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Nucleotide Polymorphism of Green-like Visual Pigment Gene from Eyed and Blind Forms of the Mexican Tetra, *Astyanax fasciatus*

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Mexican tetra (*Astyanax fasciatus*)의 녹색 시각 색소포 유전자의 염기서열 다형화 현상

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Since the end of the Pliocene, ancestral strains of *Astyanax fasciatus* have been accidently washed into different caves at the time of flooding and have lost their eyes and body pigments. Availability of this independently derived cave fish and their ancestral form within a single species provided a unique opportunity for studying the process of molecular evolution of the visual pigment gene. The nucleotide sequence comparisons of an ancestral river fish and two cave fish showed that nucleotide polymorphism of a green-like visual pigment gene between the eyed and blind form of *A. fasciatus* was much higher than that between the same blind form. Considering the number of nucleotide substitutions per nucleotide site and the direction of the nucleotide substitutions, more nucleotide substituions between the different forms of fish rather than the same one were probably due to more frequent mutations in the eyed river form. Nucleotide substitutions per site at the intron have been ocurred more than three times faster than those at the exon. This result indicates that the functional constraint has affected the green-like visual pigment gene of the blind cave fish although its eye sight is no longer required.

Key words : Nucleotide polymorphism, Visual pigment, Mexican tetra, *Astyanax fasciatus*

Introduction

Early in vertebrate evolution, fish are crucial for studying the evolution of vertebrate color vision. Many fish contain both long and short wavelength-sensitive pigments and are known for their diverse array of visual systems depending on the environment they live (Walls, 1963). Thus, fish provide an excellent model system in studying the molecular evolution of visual pigment genes.

Molecular characterization of the visual pigment genes in fish has been initiated using Mexican tetra, *Astyanax fasciatus*. Molecular analysis of the red and green visual genes in *Astyanax* shows that fish and human lineages originally had the common

long wavelength-absorbing visual pigment gene and the duplications and evolutionary changes of the duplicated genes have occurred independently in the lineages (Yokoyama and Yokoyama, 1990b). More importantly, the data analysis strongly suggested that the red visual pigment in human and fish evolved from the green visual pigment by identical amino acid substitutions in only a few key positions (Yokoyama and Yokoyama, 1990b).

Within the last one million years, the ancestral form of *A. fasciatus* had been accidently washed into different caves at the time of flooding. During this short evolutionary time span, fish in different caves have become morphologically very different from the ancestral river fish, frequently having lost both body pigments and eyes. Some cave fish, however, have some intermediate forms in blindness and body pigmentation. Since each cave has been colonized by the ancestral form of *A. fasciatus* independently, each cave fish population has its own evolutionary history. Thus, using *Astyanax* groups from different caves and their ancestral form, the effects of different cave environments on the evolution of visual pigment genes can be evaluated. Since the sight is no longer a requirement in cave dwellers, it may be expected that visual pigment genes are not required. However, an electrophysiological study demonstrated that the pineal organ of the blind cave fish still has photosensory function of both green-like pigment and rhodopsin, having the maximum abosorption at 525 nm and 494 nm, respectively (Tabata, 1982). Thus, these genes are still functional and are mediating, and It is also possible that blind cave fish are still phylogenetically young and sufficient evolutionary changes have not occurred to make these genes non-functional. If the functional constraints were relaxed for a certain visual pigment gene in the cave environment, that gene would evolve faster than those of the surface form. Therefore, the accumulation pattern of mutation in both coding and non-coding regions (including regulatory regions) of visual pigment genes will be important for studying the evolutionary processes of these genes under both constrained and relaxed environments.

Method and Material

Species to be examined

The epigean form of *Astyanax fasciatus,* belonging to the family Characidae, were originally distributed in South America. After the land bridge had been established in late Pliocene time, *Astyanax* could reach the southern part of Texas through Mexico (Myers, 1966). The blind cave fish from the Chica cave in San Luis, Mexico, was first described as a new genus and species and named, *Anoptichthys jordani* (Hubbs and Innes, 1936). This "species" differed from its nearest relative *Astyanax fasciatus* by the absence of pigmentation and eyes. The commercial blind cave fish seem to be derived from the Chica cave (Wilkens, 1988). Despite their morphological differences and independent historical background, the cave fish and eyed *Astyanax* can interbreed one another (Sadoglu, 1956) and are therefore known as the same species, *Astyanax fasciatus.* The epigean form of *A. fasciatus* were

collected from Waller Creek in Texas. The Chica fish have been obtained from local pet stores in Champaign, Illinois.

Genomic DNA preparation

The genomic DNA of *A. fasciatus* was prepared using a modified protocol of Blin and Stafford (1976). Briefly, each fish was put in liquid nitrogen and homogenized to fine powder. The powder was transferred to a 17 x100 mm sterile polypropylene tube having 100 mM EDTA, and added 2% Sarkosyl and 100 g/ml Proteinase K, and incubated overnight at 50℃. The DNA was extracted two times with phenol, and two times with chloroform : isoamyl alcohol (24 : 1), and then precipitated with 70% ethanol. After that, DNA concentration was determined with a spectrophotometer.

Primer design and PCR amplification

Eight different oligonucleotides were constructed by using the known nucleotide sequences at non-coding regions (Fig. 1). It was necessary to avoid coding regions because of the nucleotide sequence similarity observed among different visual pigment genes. In designing the primers for PCR-amplification, 50 to 60% G+C composition was considered to improve the amplification efficiency (Innis and Gelfand, 1990). The protocol for PCR-amplification was basically followed by Saiki (1990) : about 0.1 g genomic DNA was amplified in a PCR reaction mixture (final volume of 50 μl) containing 50 μM forward and reverse primers, 1x PCR buffer, 1.25 mM dNTPs, and then 0.5 μl of *Taq* polymerase was added to the mixture. Twenty-five amplification cycles were carried out by using automated PCR. With each cycle, double-stranded DNA was denaturated by 94℃ for 2 min and the primers were allowed to anneal to their complementary sequences by briefly cooling to 55 ℃ for 90 seconds, followed by heating to 72 ℃, 2 min to extend the annealed primers with *Taq* polymerase. After the 25th cycles, an additional incubation at 72℃, 7 min was carried out to ensure that the amplified DNA is double-stranded. Amplified PCR product was extracted with 50 μl of chloroform : isoamyl alcohol (24 : 1) and then electrophoresed on a 8% agarose gel.

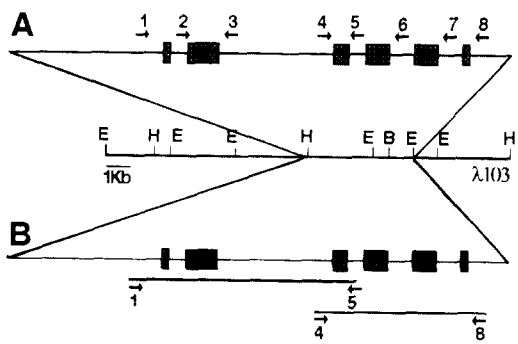

Fig. 1. The location of primers for the direct sequencing (A) and PCR amplification (B) of a green-like visual pigment gene. λ103 refers to an original genomic clone and the boxes show the locations of exons. 1 : GI1160, 2 : GI1500, 3 : GI1880, 4 : GI2407, 5 : GI2698, 6 : GI2961, 7 : GI3321, 8 : GI3953, B : *BamH*1, E : *EcoR*1, H : *Hind*3.

Direct sequencing of PCR products

The sequencing of double-stranded DNA generated by PCR using dideoxynucleotide chain termination method presents two major difficulties, namely the residual presence of primers used in PCR and template reannealing. These two problems were solved by using Gene Clean 2 Kit (from Bio 101 Inc.) and a "snap-

cooling" procedure (Casanova et al., 1990 ; Kusukawa et al., 1990), respectively. With 50 μl of PCR product, carryover primers were removed by the Gene Clean 2 protocol, and 15 μl of DNA in TE buffer was obtained. Out of 15 μl of DNA, 7 μl was mixed with 2 μl 10x sequencing buffer and 0.5 pM sequencing primer, and then boiled for 5 min. This tube was immediately placed on dry ice for 5 min. Except for reducing the sequencing reaction time, sequencing was conducted using the protocol from USB DNA Sequencing Kit.

Results and Discussions

The primers constructed for this study are shown in Table 1. Using these primers, one green-like visual pigment gene from both eyed and blind (Chica) fish has been successfully amplified (Fig. 2). The size of one fragment is about 2 Kb and it was amplified with primers, GI1160 and GI2698, whereas another one of about 1.6 Kb has been done with GI2407 and GI3953 primers. The amplified regions of two fragments covered all exons, introns and a part of 5'-flanking region. From these PCR products, direct double-strand sequencing has been accomplished

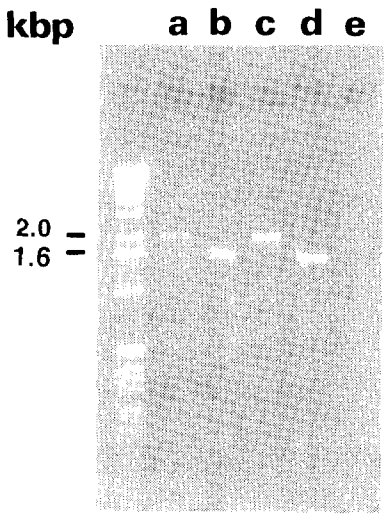

Fig. 2. PCR-amplication of green-like visual pigment gene fragments from the eyed (a,b) and blind type (c,d) of *Astyanax fasciatus*. Each sample (1 μl out of 50 μl) from amplified DNA was resolved on a 0.8% agarose gel and visualized by ethidium bromide. The primers, GI1180 and GI2698 were used for amlification in a lane a and c, whereas GI2407 and GI3953 were for b and d. Lane e was a control, in which genomic DNA was replaced by H_2O.

and clear nucleotide sequence ladders are consistently obtained. Nucleotide sequence data are readable starting from 20 to 30 nucleotides from the end of each sequencing primer. The nucleotide sequences from the whole exons and parts of introns were ob-

Table 1. Primers used for PCR

Primer #	Primers	Location	Oligonucleotides	Direction
1	GI1160	5'-flanking region	GAAGGGAGAGACTACACAAAC	Forword
2	GI1500	Intron 1	TGATTACAATATGACATGTGC	Forword
3	GI1880	Intron 2	ACTAGGAATGTCAATCATAGC	Reverse
4	GI2407	Intron 2	CCTTGCTTCCCGAAATCAATG	Forword
5	GI2698	Intron 3	AATCGCACACCTGCAATTGAA	Reverse
6	GI2961	Intron 4	TGTTGAGTGTAGTGAAAGGTT	Reverse
7	GI3321	Intron 5	CAGACACACCTGATATAACTC	Reverse
8	GI3953	3'-flanking region	CAGATGCACAGCAAACAACGG	Reverse

All sequences are written 5' to 3'. The four digit number refers to the nucleotide position originating from the start of the sequenced 5'-flanking region of GI (green-like visual pigment gene).

tained from one eyed river fish and one Chica derivative (data not shown).

For the microevolutionary study, it is necessary to know the direction of mutations because we can detect the detail process of nucleotide substitutions with it. This information sometimes provides a unique opportunity for studying the adaptive evolution (Yokoyama and Yokoyama, 1990b). In the multigene family, the present nucleotide sequence must have been derived from their ancestral sequence by gene duplications and the accumulation of mutations (Ohno, 19 70). Therefore, the rooted phylogenetic tree is very useful for the detection of patterns of the nucleotide changes. For example, there is one nucleotide substitution at the first position of the codon in exon 2 (Table 2). The phylogenetic tree (Fig. 3) including human and fish color visual pigment genes clearly indicated that the codon, GTA, of the blind cave fish has been changed to ATA in the eyed river fish because all cases exept in the eyed fish has the nucleotide G at the first position of this codon. In other words, the nucleotide change, G to A, has occurred in the eyed river fish, but not in the blind cave fish.

Using the same method mentioned, nucleotide changes and its direction were examined with the alignment of DNA sequences from one eyed river fish and one Chica derivative, as well as from another Chica derivative (Yokoyama and Yokoyama, 1990a), and it showed a small number of nucleotide differences as shown in Table 2. From the comparison of the two blind cave fish individuals, one polymorphism was found at exon 4 and two at intron 3. The nucle-

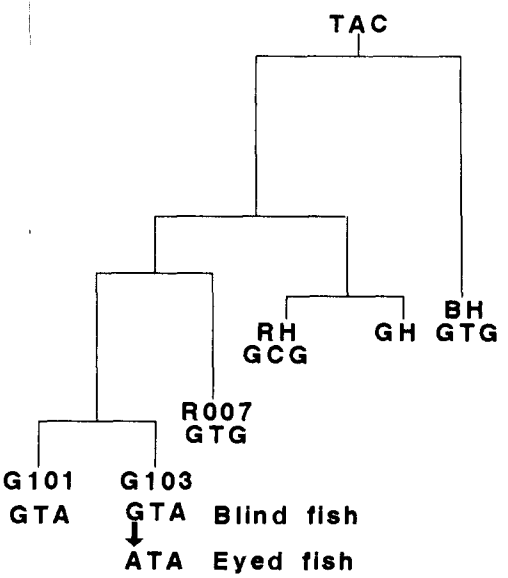

Fig. 3. Phylogenetic tree constructed for visual color pigment genes of the blind cave fish (R007, G101, and G103) and human (RH, GH, and BH) by using the number of nucleotide substitutions at all positions (d). R, G, and B refer the red-, green-, and blue-like visual pigment genes, respectively. This tree was come from Yokoyama and Yokoyama (1990b).

otide substitution at exon 3 happened at the first position of the codon, and thus does not change the amino acid. Considering the types of nucleotide changes within the eyeless form, all three differences were caused by the transitional change. On the other hand, when the eyed and eyeless forms of *A. fasciatus* were compared, the higher level of polymorphism has been detected at both exons and introns and all nucleotide changes were transitional except one case of A to C at intron 3. Interestingly, no mutations have been detected at exon 1, 5 and 6. In particular, exon 5 has been known to include important residues for specific wavel-

Table 2. Comparisons of nucleotide sequences between the different forms of *A. fasciatus*

Fish Forms	5'-flanking region	Exon 2	Eon 3	Intron 3			Exon 4	
				1	2	3	1	2
Eyed form	C	A (Isoleucine)	C	C	T	C	C	T
Blind 1	T	G (Valine)	T	C	C	A	T	C
Blind 2	T	G (Valine)	T	T	T	A	C	C

Nucleotide sequence data of Blind 2 were come from Yokoyama and Yokoyama (1990a).

ength absorption (Nathans et al., 1986 ; Yokoyama and Yokoyama, 1990b). However, it is not obvious whether this observation was due to the strong functional constraint at this domain or the small sample size. Furthermore, one nonsynonymous substitution has been detected at exon 2 as mentioned above, encoding isoleucine in the eyed fish and valine in the blind fish. However, both are nonpolar hydrophobic amino acids and thus it is not likely affect the function of the visual pigment.

With these nucleotide sequence data, the simple statistical analysis has been accomplished to know whether or not the functional constraint has affected one green-like visual pigment gene. For this purpose, the number of nucleotide substitutions per nucleotide site (d) was estimated using the method of Jukes and Canter (1969). The results are shown in Table 3. If there is the functional constraint at a gene, we can expect that the ratio of λ_e/λ_i would be less than 1, where λ_e and λ_i refers the rate of nucleotide substitution per site per year at the exons and the introns, respectively. If it is not, the ratio would be approximately 1. As a result of the estimation, the ratio (λ_e/λ_i) has been turned out approximately 0.33 when the mean value of d was used. In other words, the nucleotide substitutions at the intron have been occurred more than three times faster than those at exon. This indicates the functional constraint has affected the green-like visual pigment gene we studied.

Table 3. The number of nucleotide substitutions per nucleotide site(d) at the exons and introns of one river fish and two blind cave fish

Fish form	Eyed	Blind 1	Blind 2
Eyed		3.77(1065)	2.82(1065)
Blind 1	10.64(284)		0.94(1065)
Blind 2	9.29(325)	4.65(431)	

Values above the diagonal are from the exons ; values below the diagonal from the introns. Values in the parenthesis indicate the number of nucleotides compared.
Unit of d is $x10^{-3}$

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References

Blin, N. and D.W. Stafford, 1976. A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acid Res., 3 : 2303-2308.

Casanova, J.-L., C. Pannetier, C. Jaulin and P. Kourilsky, 1990. Optimal conditions for directly sequencing double-stranded PCR products with sequenase. Nucleic Acids

Res., 18 : 4028.

Hubbs, C.L. and W.T. Innes, 1936. The first known blind fish of the family Characidae : A new genus from Mexico. Occ. Pap. Mus. Zool. Univ. Mich., 343 : 1-7.

Innis, M.A. and D.H. Gelfand, 1990. Optimization of PCRs. *In* PCR Protocols : A guide to methods and application. Innis, M.A., D. H. Gelfand, J.J. Sninsky and T.J. White, ed., Academic Press, NY.

Jukes, T.H. and C.R. Canter, 1969. Evolution of protein molecules. *In* H.N. Murano, ed., Mammalian protein metabolism, pp. 21-132. Academic Press, NY.

Kusukawa, N., T. Uemori, K. Asada and T. Shuzo, 1990. Rapid and reliable protocol for direct sequencing of material amplified by the polymerase chain reaction. BioTechniques, 9 : 66-72.

Myers, G.S., 1966. Derivation of the freshwater fish fauna of Central America. Copeia, 1966 : 766-773.

Nathans, J., T.P. Plantanida, R.L. Eddy, T.B. Shows and D.S. Hogness, 1986. Molecular genetics of inherited variation in human color vision. Science, 232 : 203-210.

Ohno, S., 1970. Evolution by gene duplication. Berlin, Springer-Verlag.

Sadoglu, P., 1956. A preliminary report on the genetics of the Mexican cave characins. Copeia, 1956 : 113-114.

Saiki, R.K., 1990. Amplification of genomic DNA. *In* PCR Protocol : A guide to methods and application. Innis, M.A., D.H. Gelfand, J.J. Sninsky and T.J. White, ed., Academic Press, NY.

Tabata, M., 1982. Persistence of pineal photosensory function in blind cave fish, *Astyanax mexicanus*. Comp. Biochem. Physiol., 73A : 125-127.

Walls, G.L., 1963. The vertebrate eye and its adaptive radiation. Hafner Publishing Company. NY.

Wilkens, H., 1988. Evolution and genetics of epigean cave *Astyanax fasciatus* (Characidae, Pisces) : Support for the neutral mutation theory. Evol. Biol., 23 : 271-398.

Yokoyama, R. and S. Yokoyama, 1990a. Isolation, DNA sequence and evolution of a color visual pigment gene of the blind cave fish, *Astyanax fasciatus*. Vision Res., 30 : 807-816.

Yokoyama, R. and S. Yokoyama, 1990b. Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus*, and human. Proc. Natl. Acad. Sci. USA, 87 : 9315-9318.