Chapter-4
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A) Extent of polymorphism:

1) in soluble muscle protein:

In the results presented from Figs. 3-8, evidence was provided to support that the selected phenotypes in PAGE patterns of soluble muscle proteins are real variants. The two kind of marker systems (category-1 and category-2) could be identified by simple approach: 1) routine protein staining with CBB; 2) thermal incubation precipitates out unstable proteins and, 3) developing minor bands with the sensitive silver staining.

Addition of glycerol in gels of 10-12.5% for the native system and 15% for SDS containing PAGE, considerably improved resolutions can be obtained. A hypothesis has also been advanced that shows the involvement of an isolocus in a polymorphism based on codominant dimorphs. It was shown to satisfactorily explain the polymorphism of heat unstable proteins (category-1 markers).

This is to emphasize that by routine protein staining the polymorphism in soluble muscle protein extracts has seldom been reported. In one of the such reports, Tsuyuki et al. (1967) reported a diallelic polymorphism in Catostomus catostomus. In Menidia menidia (Morgan & Ulanowicz, 1976), a polymorphism was reported that was in accordance with Hardy-Weinberg prediction, and the proteins as per PAGE patterns appeared to be PA or like proteins that are classified as category-2 markers in the
present study. The polymorphism of PA has been reported in several cyprinids by Bobak & Slechta (1987; 1988) and in some species of Barbels by Huriaux et al. (1992). In a previous study from this laboratory an isoloci depended polymorphism was reported in Heteropneustes fossilis (Pandey & Hasnain, 1994). However, due to insufficient data the statistical analysis could not be carried out in that case. In the present study sample size comfortably permits such analysis that is to be presented in the appropriate sub section under this discussion.

2) in hemoglobins (Hbs):

Chandrasekhar (1959) was first to demonstrate the occurrence of multiple hemoglobins in fresh water fish species of India. High resolution of electrophoresis revealed more extensive multiplicity in some of the fish hemoglobins (Hasnain et al., 1973; 1976; Khan, 1980) than any previous study.

As explained under "Results" six polymorphs or variants were discovered within the 1350 samples of C. batrachus collected from different locations of Rohilkhand plains (Fig. 2). To exclude the possibility of the artifacts caused by autoxidation, brief frozen storage during the analysis of the samples or other variables (Giles & Vanstone, 1976; Dilorio, 1981).

The submolecular composition of the entire set of multiple Hbs constituting a particular polymorph as well as Hb eluted from each band of the individual electromorph were subjected to analysis in SDS-PAGE (15% gel) which resolve the multiple globins (Fig. 11b) rather than costacking the bands of same molecular weight as one (Fig. 11c). The
results demonstrate that two to four globin chains make one or the other electromorphs within a particular Variant (Figs. 12 & 13 and Tables 8 & 9) assigned the notation Cb-A, Cb-B, Cb-C and Cb-D to discriminate them against α- and β- mammalian homologue as adopted by Tsuyuki & Ronald (1971) for Oncorhynchus Hb globins.

A similar number (four) constituents chains was obtained if an alternate system of Schwantes & Schwantes (1970) as outlined under "Materials and Methods" and also in the legend of corresponding figure (Fig. 13). The proper stoichiometric proportion (Tables 8 & 9) could not quantitatively be achieved due to monitoring of submolecular composition keeping as the last item of the entire screening program that spanned over three years duration. However, the differing submolecular compositions of individual electromorphs of a specific polymorphs (controls) is well evident. Quantitative estimations were also made difficult and inaccurate due to heavy background of silver or benzidine staining.

Reports of multiplicity of globin chains to a magnitude of upto four chains have been published by Tsuyuki & Ronald (1971) for Oncorhynchus and for Salmo gairdneri by Ronald & Tsuyuki (1971), Iuchi & Yamagami (1971). In Sarotherodon mossambicus two to four globin chains were detected by Perez & Maclean (1976) using p-HMB method. In other instances, Manwell & Baker (1967) found three globin chains in turbot and Koehn (1969) reported a similar number in several polymorphs of a number of catostomids. SDS-PAGE revealed the existence of upto three globin chains in hemolysates of four species of genus Channa (Khan, 1980).
As already reviewed in "Introduction", most work deals with multiplicity of tetramers rather than heterogeneity of globin chains. The results of globin chains monitoring on *C. batrachus* Hb polymorphs rule out the artifacts status of any of the polymorphs. The genetic aspects of taking them as real variants have been discussed along with the variants of two other protein systems under "Statistical analysis".

3) in eye lens nuclei protein (Crystallins):

Most of the published work on whole eye lens soluble proteins of fish deals with interspecies comparison and genetic variation has seldom been demonstrated (Barrett and Williams, 1967; Eckroat and Wright, 1969). Investigations where high resolution IEF has been employed, (Basaglia and Callegarini, 1987, Basaglia, 1989; Basaglia and Di Luca, 1993) also support the observed species specificity of lens protein repertoire, majority of which are α-, β- and γ- crystallins (Piatigorsky and Wistow, 1989). Taxon specific crystallins of several elasmobranch and teleost species have been characterized as multifunctional proteins (Wistow, 1993) and a few of them have even been cloned and sequenced (Behren *et al.*, 1998; Chang *et al.*, 1988; Pan *et al.*, 1997). The patterns of soluble proteins of whole eye lenses have, however, been shown to be influenced by ontogenetic and age related changes in eye lenses of dogfish of 2-50 years of age groups (Zigman and Yulo, 1979; Smith, 1982). Benz (1980) reported the occurrence of totally different sets of bands in pups of sandbar shark as compared to those of the adults, though for adults of a specific size range (201-252 cm), bearing some quantitative variations, consistently the same number of bands was recorded. We, therefore,
perceived that ontogenetic and age related qualitative and quantitative changes might be avoidable if the sampling is confined to mature individuals of a narrow range of age or size.

Fish eye lens due to continuous growth and the crystallins involved in life long refractivity differs from that of human beings of 5.5 to 50 years of age where opacity due to an increase in insoluble proteins accompanies old age (Bours, 1980). Obviously, the nature of information obtained from electrophoretic patterns would greatly depend on the type of proteins (crystallins), their relative concentration, solubility and the techniques employed to resolve the soluble lens proteins. Genetic character of the polymorphism observed in SDS-PAGE patterns of soluble eye lens proteins of *C. batrachus* in the present study has, therefore, been ascertained after examining the data employing following criteria: 1) only adult fishes which had passed one breeding season and had an age in a range of 1-1.5 years were sampled; 2) analysis of lens nuclei was preferred since they have been shown to be more reliable source of genetic information (Smith, 1971; Smith and Clemens, 1973) rather than the whole eye lenses wherein a multitude of intraspecies non-genetic heterogeneity has been reported (references cited above); 3) in view of the tendency of aggregation of α-crystallins (Bon *et al.*, 1968; de Jong *et al.*, 1976), PAGE analysis was performed in the presence of SDS under reducing and dissociating conditions (Laemmli, 1970); 4) the data was statistically tested to examine the correlation between the observed polymorphism and the population distribution of the fish and, 5) IEF analysis of selected variants was performed to determine the number of α-, β- and γ- isoforms of crystallins which constitute one or the other
phenotype in SDS-PAGE patterns. IEF patterns of soluble proteins of whole eye lens of a sister species *C. gariepinus* are known to produce a maximum of 53 bands (Basaglia and Di Luca, 1993), whereas those of lens nuclei of *C. batrachus* resolve up to >20 bands which appears to be a reasonable number keeping in view the protein rich nature of lens cortex. The sequential deposition of crystallins as eye lens layers during development, however, is shown to follow an ontogenetic program as evident by a study on tuna species (Smith, 1982) and some other published work. In view of the above, there thus exist reasons to regard the observed eight variants as phenotypes which would have arisen due to intrinsic genetic factors specific to each population they represent.

Though any individual SDS bands (Fig. 14b) cannot be directly correlated with the type and number of crystallin isoform it is made of, IEF patterns (Fig. 14c) demonstrate that the contribution of α-crystallins in making the lens nuclei of *C. batrachus* is minimal. The comparison of these two figures also underlines the multiallelic character of crystallin isoforms.

**B) Statistical analysis:**

The data explained under subsections dealing with the polymorphism of soluble muscle proteins, hemoglobins and eye lenses has been subjected to statistical analysis to evaluate its value in describing the population structure of *C. batrachus* in the western plains of Uttar Pradesh.

Statistical analysis, using *Chi*-square (*χ²*) homogeneity test, was performed separately on muscle, hemoglobin and eye lens nuclei
polymorphs, respectively. The analysis gives some unusual results on the population substructure in the western belt of Uttar Pradesh. Out of 1350 samples in case of muscle where five polymorphs (Fig.-4) were recorded for category-1 (heat unstable proteins, Table-10) and out of 1057 samples of C. batrachus for category-2 (heat stable parvalbumin or like proteins, Table-11), theory of homogeneity of their distribution does not hold ($p<0.05$). A relative shift of low magnitude in attaining homogeneity by the population of hemoglobin polymorphs was noted wherein the population as a whole represents a perfect heterogeneous distribution (Table-12). Results exactly similar to muscle, rejecting the homogeneity hypothesis ($p<0.05$), were also obtained for eye lens nuclei proteins (Table-13) where six polymorphs (Fig.-14b) were detected and identified by SDS-PAGE.

Chi-square ($\chi^2$) homogeneity test was also performed on the distribution of total number of protein bands of soluble muscle proteins on PAGE to determine their genetic nature as a whole and to test the heterogeneous make up of the protein loci having different alleles. In Table-14 summarizes the compilation of observed and expected frequencies of protein bands (calculated on the basis of relative mobilities). $\chi^2$-test used separately on entire set most common 17 bands in the soluble muscle protein PAGE patterns demonstrate highly significant results ($p<0.05$) and thus, the hypothesis of homogeneity for protein repertoire of whole muscle extracts is not rejected. The distribution pattern of five polymorphs of category-1 (Table 10) heat unstable proteins was found to be same as that obtained for the total 17 bands (Table-15). Almost similar results as shown in Table-16, favoring
the homogeneity hypothesis \((p<0.05)\) were also recorded for the distribution of category-2 parvalbumin or like heat stable protein bands.

Some interesting results were obtained for multiple hemoglobins when the test was applied for hemoglobin tetramers as a total of 1-8 electromorphs cumulating the entire set of all the Vhbs. This in other words refers to a total of 8 bands of hemoglobin having different electrophoretic mobility on native PAGE, as detected in the 1350 samples of \(C.\ batrachus\) along the western belt of Uttar Pradesh. Among the total of 8 bands, only band \(\#\ 8\) \((p<0.05)\) and \# 2, 3, and 4 \((p<0.01)\) showed the significant relationship (homogeneity) while band \# 1, 5, 6 and 7 were found to be having a reverse relationship (Table-17), that is homogeneity hypothesis is rejected.

As already shown in Fig. 14b, the number of bands in various polymorphs of eye lens nuclei (crystallins) varied between 3-9. Band \# 1, 2, 4, 6, 7, 9, 10, 11 and 13 are frequent in their distribution while those marked \# 3, 5, 8 and 12 occur restrictively within the populations of districts other than Moradabad and Etawah in north-western and Kanpur and Lucknow in south-western part of Uttar Pradesh. Chi-square \((\chi^2)\) analysis of the total 14 protein bands (taken as loci) show that the band \# 14 is uniformly distributed throughout the investigated belt except Etah and has a significant relationship \((p<0.05)\) which appears to be a rare and unique locus within the populations.

Percent frequency distribution of all the discovered polymorphs within the investigated area was calculated and depicted in Fig.15-17. Pie diagram (Fig.15a) shows that among all the polymorphs of category-1
muscle protein loci, V-4 is the only variant that is distributed in almost all the sampling locations (55.92%) except Badaun and Etawah. Least distributed polymorph in this category-1 of muscle, V-2 (3.18%), is restricted to only Badaun, Bareilly, Kanpur and Lucknow. In case of category-2, as shown in Fig.15b, heat stable muscle protein polymorphs V-3, V-4 and V-5 inhabit the same sampling stations and occupy 28.76% while a least percent frequency distribution was noted for V-2 (4.06%) that was caught from the same sampling locations as in case of category-1.

Of all the 6 detected variants in hemoglobin, Vhb-3 (54.66%) was collected from every sampling station of Uttar Pradesh while the least recovered variant was Vhb-5 that occupies only 2.07% of the investigated belt (Fig.16).

In case of eye lens nuclei polymorphs, a sort of grouping was observed in their distribution. Two groups of maximum and minimum percent frequency distribution of polymorphs were observed as depicted in Fig.17. Maximum area of distribution is occupied by V5 (25.18%) which is very close in its distribution to the V2 (24.29%) and V6 (23.92%), respectively while 8.51% distribution was observed for V4 that is very close to the V3 (8.59%) and V1 (9.48%), respectively.

Chi-square analyses on the distribution of the variants of muscle, hemoglobin and eye lens nuclei proteins suggest a model of population structure of *C. batrachus* in the western belt of Uttar Pradesh. The data on muscle protein polymorphs belonging to either of the category (i.e. heat unstable and heat stable parvalbumin or like proteins) show that the
variants depart from the proposed hypothesis \((p<0.05)\) and strongly suggests a heterogeneous distribution (Table-10 and 11). In this respect, the case of this silurid is different from that of *Mystus nemurus* where river connections may be a source of intermixing of populations (Lessa-Nga *et al.*, 2000). We suggest that one of the reasons that explain this phenomenon is independent nocturnal migration on land, which is the characteristic of *C. batrachus* as an accessory air-breather and mixing during monsoon flooding. Had there been a significant recruitment from riverine connections, the steady decline in the output of *C. batrachus* due to induction of a better predator *C. gariepinus* as a pond culture fish would not be observed. Whereas terrestrial migration had to be instinctively a constant feature, degree of monsoon flooding varies and that will also be periodically excluded altogether if a dry spell intervened.

Results obtained for hemoglobin and eye lens nuclei protein polymorphs are exactly same as that of muscle and reject the hypothesis of homogeneity \((p<0.05)\) (Table-12 and 13). A comparative data of the homogeneity test applied on muscle, hemoglobin and eye lens nuclei protein polymorphs distribution along the western belt lead to the conclusion that variants may be related with one another in distribution pattern and they do not seem to be influenced by any of the external factors like temperature of water body and latitude.

*Chi*-square homogeneity test performed separately on the total of 17 muscle protein bands and, category-1 heat unstable as well as category-2 heat stable parvalbumin or like muscle protein bands favors the hypothesis \((p<0.05)\) as shown in Tables 14-16. The protein loci (having alleles) belonging to either of the categories, are invariably homogeneous
within the population that may only be possible when the subpopulations tend to intermix and disturb the population structure of a region that is in agreement with our previous conclusions described in the above paragraph.

Single band of hemoglobin (i.e. bands #2, 3 and 4) having different electrophoretic mobilities showed significant relationship ($p<0.01$), including band # 8 ($p<0.05$) that seems to be a rare band of relatively high electrophoretic mobility and homogeneous in distribution (Table 17). Bands #1, 5, 6 and 7 are statistically non-significant (reject the hypothesis of homogeneity), and therefore, these should be heterogeneously distributed in the population like band #1 and 7. But bands # 5 and 6 do not follow this trend of distribution pattern and exists almost homogeneously in the polymorphs detected in the belt. A possible explanation to this controversy may either be a lack of high level accuracy in statistical approach applied exclusively for this protein or there is an inaccuracy in measuring the electrophoretic mobility of bands due to their unavoidable high thickness. Intermixing that has already been discussed (in the first para of statistical discussion) may plausibly explains the heterogeneous distribution of polymorphs within the belt. It can be concluded that as the favorable conditions approached intermixing populations may experience interbreeding among the subpopulations of *C. batrachus* that happened to be responsible to shapen the genetic structure of a population. Therefore, to conclude that the homogeneous distribution of protein bands (either of muscle or hemoglobin) is a consequence of interbreeding that seems to play a role in giving a
homogeneous distribution of genetically determined protein bands, might be a correct and possible explanation to the present case.

Chi-square ($\chi^2$) test suggests a heterogeneous distribution of eye lens nuclei protein loci within the investigated western belt. Out of a cumulative estimate of 14 protein bands detectable in SDS-PAGE patterns of one or the other phenotype (Fig. 14b), phenotype # 2 (3 bands) appears to have a rare locus. It is distributed mainly in Firozabad, Agra, Etawah, Kanpur and Lucknow and is not affected by latitudinal distribution suggesting its recognition as a unique genetic marker suitable for further phylogenetic and microgeographical studies. This unique locus appears to be conserved one and may be more close to an ancestral form.

Percent frequency distribution of all the polymorphs belonging to different categories of proteins is depicted in Pie diagrams (Figs. 15-17). Figs. 15 and 16 (i.e. category-1 heat unstable muscle proteins and hemoglobin variants) do not suggest any perfect system of their distribution in the inhabited area and a very simple conclusion can be drawn about the heterogeneous distribution of polymorphs. Fig. 15b, shows the percent frequency of category-2 heat stable muscle proteins. Variants V-3, V-4 and V-5 which were recorded in all the districts other than Rampur and Lucknow, have an equal percent frequency (28.76%) distribution. Minimum value (4.06%) was obtained in case of variants V-2 though it occupies nearly 30% sampling stations or locality which is higher than V-1 (Table 11). Analyses of the data again reject the hypothesis ($p<0.05$). Two groups of populations were also recognized in case of eye lens nuclei protein having sets of closely related distribution frequencies (Fig. 17), one is maximum (V5, V2 and V6 ≥ 23.92%) and the
other is minimum (V4, V3 and V1 ≤ 9.48), and none of the group was observed in between. It can be concluded that the eye lens nuclei protein variants tending to achieve a kind of group separation that can be understand by their nature of distribution particularly their heterogeneous make up within the investigated belt of western Uttar Pradesh.