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Partial Biochemical Characterization and Iron Binding Capacities of Transferrin Variants of Air-breathing Murrel *Channa punctata* (Channidae: Channiformes)

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Abstract

Partial biochemical characterization of highly purified homozygous serotransferrin (Tf) phenotypes AA, BB and CC of air-breathing murrel, *Channa punctata*, was conducted. Molecular weight of each pure Tf variant was 75 kDa. Each Tf variant also had sialic acid as carbohydrate moiety of 10.5 kDa. Difference in spectra of iron-apotransferrin complexes of *C. punctata* and human Tf were similar. Complex of each apoTf with iron had its maximum at 460 nm. Lack of a shoulder at 480 nm was characteristic of Tf*BB*, while TfCC and Tf*AA* had one shoulder at 480 nm and the other at 510 nm. The Tf*BB* also had the highest total iron-binding capacity followed by Tf*CC* and Tf*AA*, respectively, though total saturation for each Tf was reached at 14 μM FENTA. Interestingly, the relative total iron-binding capacities of Tf*BB* and *CC* showed a positive correlation with the allelic composition of populations, which were the sources of sera. The significance of iron binding capacities of Tf variants along with some other factors on the population structure of *C. punctata* is discussed.

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Introduction

Due to its significance as genetic markers, transferrin electromorphs continue to draw attention for investigating teleost populations (Csizmadia et al. 1995; Van-Doornick and Milner 1996; Calcagnotto and Toledo-Filho 2000; Teixeira et al. 2002). We have recently reported Tf polymorphism in *Channa punctata* populations of the Rohilkhand plains (Nabi et al. 2003). These populations were unique for having homozygous transferrin phenotype BB in unexpected excess of not only homozygote AA but also heterozygote AC and its allele designate Tf*Bcp* was also in a correspondingly high frequency. This was a departure from the general trend among teleost populations. The report of Christiansen (1980) showed that even if there was a deficit in heterozygotes during early life of eelpout, population achieved balance later on. *C. punctata* Bloch is a widely distributed food fish of bimodal respiration that also contributes to integrated aquaculture in India and other Asian countries (Jhingran 1991).

Biochemical characterization of various Tf phenotypes identified in sera samples collected during the above cited study on *C. punctata* (Nabi et al. 2003) is being reported here. So far, only the partial purification of Tf phenotypes of this species has been described (Sahoo and Khuda Bukhsh 1989), while no report is available on the structural and functional aspects of its Tf variants. Therefore, a prime objective of the biochemical analyses was to compare the iron-binding capacities of homozygous Tf variants, since iron-binding is the main functional property of transferrin. We could find only one report on teleosts where biochemical analysis of Tf variants included iron binding (Hershberger 1970). Iron binding is the crucial functional property of transferrins (Baker et al. 2002). By binding out free iron of plasma, Tf prevents iron toxicity as well as bacterial growth (Winter et al. 1980; Hirono and Aoki 1996; Baker et al. 2002).

Materials and Methods

Collection of fish and serum samples

Live *C. punctata* were purchased from local fish markets of several towns of Aligarh district. The procedure of sera collection and screening has already been detailed in the previous report (Nabi et al. 2003). Briefly, using sterilized syringe, blood was collected through cardiac puncture and

transferred to sterilized eppendorf tubes of 2.0 ml capacity. Blood was allowed to clot at room temperature. Serum separated following shrinkage of clot was pipetted out and cleared of contaminating blood cells by centrifugation at 4000xg. The samples were stored freezing at -20°C.

Electrophoresis

Phenotyping was carried out by native 7.5 % polyacrylamide gel electrophoresis (PAGE) in a modified system lacking SDS (Nabi et al. 2003). Lower gels were 0.375 M in Tris-HCl (pH. 8.6), the upper 3% in 0.125 M Tris-HCl (6.8), while the runs were made in Tris-glycine (0.025 and 0.25 M, respectively). The Tf bands were identified by a combination of specific staining with nitroso-R and protein staining with coomassie brilliant blue R-250.

The SDS-PAGE was carried out in the porous system of Doucet and Trifaro (1988). Stock solution contained acrylamide: bisacrylamide in a ratio of 40:0.4 instead of 30:0.8 (Laemmli 1970). All buffers contained 0.04% SDS. Lower separating 10% gel was cast in buffer containing 0.2 M Tris and 0.1 M glycine (pH 9.0). The upper stacking gel was 4%, containing 4 mM EDTA, 5% glycerol and 70 mM Tris-HCl (pH 6.7). Upper tank buffer contained 0.04 M Tris with 0.06 M glycine (pH 8.5). Lower tank buffer was made by one time dilution of upper buffer.

Purification

Partial purification of transferrin was carried out essentially as described by Sutton and Karp (1965). Pooled sera of a specific phenotype were mixed with 0.5, 1, 1.5, 2 and 2.5 parts (v/v) of rivanol (2-ethoxy-6,9-diaminoacridine lactate, Sigma, U.S.A.) in 5 mM Tris-HCl buffer of pH 8.8. Under these experimental conditions, the best partial purification of Tfs was achieved at the ratio of 1:2 serum to 0.4% rivanol. For biochemical analysis, partially purified identical phenotypes were further purified to homogeneity by native preparative-page in 7.5 % gels as detailed above. The major band of human Tf (Fluka, Switzerland) that served as a standard reference was also purified to homogeneity by preparative PAGE. Electroeluted pure phenotypes were exhaustively dialyzed against distilled water, monitored by native PAGE and freeze-dried.

Digestion with neuraminidase

This was done according to protocol of Stratil et al. (1983). Lyophilized pure Tf variants and human Tf were dissolved in 0.2 M sodium acetate buffer (pH 3.7) and pH adjusted to 5.8. The same buffer was used to

dissolve neuraminidase (final concentration 2 µg/µl). Digestion was carried out at a ratio enzyme to Tf 1:2 at 37°C. At 2, 4, 8, 10 and 12 hr, 20 µl aliquots were drawn and incubated for 10 min at 100°C with ¼ volumes of sample buffer containing SDS (Doucet and Trifaro 1988).

Conversion to apotransferrin

Highly purified Tf solutions were converted to apotransferrin by removing bound iron by exhaustive dialysis of the samples against several changes of 1.0 M citric acid. The contents were centrifuged at 4°C and 15,000xg and the supernatant dialyzed against distilled water followed by freeze drying. The calculations of the iron-binding by individual apotransferrin isoform were made according to Welch (1990) using human apotransferrin as the control. The system makes use of FENTA or ferric nitrilotriacetic acid containing 0.1 µg of Fe/µl [composition: nitrilotriacetic acid, 0.19 g per 3 ml of distilled water; 1M NaOH (2ml); 0.5 M ferric chloride (2.0 ml) L^{-1}]. It was added to lyophilized samples dissolved in 25 mM Tris-HCl pH 7.5 containing $NaHCO_3$ and the absorbance monitored at 460 nm (Palmour and Sutton 1971) on Spectronic-1001. Monitoring of the binding was continued till the transferrin was totally saturated with iron as indicated by a constant value of absorbance. Iron binding capacity was calculated according to the following formula:

Fe atoms bound/Tf molecule = mole of Fe/mole of Tf

In this equation, 1 mole = weight of substance used per M_r of that substance.

Results and Discussion

Co-dominant alleles are conventionally denoted with the two letter system. In this report genotype of homozygous Tf variants of *C. punctata* have been designated *AA*, *BB* and *CC*, while phenotypes in non-Italics. Under our experimental conditions, the best partial purification of three transferrin phenotypes of *C. punctata* was achieved at the ratio of 1:2 serum to 0.4% rivanol (Fig. 1a, lane-2). Lanes 3-5 in the same figure show purification of the three Tf phenotypes AA, BB and CC to homogeneity following preparative-PAGE. Figure 1b shows that a mixture of all three isotypes (A, B and C) of *C. punctata* resolved as a single band migrating slightly faster than pure human serotransferrin. Its molecular weight value was 75 kDa as compared to 80 kDa of human Tf.

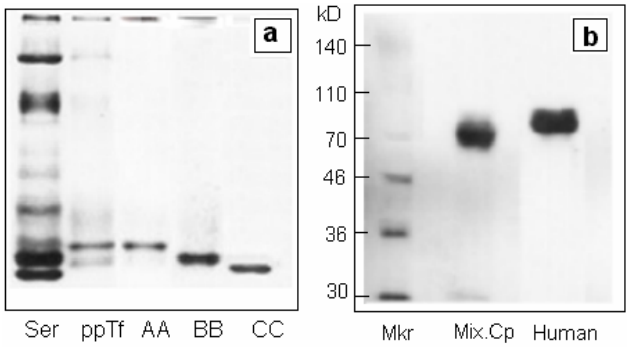

Fig. 1(a). Native PAGE patterns of whole serum (lane-1), partially purified with rivanol (lane-2) and preparative PAGE purified transferrin phenotypes AA, BB and CC of *Channa punctata* (lanes 3, 4 and 5, respectively). Electrophoresis was carried out according to Laemmli (1970) with the modification that no buffer contained SDS. (b). SDS-PAGE profile of transferrins demonstrating purity and M_r values (lane-3). Lanes from left to right show: M_r marker polypeptides (lane-1); mixture of Tf phenotypes AA, BB and CC of *C. punctata,* Mix.Cp (lane-2). Electrophoresis was carried out in porous polyacrylamide gel system of Doucet and Trifaro (1988). All buffers contained SDS.

A value of M*r* 75 kDa of *C. punctata* transferrin phenotypes is in reasonable agreement with the apparent range of 70-81 kDa documented for teleosts as well as mammalian Tfs (Aisen et al. 1972; Hershberger 1970; Hara 1984; Boback et al. 1984; Welch 1990). *C. punctata* transferrins are glycoproteins, since the variants did not stack properly in routine SDS-PAGE (Laemmli 1970) and required a porous system designed for glycoprotein separation (Doucet and Trifaro 1988). The glycoprotein nature of Tf of *C. punctata* was confirmed by neuraminidase digestion of the variants. As shown in figure 2, bulk of 75 kDa band of pooled Tf variants was converted into a band of 64.5 kDa following 12 hr long incubation with neuraminidase. Therefore, Tf variants of *C. punctata* have sialic acid as the carbohydrate moiety that adds 10.5 kDa to the mass of intact Tf. In this respect, Tfs of *C. punctata* are different from some other teleosts which are devoid of carbohydrate moiety (Stratil et al. 1983; Yang et al. 2004). According to molecular cloning data, Tf variants of goldfish display M*r* differences with no glycosylation (Yang et al. 2004).

The difference spectra (DS) of iron-apotransferrin complexes of pure variants AA, BB and CC of the three homozygotes are compared in figure 3. Apart from having a very shallow trough at 450 nm, spectral behavior of human iron-apoTf resembled closely with iron-apotransferrin complexes of *C. punctata* variants. The main peak at 460 nm was common to all transferrins. Shoulders of iron-apoTf*AA* and Tf*CC* complexes at 480 and 510 nm were similar to those visible in DS of human iron-apoTf complex (Fig. 3). DS of iron-apoTf*BB* complex was, however, devoid of a prominent shoulder at 480 nm. Minor substructural differences with Tf*CC*

and Tf*AA* are thus likely. In general, the DS of *C. punctata* and human apotransferrin-iron complexes resembled closely, suggesting submolecular and conformational similarities. In fact, a high degree of sequence identity has been demonstrated among DNA sequences of cloned genes of transferrins of mammals and teleosts (Hirono and Aoki 1996; Ford 2000; Antunes et al. 2002; Yang et al. 2004). In addition, positions of iron binding and cysteine residues have also been conserved (Williams 1982; Baker et al. 2002). Thus, similarity in DS profiles of iron-apoTf complexes (Fig. 3) has to have a basis at the level of primary structure.

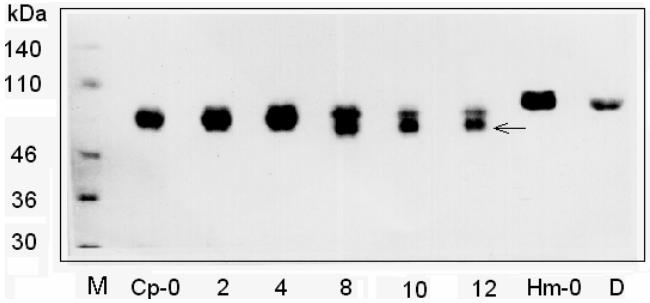

Fig. 2. SDS-PAGE profiles of neuraminidase digests of pure Tf variants (AA + BB + CC mixed in equal amounts) of *C. punctata*. Gradual conversion of 75 kDa Tf band into 64.5 kDa band by neuraminidase is shown by arrow. From left to right, the lanes are: M (molecular weight markers); undigested control of mixed Tf variants (Cp-0); lanes 3 to 7 show duration of neuraminidase digestion at 2, 4, 8, 10 and 12 hr, respectively. Lane 8 (Hm-0) is human control and its 12 hr digest (lane 9).

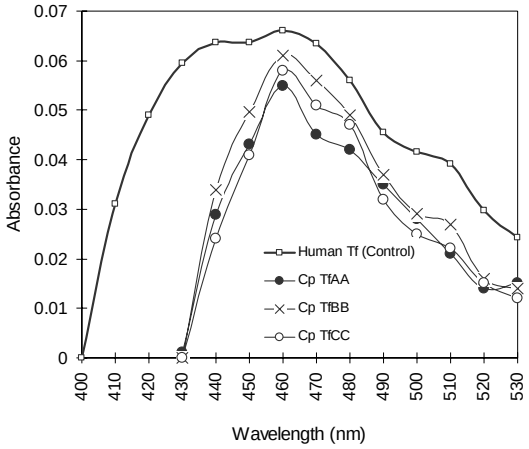

Fig. 3. Difference spectra (DS) of iron-apotransferrin complexes of human and pure Tf variants of *C. punctata*. Spectral profiles represent absorbance at 400-530 nm after adding 14 µM FENTA (ferric nitrilotriacetic acid). Lyophilized powder of proteins (0.76 mg) of each apotransferrin Tf genotype was dissolved in 1 ml of 25 mM Tris-HCl buffer of pH 7.5. It also contained 30 mM sodium bicarbonate which is essential for iron-binding.

Total iron binding capacities of homozygous Tf variants of *C. punctata* are compared in figure 4. Though saturation for either of the variants was achieved at 14 µM FENTA, relatively higher absorbance of apoTf*BB* indicated its better iron binding capacity. As per calculation given under Materials and Methods, however, each apoTf bound two iron atoms per molecule. These results suggested that the number of iron-binding sites

was identical and there also existed substantial conformational similarity around the binding sites.

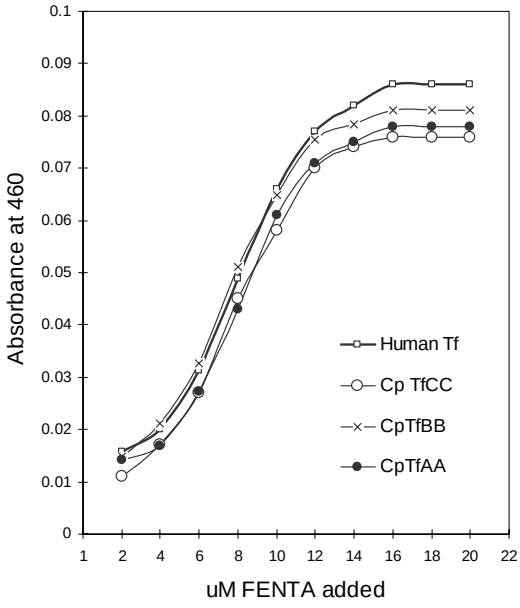

Fig. 4. A comparison of iron-binding capacities of human and homozygous *C. punctata* Tf genotypes at various concentrations of the FENTA showing differences at a final concentration 1 mg protein/ml. Peak values taken at 460 nm following addition of increasing amounts of FENTA (ferric nitrilotriacetic acid)` χονταινινγ 0.1 μg of Fe/μl.

Kirpichnikov (1981) had emphasized the importance of iron-binding properties of transferrin variants in teleost evolution that involved gene duplication and polyploidy. The DNA sequence data on salmonids and goldfish support that occurrence of polyploidy event was followed by selection of the beneficial mutants (Ford 2000; Yang et al. 2004). More importantly, the selection pressure exerted by 'limiting iron availability' to bacterial pathogens has been implicated in the evolutionary selection of new Tf alleles in salmonids and brown trout (Ford 2000). Since transferrin is the principal iron-binding protein, differences in availability of free iron in serum will obviously depend on total iron-binding (removal from serum) capacity of a specific Tf variant. In rainbow trout Tf heterozygotes were shown to retain more iron (Hershberger 1970), while Tf and lactate dehydrogenase of heterozygotes has been reported to display better functional adaptability as compared to homozygotes (Kirpichnikov 1981). Since, total iron binding capacities of different Tf phenotypes of *C. punctata* can as such predict the reported excess of allele TfB*cp* followed by TfC*cp* or TfA*cp* (Nabi et al. 2003), functional property of iron-binding is identified here as one of the important adaptive elements for survivorship of this species.

A number of factors such as the latitude, temperature fluctuations and duration of growth season also affect allelic frequencies of Tf and several other proteins, iso- and allozymes by way of functional properties (Kirpichnikov 1992). Most of the studies included in that review dealt with

temperate water teleosts, while information on those aspects of *C. punctata* is yet to be published. However, combining our previous study on population structure of Tf variants of *C. punctata* (Nabi et al. 2003) and the functional aspects presented in this report, we rule out at least three of the factors important to relative viability of the progeny that may lead to variations in population structure. First of all, *C. punctata* is not a polyploid (Ponniah and John 1998); therefore, selection mechanics of beneficial Tf mutants during evolution might not have been identical to positive selection discovered in polyploid salmonids (Ford 2000). However, better total iron binding capacity of Tf*BB* supports the crucial role assigned to 'limited iron availability' in evolution of transferrins (Ford 2000). Second, the possibility of differences in fertility of Tf phenotypes in *C. punctata* can also be ruled out, since all of Tf alleles are well accounted in heterozygotes (Nabi et al. 2003). Third, the role of accessory air-breathing in enhancing the viability of early larval stages may also be ruled out, since the partitioning to bimodal breathing occurs on the 14-15th day post-hatching, when larvae achieve adult fish-like morphology and disperse out by giving up the larval behavior of swimming in shoals (Ahmad and Hasnain 2006).

Involvement of different types of receptors is a strong possibility that is also directly concerned with iron transport, since iron bound by Tf is transported to hematopoeitic tissues via receptor mediated endocytosis (Lim and Morgan 1984). Better retention of bound iron by homozygous Tf*BB* of *C. punctata* in the range of ~10-14 µl suggested that the removal (dissociation) of Tf bound iron might require different types of receptors or physiological conditions.

Population of *C. punctata* in several parts of India is declining due to destruction of habitat and other environmental changes. Therefore, a better appreciation of other factors influencing its population structure is highly desirable. Research should be extended not only to Tf receptors, but to other easy-to-monitor genetic markers as well.

Conclusions

The M*r* values, glycoprotein nature and spectral behavior during iron-binding favors a basic structural and functional similarity between Tf variants of *Channa punctata* and transferrins of other vertebrates. Intraspecies differences in total iron-binding capacities, however, exist and support the crucial role of 'limiting iron availability' to bacterial pathogens as one

of the key elements in selection of beneficial Tf mutants during teleost evolution. While some other factors may influence the population structure of *C. punctata*, arguments have been put forth here that rule out differences in fertility and the benefit of accessory air-breathing to survival of less grown larvae.

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References

Ahmad, R. and Hasnain, A. 2006. Differential expression of G6PD and alkaline phosphatase isozymes associated with ontogeny and air-breathing transition in *Channa punctata*. Asian Fisheries Science, 19**:**141-148.

Aisen, P., Leibman, A. and Sia, C.L. 1972. Molecular weight & subunit structure of hagfish transferrin. Biochemistry, 11:3461-3464.

Antunes, A., Templeton, R., Guyomar, R. and Alexanderino, P. 2002. The role of nuclear genes in intraspecific evolutionary inference: genealogy of the transferring gene in the brown trout. Molecular Biology and Evolution, 19:1272-1287.

Baker, E.N., Heather, H.M. and Kidd, R.D. 2002. Lactotransferrin and transferrin: Functional variations on a common structural framework. Biochemistry and Cell Biology, 80:27-34.

Boback, P., Stratil, A. and Valenta, M. 1984. A comparison of molecular weights of transferrins of various vertebrates. Comp. Biochemistry and Physiology, 79B:113-117.

Calcagnotto, D. and Toledo-Filho, S.A. 2000. Loss of genetic variability at the transferrin locus in five hatchery stocks of tambaqui (*Colossoma macropomum*). Genetics and Molecular Biology, 23:127-130.

Christiansen, F.B. 1980. Studies on selection components in natural populations samples of mother-offspring combinations. Heriditas, 92:129-203.

Csizmadia, C., Jeney Z., Szerecses and Gorda, S. 1995. Transferrin polymorphism of some races in a live gene bank of common carp. Aquaculture, 129:193-198.

Doucet, J.P. and Trifaro, J. M. A. 1988. discontinuous and highly porous sodium dodecyl sulphate polyacrylamide slab gel system of high resolution. Analytical Biochemistry, 168**:**265-271.

Ford, M.J. 2000. Molecular evolution of transferrins: Evidence for positive selection in Salmonids. Molecular Biology and Evolution, 18:639-647.

Hara, A. 1984. Purification and some physicochemical characterization of chum salmon transferring. Nippon Suisan Gakkaishi, 50:713-719.

Hershberger, W. K. 1970. Some physicochemical properties of transferrin in brook trout. Transactions of American Fishery Society, 1970:207-218

Hirono, I. and Aoki, T. 1996. Characteristics and genetic analysis of fish transferrin. Journal of Fish Pathology, 30:167-174

Jhingran, V.G. 1991. Fish and Fisheries of India, 3rd ed. Hindustan Publishing Corporation, India.

Kirpichnikov, V.S. 1981. In: Genetic basis of fish selection (Translated by Gause, G.G.), Springer-Verlag, Berlin, Heidelberg, New York, 141 p.

Kirpichnikov, V.S. 1992. Adaptive nature of intrapopulational biochemical polymorphism in fish. Journal of Fish Biology, 40: 1-16.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685.

Lim, B.C. and Morgan, E.H. 1984. Transferrin endocytosis and the mechanism of iron uptake. Comparative Biochemistry and Physiology, 79A:317-323.

Nabi, N., Arif, S. H. and Hasnain, A. 2003. Genetic structure of natural populations of air-breathing murrel *Channa punctatus* Bloch in the Rohilkhand plains of India, Asian Fisheries Science, 16**:**77-84.

Palmour, R. M. and Sutton, H E 1971. Vertebrate transferrins: Molecular weights, chemical compositions and iron binding studies. Biochemistry, 10:4026-4032.

Ponniah, A.G. and John, G. 1998. In: Fish chromosome atlas, (eds. Ponniah, A.G. and John, G.) National Bureau of Fish Genetic Resources (ICAR), Pub.1. Special Publication, Lucknow, India.

Sahoo, P.K. and Khuda-Bukhsh, A.R. 1989. A study of plasma hemoglobin and transferrin protein pattern in three species of *Channa.* Perspectives in Cytology and Genetics, 6:69-73.

Stratil, A., Bobak, P., Valenta, M. and Tomasek, V. 1983. Partial characterization of transferrins of some species of the family Cyprinidae. Comparative Biochemistry and Physiology, 47B:603-610.

Sutton, H.E. and Karp, G.W. 1965. Adsorption of rivanol by potato starch in the isolation of transferrins. Biochim et Biophysica Acta, 107:153-155.

Teixeira, A. S., Jamieson, A. and Raposo, J. C. P. (2002). Transferrin polymorphism in Central Amazon populations of pescada, *Plagioscion squamosissimus*. Genetics and Molecular Research, 1:216-226.

Van-Doornick, D.M. and Milner, G.B. 1996. Transferrin polymorphism in coho salmon, *Oncorhynchus kisutch*, and its applications to genetic stock identification. Fishery Bulletin, 94:566-575.

Welch, S. 1990. A comparison of the structure and properties of serum transferrin from 17 animal species. Comparative Biochemistry and Physiology, 97B:417-421.

Williams, J. 1982. The evolution of transferrin. Trends in Biochemical Sciences, 7:394-397.

Winter, G.W., Schreck, C.B. and McIntyre, J.D. 1980. Resistance of different stocks and transferrin genotypes of coho salmon *Oncorhynchus kisutch* and steel-head trout. *Salmo gairdnerii* to bacterial kidney disease and vibriosis. Fishery Bulletin, 77:795-802.

Yang, L., Zhou, L. and Gui, J.F. 2004. Molecular basis of transferrin polymorphism in goldfish (*Carassius auratus*). Genetica, 121:303-313.

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