<td>± 0.040</td>
</tr>
<tr>
<td>TfBcp</td>
<td>0.6</td>
<td>0.52 - 0.66</td>
<td>± 0.051</td>
</tr>
<tr>
<td>TfCcp</td>
<td>0.3</td>
<td>0.27 - 0.33</td>
<td>± 0.024</td>
</tr>
</tbody>
</table>

Number of fish examined = 650  
Number of lots = 30  
Sample size / lot = 20-25 fish

TABLE 6: Frequency of alleles TfAcp, TfBcp and TfCcp of *Channa punctatus* in Bulandshahr

<table>
<thead>
<tr>
<th>ALLELE</th>
<th>MEAN</th>
<th>RANGE</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfAcp</td>
<td>0.11</td>
<td>0.09 - 0.14</td>
<td>± 0.052</td>
</tr>
<tr>
<td>TfBcp</td>
<td>0.59</td>
<td>0.55 - 0.63</td>
<td>± 0.040</td>
</tr>
<tr>
<td>TfCcp</td>
<td>0.3</td>
<td>0.27 - 0.35</td>
<td>± 0.043</td>
</tr>
</tbody>
</table>

Number of fish examined = 100  
Number of lots = 6  
Sample size / lot = 15-19 fish
### TABLE 7: Frequency of alleles TfAcp, TfBcp and TfCcp of *Channa punctatus* in Badaun

<table>
<thead>
<tr>
<th>ALLELE</th>
<th>MEAN</th>
<th>RANGE</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfAcp</td>
<td>0.07</td>
<td>0.06 - 0.08</td>
<td>± 0.010</td>
</tr>
<tr>
<td>TfBcp</td>
<td>0.58</td>
<td>0.56 - 0.59</td>
<td>± 0.017</td>
</tr>
<tr>
<td>TfCcp</td>
<td>0.35</td>
<td>0.33 - 0.38</td>
<td>± 0.026</td>
</tr>
</tbody>
</table>

Number of fish examined = 78  
Number of lots = 7  
Sample size / lot = 10-15 fish

### TABLE 8: Frequency of alleles TfAcp, TfBcp and TfCcp of *Channa punctatus* in Bareilly

<table>
<thead>
<tr>
<th>ALLELE</th>
<th>MEAN</th>
<th>RANGE</th>
<th>STANDARD DEVIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>TfAcp</td>
<td>0.08</td>
<td>0.06 - 0.11</td>
<td>± 0.024</td>
</tr>
<tr>
<td>TfBcp</td>
<td>0.58</td>
<td>0.54 - 0.62</td>
<td>± 0.033</td>
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<tr>
<td>TfCcp</td>
<td>0.34</td>
<td>0.32 - 0.37</td>
<td>± 0.024</td>
</tr>
</tbody>
</table>

Number of fish examined = 150  
Number of lots = 10  
Sample size / lot = 12-18 fish
TABLE 9: Data showing iron binding capacities of *C. punctatus* transferrin bands A, B and C. Human transferrin is included as control. Ferric nitrilotriacetic acid (2mM) containing 0.1 µg of Fe/µl was used as standard iron solution for titration. (Other details under Materials and Methods)

<table>
<thead>
<tr>
<th>Transferrin</th>
<th>Protein content (mg/ml)</th>
<th>µl of Fe solution added</th>
<th>µg of Fe bound / mg of protein</th>
<th>Atoms of Fe bound / molecule of transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>1mg/ml</td>
<td>16</td>
<td>1.6</td>
<td>2.22 (79,550)</td>
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<tr>
<td><em>C. punctatus</em> (A band)</td>
<td>1mg/ml</td>
<td>16</td>
<td>1.6</td>
<td>2.15 (75,000)</td>
</tr>
<tr>
<td><em>C. punctatus</em> (B band)</td>
<td>1 mg/ml</td>
<td>16</td>
<td>1.6</td>
<td>2.15 (75,000)</td>
</tr>
<tr>
<td><em>C. punctatus</em> (C band)</td>
<td>1 mg/ml</td>
<td>16</td>
<td>1.6</td>
<td>2.15 (75,000)</td>
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</table>
Table 10: Microcomplement fixation (MC'F) protocol and the results of a typical experiment on transferrin of *C. punctatus*

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Diluent ml</th>
<th>Antiserum (1ml)</th>
<th>C ml</th>
<th>Antigen (1ml)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ant conc.</td>
<td></td>
<td></td>
<td>%C' fixed</td>
</tr>
<tr>
<td>1.</td>
<td>3.0</td>
<td>Anti 1/3,200</td>
<td>1.0</td>
<td><em>C. punctatus</em> TBB</td>
<td>1,5000  0.594  13</td>
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<td>2</td>
<td></td>
<td><em>C. punctatus</em> TBB</td>
<td>167</td>
<td>500 0.539 22</td>
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<td>3</td>
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<td>55.5</td>
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<td>0.620 9</td>
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<td>18</td>
<td></td>
<td></td>
<td>1500</td>
<td>0.658 3</td>
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<td>500</td>
<td>0.620 9</td>
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<td>0.432 39</td>
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<td>0.400 44</td>
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<tr>
<td>24</td>
<td></td>
<td></td>
<td>1500</td>
<td>0.656</td>
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</tr>
<tr>
<td>25</td>
<td>4.0</td>
<td>1/3,200</td>
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<td><em>C. punctatus</em> TBB</td>
<td>1,5000  0.656 –</td>
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<tr>
<td>26</td>
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<td>– – 0.673 –</td>
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<td>– – 0.659 –</td>
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<td>5.0</td>
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<td>– – 0.611 –</td>
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<tr>
<td>39</td>
<td>6.0</td>
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<td>– – 0.049 –</td>
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<td>40</td>
<td></td>
<td>– –</td>
<td></td>
<td>– – 0.051 –</td>
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</table>
**TABLE 11**: Data showing final results of microcomplement fixation (MC′F) analysis against Tfs of all four species of genus *Channa*

(A) *Channa punctatus*

<table>
<thead>
<tr>
<th>Antiserum concentration (y)</th>
<th>% C′ fixed at peak</th>
<th>Log (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3.200</td>
<td>77</td>
<td>-3.505</td>
</tr>
<tr>
<td>1/3.800</td>
<td>63</td>
<td>-3.579</td>
</tr>
<tr>
<td>1/4.400</td>
<td>52</td>
<td>-3.643</td>
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<tr>
<td>1/5.000</td>
<td>44</td>
<td>-3.698</td>
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Slope = 142.487

(B) *Channa striatus*

<table>
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<tr>
<th>Antiserum Concentration (y)</th>
<th>% C′ fixed at peak</th>
<th>Log (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/2.800</td>
<td>76</td>
<td>-3.447</td>
</tr>
<tr>
<td>1/3.400</td>
<td>58</td>
<td>-3.531</td>
</tr>
<tr>
<td>1/4.000</td>
<td>50</td>
<td>-3.602</td>
</tr>
<tr>
<td>1/4.600</td>
<td>42</td>
<td>-3.662</td>
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</table>

Slope = 158.13
### (C) *Channa gachua*

<table>
<thead>
<tr>
<th>Antiserum concentration (y)</th>
<th>% C' fixed at peak</th>
<th>Log (Y)</th>
</tr>
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<tbody>
<tr>
<td>1/2,900</td>
<td>75</td>
<td>-3.462</td>
</tr>
<tr>
<td>1/3,500</td>
<td>60</td>
<td>-3.544</td>
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<tr>
<td>1/4,100</td>
<td>54</td>
<td>-3.612</td>
</tr>
<tr>
<td>1/4,700</td>
<td>43</td>
<td>-3.672</td>
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Slope = 152.38

### (D) *Channa marulius*

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<tr>
<th>Antiserum concentration (y)</th>
<th>% C' fixed at peak</th>
<th>Log (Y)</th>
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</thead>
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<tr>
<td>1/2,500</td>
<td>74.8</td>
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<tr>
<td>1/3,100</td>
<td>57</td>
<td>-3.491</td>
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<tr>
<td>1/3,700</td>
<td>42</td>
<td>-3.568</td>
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<tr>
<td>1/4,300</td>
<td>33</td>
<td>-3.633</td>
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Slope = 173.70
TABLE 12: Final values of immunological distance (ID) and percentage sequence differences (P) as calculated for four channid species (Relevant formulae given under Materials and Methods)

<table>
<thead>
<tr>
<th>Combinations among Four Channid species</th>
<th>Immunological distance (ID)</th>
<th>Percentage sequence differences (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. punctatus</em> - <em>C. gachua</em></td>
<td>4.275</td>
<td>0.85</td>
</tr>
<tr>
<td><em>C. punctatus</em> - <em>C. striatus</em></td>
<td>5.799</td>
<td>1.16</td>
</tr>
<tr>
<td><em>C. punctatus</em> - <em>C. marulius</em></td>
<td>10.720</td>
<td>2.14</td>
</tr>
<tr>
<td><em>C. gachua</em> - <em>C. striatus</em></td>
<td>1.523</td>
<td>0.30</td>
</tr>
<tr>
<td><em>C. gachua</em> - <em>C. marulius</em></td>
<td>6.445</td>
<td>0.98</td>
</tr>
<tr>
<td><em>C. striatus</em> - <em>C. marulius</em></td>
<td>4.921</td>
<td>1.29</td>
</tr>
</tbody>
</table>
TABLE 13: Operational taxonomic units (OTUs) generated after taking into account immunological distance (ID) values from MC'F data for channid species

Table 13 (a)

<table>
<thead>
<tr>
<th>OTU 2 (C. gachua)</th>
<th>OTU 3 (C. striatus)</th>
<th>OTU 4 (C. marulius)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU 1 (C. punctatus)</td>
<td>4.275</td>
<td>5.799</td>
</tr>
<tr>
<td>OTU 2 (C. gachua)</td>
<td>1.523</td>
<td>6.445</td>
</tr>
<tr>
<td>OTU 3 (C. striatus)</td>
<td>4.921</td>
<td></td>
</tr>
</tbody>
</table>

Table 13 (b)

<table>
<thead>
<tr>
<th>OUT 2 / 3</th>
<th>OTU 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>OTU 1</td>
<td>5.037</td>
</tr>
<tr>
<td>OTU 2 / 3</td>
<td>6.683</td>
</tr>
</tbody>
</table>
Photograph of a live anaesthetized specimen of *Channa punctatus* Bl. Present investigations mainly deal with subpopulation genetics and biochemical characterization of transferrin (Tf) phenotypes of this species.

Line diagram of the same showing external discriminative characters:

(i) 15-20 scales between snout and the origin of the dorsal fin. (ii) 9-10 scales between the orbit and the angle of preopercle (iii) Ventral 2/3 to 3/4 length of pectoral (iv) Pectoral without transverse bands and with uniform coloration. (v) Several bands or patches from back pass down the abdomen.
Fig. 2 Location map of Channa punctatus samples.
**Fig. 3a** Typical polyacrylamide gel electrophoretic (PAGE) patterns of sera samples of *C. punctatus* showing representative phenotypes.

Gel was 7.5% and run essentially as described by Laemmli (1970) (See Materials and Methods) with the modification that SDS was not added to any solution or buffer. Protein bands were visualized by staining with coomassie brilliant blue G250. Since identical polypeptides comigrate, a pair of homologous monomeric protein will stack as a single band viz AA, BB and CC, presuming codominant inheritance of Tf alleles. Therefore, the random heteromic combinations of the above monomers will constitute observed heterozygotes AB, AC and BC.
Fig.: 3b  A line diagram of various Tf phenotypes detected in *C. punctatus* showing comigration of double band heterozygous Tfs (AB, AC and BC) with single band homozygous Tfs (AA, BB and CC).
Fig. 4  Frequency (Range, Mean and Standard deviation (S.D.)) of transferrin alleles Tf Acp, Tf Bcp and Tf Ccp of *C. punctatus* in different districts.
Fig. 5 Typical PAGE patterns of sera samples of *C. puntatus* showing Tf phenotypes as visualized after specific staining with nitroso-R. Other details were the same as in Fig. 3a.
Fig. 6  PAGE patterns of partially purified Tf phenotype “BB” of \(C\) \textit{punctatus}\ after adding 0.4% rivanol at different ratios

Electrophoretic conditions were the same as in Fig 3a
Fig. 7: PAGE patterns of highly purified single bands A and C following preparative PAGE of corresponding Tf phenotype (AC) partially purified with rivanol.

Electrophoretic conditions were the same as in Fig 3a. Gels were visualized following silver staining.
Fig.: 8 Isoelectric focusing (IEF) patterns of Tf's of *C. punctatus* along with human Tf showing respective pI values.

Lane from left to right are: 1, IEF marker; 2, human Tf; 3, Tf-AC; 4, TfAA and TfAB of *C.punctatus*. 
Fig. 9  SDS-PAGE of highly purified Tf isoforms of *C. punctatus* and human Tf, run along with markers. The gel was 10%, cast and run essentially as described by Doucet and Trifarro (1988).

Lanes from right to left are 1, markers; 2, human Tf; 3, TfC; 4, Tf B; 5, TfA.
Fig.: 10  Semilogrithmic graph showing the correlation between log molecular weights and relative electrophoretic mobilities of marker polypeptides and Tf bands A, B and C of \textit{C. punctatus}.
Fig.: 11. Absorption spectra of purified transferrin (Tf) bands A, B, and C of *C. punctatus*. Human Tf is included as control.
Fig.: 12. A comparison of iron binding capacities of transferrin bands A, B and C of *C. punctatus*. Human transferrin is included as control.
Fig.: 13 Photograph of immunodiffusion pattern obtained by reacting rabbit anti-TfBB of *C. punctatus* serum (Ab) with purified *C. punctatus* Tf "BB" (Ag). The control (C) is the rabbit serum obtained before immunization.
Fig.: 14 The effect of antiserum concentration on complement fixation. The test antigen was *C. punctatus* transferrin (BB phenotype). The antiserum used was rabbit anti *C. punctatus* (BB) transferrin.
Fig.: 15 Typical diagram showing the dependence of peak height of the complement fixation curve on the antiserum concentration. Each point of the graph represents the peak height of a complement fixation curve obtained from Fig. 14 for a particular antiserum concentration.
The dependence of peak height of the complement fixation curve on the concentration of *C.punctatus* (BB) antiserum. Each point represents the peak height of complement fixation curve for a particular antiserum concentration (shown in table-11).

The antigen used were:

a) *C.punctatus* Tf (BB)
b) *C.gachua* Tf
c) *C.striatus* Tf
d) *C.marulius* Tf
Fig. 17 Dendrogram showing the intrageneric relationship between four species of genus *Chaenometa* based on the MCF analysis of their transcripts (OTU1: *C. punctatus*, OTU2: *C. gocinoida*, OTU3: *C. virantis*, and OTU4: *C. marilis*).
CHAPTER - 4
DISCUSSION

*Channa punctatus* Bloch, the species whose sera transferrins are primarily investigated in this study is a bony fish belonging to the Family Channidae (Order: Channiformes; Class : Osteichthyes). Due to profile of the head and scale patterns there on, species comprising this family have been given the common name of snake-heads or murrels (Day, 1958; Srivastava, 1980). They possess specialized accessory organs for airbreathing which makes them physiologically and evolutionarily distinct from other species of the class Osteichthyes. Beside *C. punctatus*, three other species of this family , namely : *C. gachua*, *C. marulius* and *C. striatus* which have been included solely to work out the phylogenetic interrelationships, also dwell fresh waters of the area covered during this study. All the members of genus *Channa* are important food fishes of India and several other South Asian countries. They not only make a significant contribution to capture fisheries in these countries but carry importance in paddy-cum-fish aquaculture practices also. They have, thus, a direct bearing upon viability of commercial fishery of freshwaters in these countries. The documentation of genetic diversity and resources are important from the point of view of their conservation as well as better management.

In view of the general paucity of information on biochemical genetics of this important group of fishes, investigations comprising the following aspects were carried out : (a) to determine the extent of polymorphism at transferrin locus by typing Tf isoforms electrophoretically and assess the significance of obtained data in discerning population genetics of *C. punctatus* Bl.; (b) to characterize its Tf isoforms
biochemically and (c) to trace phylogenetic interrelationships between Tfs within the genus *Chauna* by microcomplement fixation (MC'F) analysis using selected purified Tf phenotype of the main reference species as immunogen.

Generally, the typing of transferrin isoforms of vertebrates has been performed by gel electrophoresis of these proteins. In most of the instances, extensive polymorphism has been discovered at Tf locus (Klucinski, 1975; Hartle and Ferrand, 1993). Fishes are no exception to this, (Kirpichnikov, 1973). As for instance polymorphism of tuna Tfs has been traced to a 3-allele system (Fujino and Kang, 1968). Three isoforms which corresponded to these alleles differed in their relative electrophoretic mobilities in a descending order and were designated as A, B and C, respectively. In all, five Tf phenotypes were discovered in this fish which on the basis of electrophoretic comigration with isoforms (corresponding to three recognized alleles) contributed to phenotypes AB, BB, BC, CC and AC; short of one possible random combination AA. The occurrence of homozygote AA in tunas appears to be rare and reported only in selected localities of the above cited papers (Barrett and Tsuyuki, 1967; Fujino and Kang, 1968). Recently, Van-Doornik and Milner (1996) have detected a 4 allele system in coho salmon. Still more extensive polymorphism is displayed by Tfs of European carp (*Cyprinus carpio* L.) where as many as 7 alleles A, B, C, D, E, F and G have been identified by Valenta *et al.* (1976).

During the present studies also a polymorphism of high magnitude was recorded at Tf locus of *C. punctatus*. As many as six phenotypes were discovered within the samples of this species which were collected from Aligarh, Badaun, Bulandshahr and Bareilly districts of western Uttar Pradesh covering an area of about 73,815
sq. kms. (Fig. 2). Genotypes of Tf isoforms of *C. punctatus* were assigned capital Roman letters after the conventional approach as followed in the above examples on fish transferrins (Fujino and Kang, 1968, Utter et al., 1970). As shown in Fig. 3, three Tf phenotypes out of a total of six were represented by single bands and taken as homozygotes of three isoforms A, B and C which differed in their relative electrophoretic mobilities in an increasing order. Since homozygous isoforms are identical polypeptides, they stack as single superimposed bands (Fig. 3a, lanes 6, 8 and 9) that types single band variants of Tfs to AA, BB or CC homozygotes. Double band Tf phenotypes depending on the presence of bands comigrating with any two of A, B or C isoforms have to be accordingly marked as heterozygotes AB, BC or AC (Fig. 3a : lanes 1, 3 and 5). Three alleles of Tf locus designated TfAcp, TfBcp and TfCcp should, therefore, control the expression of three Tf isoforms A, B and C. Phenotype AA was extremely rare, as out of a total of 978 sera samples of *C. punctatus* analyzed here only 5.0 samples belonged to this category. All populations of *C. punctatus* follow the similar pattern of distribution frequencies of various phenotypes in different districts screened for Tf polymorphism i.e. in the entire 73,815 sq. kms. there is an excess of phenotypes BB and BC whereas AB ranks third in relative abundance (Table-3).

Conventionally, homozygous monomeric proteins resolving as single bands (superimposed isoforms) in native gel electropherograms and representing a two band heterozygous combination as encountered here, have been conceived under control of codominant alleles (Ferguson, 1980a). As already pointed out, the same polymorphic locus appears to control the expression of different isoform in
C. punctatus. Though among fishes polyploidy is not uncommon which may add another locus as suspected in carp (Valenta et al., 1976), they are bound to be lost during evolution because of the vital and specific function assigned to Tf (Kirpichnikov, 1981). C. punctatus among channids in any case has the least number of chromosomes, 2n=32 (Fish chromosome atlas, NBFGR, 1998), and may not be a polyploid, specifically in comparison with C. gachua which has been phylogenetically considered most generalized form of remote origin (Chandy, 1955) and has a 2n value of 78. The values of chromosome number (2n) for C. marulius and C. striatus are 44 and 40 respectively as reported in the above reference. This fact again indicates that in comparison with other channids, C. punctatus is evolutionarily of recent origin as suggested by morphometric data (Chandy, 1955). Though the data on Tf polymorphs of other channid species has not been included in this work because of lesser number of samples screened, an initial estimate indicates that among all of them Tf locus of C. punctatus is most polymorphic.

Published evidence suggests that the relative abundance of various phenotypes of fish populations depends on: (a) differences in fertility (Ashton, 1959) and (b) post-hatching viability of progeny as shown by Hershberger (1970) for Tf phenotype AA in brook trout where the absence of this combination was attributed to mortality that occurred before the breeding. The homozygous condition thus had a low survivability. There is no published report on differential survival rates of any stock of C. punctatus and breeding experiments were not part of the present programme either. The occurrence of all possible combinations of Tf genotypes in C. punctatus, however, indicates that none of the homo or heterozygous condition is lethal in this
fish. Moreover, at least the homozygotes BB are not at a disadvantage, since they are similar in abundance as heterozygotes BC (Table-3). It also suggests that variations in fertility if any of at least BB or CC phenotypes may not be very remarkable, because their heterozygote BC is quite in abundance and the population of this heterozygote represents a pool of inbreds of BC as well as those arising from crosses between BB and CC parents. The observed variations in the relative abundance of Tf phenotypes within populations of *C. punctatus* may thus depend more on viability rather than any other factor. The discussion that on functional properties of Tf isoforms which follows in the third paragraph, hereafter, favours receptor related variations in the viability.

For the purification of transferrins, rivanol (2-ethoxy-6,9- diaminoacridine lactate) has served as the principal precipitant of non-transferrin proteins ever since the first report published by Boettcher *et al.* (1958). Apparently, due to variations in conformations or the charge on albumins, γ-globulins and Tfs from different species, for the best purification of Tfs differing ratios of serum to rivanol are required. A review of literature shows that as compared to the ratio of 1:3.5 serum to rivanol (0.4%) is required for human Tf, whereas, 1:3 is required for that of cattle. Fish Tfs required still different ratios of serum:rivanol such as 1:2 for brook trout (Hershberger, 1970) and Atlantic salmon (Roed *et al.*, 1995). In case of channids, Sahoo and Khuda-Bukhsh (1989) reported total purification of Tfs at a ratio of 1:4 serum to rivanol but used a concentration of 0.65% instead. In our hands, however, best results were obtained by mixing 2 volumes of 0.4% rivanol with 1 volume of serum (Fig. 6). In the course of present studies at any tested ratio, trace impurities were
always visualized after high resolution PAGE of Laemmli (1970). The discrepancies 
between the reported ratio on *Channa* sp, and our experiments may partly be 
attributed to resolving powers of PAGE protocol of Davis (1964) employed by the 
above authors and modified system of Laemmli (1970) as used here, which may not 
produce identical results. Besides, the origin and purity of rivanol and pH variations 
during precipitation may be among other reasons.

Serum proteins are rarely purified by a single step of purification and the general 
principle of protein purification employing a combination of the techniques is likely 
to produce better results (Roop and Putnam, 1967). As per experience during the 
present investigations, a comprehensive protocol of serotransferrin purification 
consists of the following steps: (a) stabilization of Tfs by saturation with iron (Sutton 
and Karp, 1965; Bron *et al.*, 1968); (b) partial purification by rivanol (Boettcher 
et al., 1958; Sutton and Karp; 1965; Roed *et al.*, 1995) and (c) the final purification 
by gel filtration (Palmour and Sutton, 1971), ion exchange chromatography (Welch, 
1990; Roed *et al.*, 1995) or by preparative gel electrophoresis (Palmour and Sutton, 
1971). Our results on Tfs of *C. punctatus* are consistent with this scheme. Rivanol 
fractionation of iron saturated sera of this fish still contained a few protein bands, in 
addition to those of Tfs, as impurities (Fig. 6) which were completely eliminated 
following the purification by preparative PAGE (Fig. 7). The isoforms were purified 
to homogeneity is also supported by isoelectric focusing patterns of these 
preparations (Fig. 8). Therefore, Tf preparations of *C. punctatus* as used in the 
present investigations for iron binding, molecular weight estimations and 
immunological cross-reactivity were of highest purity.
There is some controversy concerning the range of isoelectric point of fish transferrins. Whereas Boffa et al. (1966) gave the values of 9.0 and 8.9 for petromyzon and a shark Tfs, Valenta et al. (1976) reported similar value of pl = 5.0 for carp and mammalian Tfs. To establish this value, the latter authors used starch gels with buffers of differing pH values. Employing IEF for human Tf, a pl value of 5.35 has been reported by Welch (1990). For human Tf a pl value of 5.30, rather close to the value reported by the latter author, was obtained in the present study (Fig. 8). Tf isoforms A, B and C of C. punctatus have pl values of about 5.30, 4.90 and 4.0 reflecting significant structural differences between the variants involving the interactions which determine the charged state of them.

The lowest ever value of molecular weight of 44 kD was reported for primitive hagfish (Palmour and Sutton, 1971), and controversy still prevails whether it has two subunits or one as per the molecular weight of 77 kD determined by Aisen et al. (1972). Among few other reports, which have dealt with this aspect of fish, are the findings of Hershberger (1970) on Tfs of brook trout and Valenta et al. (1976) on that of carp. On the basis of the number of peptides during finger printing or gel filtration, respectively the values of approximately 78 kD and 70 kD were obtained for Tfs of the above two species. SDS-PAGE gave slightly higher value of 80 kD (Hara, 1984). Though for mammalian Tfs estimates were initially on the higher side (89-93 kD), which among others were derived by sedimentation equilibrium as one of the techniques (Koechlin et al., 1952; Aisen et al., 1966; Bezkorovainy et al., 1963), following the same approach Charlwood (1963) recalculated the value to 76.5 kD. This value has recently been supported by SDS-PAGE also (Welch, 1990).
In general, for fish as well as mammal and other vertebrates, Tf isoforms appear to carry molecular weights within the consensus range of 70-81 kD (Welch, 1990). Tfs of *C. punctatus* also fall in the same range because in SDS-PAG the fish as well as human Tfs migrated to the similar distance (Fig. 9). It is noteworthy that for this purpose SDS-PAGE system specially suitable for estimating molecular weights of glycoproteins (Doucet and Trifaro, 1988) had to be employed. In the system of Laemmli (1970) and other similar systems, apparent molecular weights are influenced by size and configuration of glycan moiety (Leach *et al.* 1980). The discrimination of Tf isoforms of *C. punctatus* by these two systems of electrophoresis supports the glycoprotein nature of these proteins.

Iron binding capacity of highly purified Tfs of *C. punctatus* was measured by monitoring changes in O.D., which showed the maximum at 460 nm, after adding ferric-nitrilo-triacetic acid (FENTA) in a fixed amount (2 μl). The results on iron-binding capacities of electromorphs A, B and C (Table-9) did not demonstrate any significant differences and in either case a value of $\pm 2$ atoms of Fe/Tf molecule could be obtained. Since vertebrate serotransferrin unless proved otherwise, is a molecule with two sites, one for binding iron (comprising two domains for binding one iron atom each) and the other for attachment with the receptor on the cell that is the likely site of unloading bound iron (Davis *et al.*, 1962), the wide variations observed in the relative abundance of various phenotypes specifically the rare phenotype AA can not be correlated with the iron binding capacity. Whereas the selective advantage in the rate of iron binding was correlated with the relative abundance of a particular Tf heterozygote in rainbow trout (Hershberger, 1970), the situation is somewhat different with Tfs of *C. punctatus*. In this case not only heterozygote BC but
homozygote BB was also in a compatible abundance. Variations in the site of attachment of Tf isoforms with the cellular receptors offer the alternative explanation which should also have a bearing on the survival rate of low abundance phenotypes. This may rule out the low fertility as a factor responsible for limiting the population of a particular phenotype, as already pointed out in the preceding part of this discussion.

The functional properties of binding and unloading iron has recently figured in microbiological studies, where for iron uptake secondary pathogens such as Aeromonas sp. and other bacteria may depend on serotransferrins (Hirst and Ellis, 1996). Whereas no such literature is available on Tfs of genus Channa at the moment, it is well established that the above bacteria which act as secondary pathogens are currently under such investigations affect species of genus Channa as well. Further, as compared to other fishes of southeast Asia, Channa sp. belong to a group which is highly susceptible to ulcerative disease related secondary infections (Nabi et al., 2000).

Immunological comparison provides a mean to readily obtain reliable quantitative information on relatedness of proteins (Champion et al., 1975). Evidence suggests that the antibodies raised against the reference protein of one species can be successfully used to explore the phylogenetic relationship with homologous proteins from other species also (Arnheim and Wilson, 1967; Fink et al., 1970). However, due to similarities with human Tfs, the interest has primarily been confined to primate homologues. In an extensive investigation on Tfs of primates, the evolution of antigenic determinants was found to be in good correspondence with the taxonomic data
(Wang et al., 1968). On the basis of microcomplement fixation (MC'F), Prager and Wilson (1971) demonstrated that MC'F determinations may be valid for proteins differing in <30-40% in their primary sequence. Champion et al. (1975) demonstrated the dependence of cross-reactivity upon sequence resemblance of bacterial azurins and predicted the successful application of the approach in defining immunological relatedness of globular monomeric proteins. Suitability of MC'F for phylogenetic studies on transferrins was supported by the data of Prager et al., (1974) which showed slow evolution of this protein and albumins in flightless birds. Though MC'F provides a highly sensitive non-radioactive method of determining evolutionary interrelationships of proteins, no report is available on this aspect of fish Tfs. The only report that deals with immunological phylogeny of fish Tfs concerns with cartilagenous species (Lawson et al., 1995), but an alternate methodology was employed in that case.

During present investigations, antibodies raised against the most abundant phenotype BB of C. punctatus were chosen to determine the immunological relatedness between Tfs of four species of genus Channa. As determined by MC'F at the antibody titre of 1:3200 reactions of the amplitude resembling to that for BB were observed for AA and CC phenotypes of this species. However, to obtain similar peak values of C' (complement) fixed, the concentration of antiserum has to be raised to 2,900; 2,800; and 2,500 for the other three species, i.e. C. gachua, C. striatus and C. marulius (Table-11, Fig. 16) respectively.

For the above four species of genus Channa, the values of immunological distance (ID) predicted on the basis of MC'F results range from 1.5 to 10.7 (Table-12).
When ID values were converted to percentage sequence differences, the minimum difference obtained between *C. gachua* and *C. striatus* was 0.30 and between *C. punctatus* and *C. marulius* was 2.14, the maximum. Fig. 17 demonstrates the pattern of branching derived from the data given in Table-13. The relatedness between immunological determinants of TfS within genus *Channa* appears to be of the following order: *C. punctatus* > *C. gachua* > *C. striatus* > *C. marulius*. Thus, on the basis of this dendrogram TfS of *C. gachua* and *C. striatus* are closest in comparison with the others. This is in contradiction with the morphometric estimates which conceived *C. marulius* and *C. striatus* to be the closest (Chandy, 1955). Apparently, additional information is needed to settle this discrepancy. This is, however, noteworthy that *C. gachua* appears to have undergone ploidy as it possesses 78 chromosomes against 40 of *C. striatus*. Though additional Tf loci as per suggestion of Kirpichnikov (1981) may have lost during evolution, its effect on the properties of immunological determinants has yet to be considered. A chromosome compliment which is nearly twice that of *C. marulius* and *C. striatus* and about three times that of *C. punctatus* may point to its longer evolutionary history, which in other words would make it the species of remote origin, a suggestion which is in accordance with the morphometric data (Chandy, 1955). Similarly, a recent origin of *C. punctatus* is also supported by immunological data. Right now no plausible explanation for the observed immunological relatedness between TfS of *C. gachua* and *C. striatus* is available. It appears that similar evolutionary mechanics modified the antigenic determinants of TfS of these two species.
CONCLUSIONS

On the basis of present investigations on Tf of *Channa punctatus* Bl., the following inferences can be made:

1) Extensive genetic polymorphism is displayed by Tf of *C. punctatus* which is applicable in identifying and typing its populations to monitor their dynamics.

2) The abundance of a homozygous phenotype, as reported for Tf BB of this fish, is an exception to the general observation made so far that heterozygote excess is because homozygotes are less viable in comparison with heterozygotes.

3) A similarity in iron binding rates displayed by BB and excess heterozygote BC suggests a role for the second functional site of Tf, i.e. the cell receptor binding site in determining the viability of these phenotypes.

4) Polymorphism displayed by *C. punctatus* Tf is under the control of a single locus, variants of which encode isoforms in the combinations typical of one or the other phenotype.

5) Inspite of recent origin of *C. punctatus*, its Tf locus has undergone extensive mutations giving rise to atleast three different co-dominant alleles and their random combinations. Though *C. punctatus* is a highly specialized fish, its Tf are structurally and functionally similar to those of other vertebrates. All Tf studied so far invariably possess the same iron binding capacity indicates that the number of binding sites per molecule and most probably the structure of the relevant sites have been substantially conserved during evolution.
6) MC'F data suggests some agreement between classical morphometric data and evolutionary relatedness of immunological determinants of species other than that shown by Tfs of *C. gachua* and *C. striatus*. As originally proposed by Chandy (1955), *C. gachua* might have been the most generalized form, but immunologically its Tfs are more close to those of *C. striatus* indicating that during evoluation similar changes were incorporated in their antigenic determinents. Divergence of *C. punctatus*, was ahead of these two i.e. *C. gachua* and *C. striatus*. 
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