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Genetic Divergence and Speciation of Eurasian and American Yellow Perch Based on the Nucleotide Sequence of Cytochrome *b* Gene

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Eurasian yellow perch (*Perca fluviatilis*) and American yellow perch (*Perca flavescens*) are known to be endemic species in Eurasia and North America, respectively. The presence of endemic species on each continent suggests their independent evolutionary history. However, because of the morphological similarity, distribution pattern, and only recent fossil record, their divergence time and speciation of the two *Perca* species has long been controversial. Here, from the comparison of the entire nucleotide sequences of cytochrome *b* gene, large genetic divergence between the two *Perca* species is observed although they are morphologically similar each other. Among 1,140 base pairs, interspecific nucleotide differences are found at 130 sites (11.4%). The differences varies with codon position, showing 22 sites in the first, 5 sites in the second, and 103 sites in the third codon position. Considering the types of nucleotide changes, transitional differences are much more than transversional differences and its ratio turned out to be 5.19. The estimated divergence time of the two *Perca* species indicates that they were separated each other approximately in the late Miocene period, which implies the long history of speciation. With comparison of the inferred amino acid sequences, strong structural and functional constraints which seem to be maintained by the highly conservative amino acid residues or protein regions, as found in other taxonomic groups of organisms, are also recognized in the cytochrome *b* of the fishes examined.

Key words : genetic divergence, *Perca*, cytochrome *b*, nucleotide sequence

Introduction

Mitochondrial DNA (mtDNA) is a single, duplex (light and heavy chain), closed-circular, maternally (clonally) inherited molecule that appears to have much less frequent sequence rearrangement than does nuclear DNA (Brown, 1983). Infrequent rearrangement of mtDNA together with its rapid rate of base substitutions has made animal mtDNA popular in genetical, populational, and evolutionary studies at and below the species level (Greenberg et al., 1983; Ferris et al., 1983). Early evolutionary studies using mtDNA were done using restriction endonucleases (Brown et al., 1979) because conventional cloning and sequencing were difficult and extremely time-consuming. Recently, the polymerase chain reaction (PCR) method was developed (Saiki et al., 1985) and information on complete base sequences from the human (Anderson et al., 1981), cow (Anderson et al., 1982), mouse (Bibb et al., 1981), *Xenopus* (Roe et al., 1985), and *Drosophila* (Clary and Wolstenholme, 1984) has been accumulated. Sequence data on these organisms allow us to design primers necessary for PCR amplification in other groups of organisms and thereby to extend the studies using nucleotide sequences.

From studies on various multicellular animals, it is known that the animal mitochondrial genome has 37 genes, including 22 tRNAs, 13 mRNAs, and 2 rRNAs (de la Cruz et al., 1984). Among the 13 protein-encoding genes, the cytochrome *b* gene encodes a protein involved in complex III of the mitochondrial oxidative phosphorylation system that is incorporated

into the inner membrane of the mitochondrion (Hatefi, 1985). Because of its important function related to energy production, cytochrome *b* is one of the structurally and functionally best-characterized protein. Such detailed information has the potential to provide insight into the evolutionary history of the gene. In addition, the nucleotide sequences in the cytochrome *b* gene appeared to be highly conserved and easily aligned between even very distantly related species, presumably because of its structural and functional constraints at the protein level. For example, sequence data from a sturgeon (Brown et al., 1989), mammals (Irwin et al., 1991), and even *Drosophila* (Clary and Wolstenholme, 1984) are easily aligned with those from the teleost. Unambiguous sequence alignment is an important prerequisite for nucleotide sequence analysis. Because of its advantages, the cytochrome *b* gene has been used in evolutionary studies on placental mammals (Kocher et al., 1989), marsupials (Thomas et al., 1989), birds (Kocher et al., 19 89), lizards (Hedges et al., 1991), and fishes (Meyer et al., 1990; Normark et al., 1991). However, only in the study on the placental mammals was the entire cytochrome *b* gene sequenced. In the other studies only 300~400 base pairs were sequenced and used.

Perca flavescens (American yellow perch) is widely distributed in North America, whereas *Perca fluviatilis* (Eurasian yellow perch) is distributed throughout temperate regions of Eurasia (Craig, 1987). The specific distinctiveness of *P. flavescens* from *P. fluviatilis* is controversial because morphologically they have been diagnosed by only slight differences in body shape and in the position of the predorsal bone (Collette and Banarescu, 1977). Due to their morphological similarity and distribution on two continents, several hypotheses about their biogeographic history have been proposed (Cihar, 1975). Therefore, the comparative study has been performed using the entire cytochrome *b* nucleotide sequences to know the genetic divergence and specific distinctiveness of the two *Perca* species.

Materials and Methods

Isolation of total DNA

DNA isolation followed the modified protocol of Lee and Taylor (1990) because their method was much simpler than other method reported by Blin and Stafford (1976) and it can be performed in microcentrifuge tubes at room temperature. For DNA isolation, 700 μl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, and 3% SDS) was added to a 1.5 ml eppendorf microcentrifuge tube which was filled with about 0.1 g ground tissue that had been frozen in liquid nitrogen, and the mixture was vortexed until homogeneous. After incubating the tube at 65℃ for 1 hour, 700 μl of chloroform : phenol (1 : 1) was added and the mixture was vortexed for about 10 seconds. The tube was microcentrifuged at 12,000 rpm at room temperature for 10 minutes or until the aqueous phase was clear. Then, 10 μl of 3 M NaOAc and 0.54 volumes of isopropanol were added to the aqueous phase in the new tube, which was gently inverted and microcentrifuged at 14,000 rpm for 6 minutes. After pouring off the supernatent, the DNA pellet was rinced once with cold 70% ethanol, dried, and resuspended in 50 μl of distilled water. After the DNA pellet was completely dissolved, 2 μl of DNA and 1 μl of DNA marker (1 Kb ladder, 50 ng/μl) were electrophoresed on 0.8 percent agarose gel. The DNA concentration was adjusted for PCR amplification.

Primer design and PCR amplification

Primers for amplification were designed to correspond to conserved regions of mitochondrial tRNA genes adjacent to the cytochrome *b* gene that have been identified in humans (Anderson et al., 1981), mammals (Kocher et al., 1989), cichlid fishes (Meyer et al., 1990), and the common carp (Araya et al., 1984). From comparisons among the different organisms (some nucleotide variation occurs even at highly conserved regions of the tRNA gene), degenerated primers for PCR amplification were designed first and

then refined to the specific primers based on sequencing results obtained from using vector sequencing primers (M13 or SK primer). The entire cytochrome *b* gene and parts of the tRNA regions were amplified more efficiently with the specific primers than the degenerate primer. The primers used for the amplification of the entire cytochrome *b* gene were H14724F and L15915R and the details of primer sequences have been reported in Song (1994).

The protocol for PCR amplification is basically that of Saiki (1990): about 0.1 μg total DNA is used in a reaction mixture (final volume of 25 μl) containing 1 μM each of forward and reverse primers, 1X PCR magnesium-free buffer, 2 mM magnesium chloride, 0.4 mM each of the dNTPs, and 0.2 μl of Taq DNA polymerase (1 unit, Promega). Twenty-nine amplification cycles were performed using an automated DNA thermal cycler (Perkin-Elmer). The temperature conditions for amplification were: 90℃, 1 min. for DNA denaturation, 42℃, 1 min. for primer annealing, and 72℃, 2 min. for primer extension, plus a final extension at 72℃ for 7 min. For each amplification, the possibility of PCR contamination was checked with a negative control. Amplification efficiency was monitored by electrophoresing 10 μl of PCR product through a 0.8% agarose gel.

Cloning the PCR product

Depending on the efficiency of the PCR reaction, 150 to 250 μl of pooled PCR products were used for cloning. The remaining primers and non-specific products were removed by low-melting temperature agarose gel isolation using the Gene Clean II Kit (Bio 101, Inc). Isolated DNA was treated with the enzyme Klenow Fragment (Promega) for 15 min. at 30℃ to make the DNA completely blunt-ended. After ethanol precipitation of the blunt-ended DNA with the pBluscript SK$^-$ vector, the combined DNA was ligated with T4 DNA ligase at 15℃ for over 16 hours. To transform the ligated DNA into XL1-Blue competent cells, both the ligated DNA and competent cells were heat-shocked for 90 seconds at 42℃ in the same tube and then they were grown in LB media for 45 min. at 37 ℃. Transformed colonies were screened on LB plates containing ampicillin, X-gal, and IPTG. Mini-prep DNA of potential clones was then digested with restriction enzymes, *Eco*RI and *Xho*I, to check for the inserted fragment of the expected size. About 5 percent of the total colonies usually appeared to have the right inserted fragments using this method.

DNA sequencing

The sequencing reaction was conducted using a USB sequenase version 2.0 kit according to the manufacturer's protocol. M13 universal and SK primers were used for double-stranded sequencing, and SK primer for single-stranded sequencing. For subsequent sequencing, internal sequencing primers were designed based on the sequencing alignment of preliminary data. Saiki et al. (1988) reported that cloned materials amplified via PCR typically contain mutations. To detect the presence of PCR and cloning artifacts, I initially sequenced three independent clones from each of two species as recommended by Irwin and Wilson (1990).

Results

Comparison of cytochrome *b* nucleotide sequences

DNA fragments about 1.2 Kb in length, including the entire cytochrome *b* gene and portion of the two tRNA genes for glutamic acid and Threonine, were amplified and sequenced (Fig. 1). The number of nucleotide differences (less than 0.2%) between clones within an individual was less than the typical error frequency in PCR amplification reaction (about 0.25 %), and all were transition changes. If differences existed in the nucleotide sequences in the two orientations, another clones were sequenced additionally to determine the real sequence. No size difference in

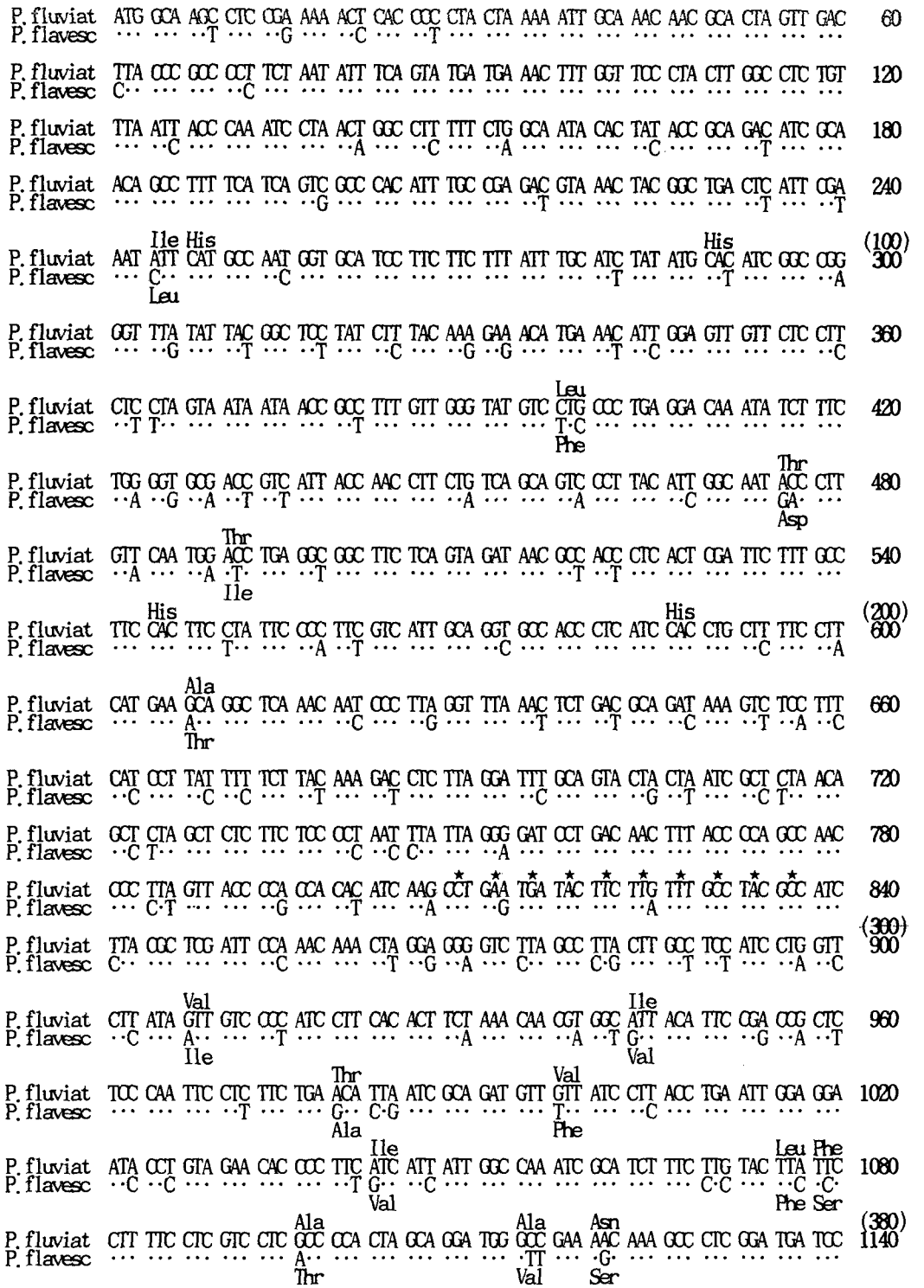

Fig. 1. The nucleotide sequences for the cytochrome *b* gene of the two *Perca* species. The positions either showing amino acid differences or being functionally important are depicted by the three-letter amino acid codes. The number in the parenthesis reveals amino acid position and ★ indicates the PEW region.

the cytochrome *b* gene was observed between two *Perca* species. Among 1,140 base pairs, interspecific nucleotide difference were found at 130 sites (11.4%) revealing both transitional and transversional mutations. These differences varied with codon position, showing 22 sites in the first, 5 sites in the second, and 103 sites in the third codon position. Number of transitional differences (109 sites) were much larger than that of transversional differences (21 sites) and its ratio turned out to be 5.19. As shown in Table 1, the transitional nucleotide changes between A and G occurred at 33 sites, and those between T and C at 76 sites, whereas the transversional changes between A and T, A and C, T and G, C and G were 9, 7, 2, and 3 sites, respectively.

Table 1. The number of nucleotide substitutions depending on its type and codon position

Codon position	Transition		Transversion			
	T-G	A-G	A-T	A-C	T-G	C-T
First	13	7	–	1	1	–
Second	3	1	–	1	–	–
Third	59	25	9	6	1	3

Base compositions

Base compositions and bias indices of compositional differences in the cytochrome *b* gene of two *Perca* species examined are shown in Table 2. The bias index was calculated following the method of Prager and Wilson (1988). There were not much difference in the base composition between two *Perca* species. However, considering mean bias indices calculated from the different codon positions, the first codon position showed a relatively unbiased composition, with a bias index of 0.032. The second and third positions had relatively high biases, with indices of 0.215 and 0.261, respectively. Biases from the expected frequency of 25% resulted mainly from high frequencies of T and low frequencies of G at the second codon position, and from high frequencies of C and low frequencies of G at the third position.

Putative cytochrome *b* protein sequence

The predicted translation products were 380 amino acid in length, beginning with a conserved initiating ATG methionine codon and putatively terminating with an TGC stop codon which will be reported elsewhere. Among 380 amino acid translated using the mammalian mitochondrial genetic code, there were 15 differences (3.9%) between two *Perca* species (Fig. 1). The only amino acid change of electrical charge were observed at residue 159, where threonine, polar amino acid having uncharged R group, was altered to the strongly and negatively charged amino acid, aspartic acid. There were, however, no charge differences found at the rest of changes. *Perca* cytochrome *b* protein possessed the heme-ligating his 83 and his 182 residues that was determined from the comparative studies using the several organisms such as trypanosome, spinach, *Rhodobacter,* wheat, yeast, *Drosophila,* and mouse (Howell, 1989), as well as his 97 and his 196 residues. In addition, there was the PEW region containing the invariant amino acid triplet proline-glutamic acid-tryptophan at the positions 270-272.

Table 2. Comparison of base compositions and bias indices at three different codon positions

Species	First				Second				Third			
	A	T	C	G	A	T	C	G	A	T	C	G
P. fluviatilis	23.2	25.5	24.2	27.1	19.7	40.2	25.8	13.9	29.5	25.5	39.7	5.3
P. flavescens	22.6	25.0	25.3	27.1	19.7	40.8	25.3	14.2	29.7	26.1	38.7	5.5
Mean	22.9	25.3	24.7	27.1	19.7	40.5	25.5	14.1	29.6	25.8	39.2	5.4
Bias	0.032				0.215				0.261			

Discussion

Lacking fossil evidence, several hypotheses on the divergence time of percid fishes have been offered based on comparative anatomy, distribution patterns, and geological events. From his study on the anatomy of scales, McCully (1962) suggested that percids originated from anadromous serranids (including species now in Moronidae) during the Cenozoic somewhere in Europe or North America. About the time and routes of invasion of *Perca* species from Eurasia to North America, because of the morphological similarity of two *Perca* species, their distribution pattern, and the presence of only recent fossil records, Cichar (1975) suggested that *Perca* species invaded from Europe through the Atlantic in brackish water along the thawing Arctic ice during the late Pleistocene or early Holocene, 12 to 15 thousand years ago. In North America, the earliest fossils of *Perca* were from Pleistocene deposits in McPhaerson County, Texas (Semken, 1966) and Beaver County, Oklahoma (Smith, 1954). In Eurasia, fossils of *Perca* have been dated from the Eocene to recent. However, Cavender (1986) has questioned the identification of the Eurasian fossils and stated that at least some of the fossils clearly are not *Perca*. Later, Collette and Banarescu (1977) hypothesized that species of *Perca* and *Stizostedion* subsequently dispersed from Siberia to North America via a North Pacific route in the Neogene.

From the comparison of nucleotide sequences and the fossil records of centrarchids and moronids, the divergence rate of transversion differences at the third codon position to the total number of nucleotide (1,140 bp) of these fishes was estimated to be 0.22% per one million years (Song, 1994). Since sequence divergence between two *Perca* species was 1.7% considering transversional changes at the third codon position, two *Perca* species might be separated each other approximately 8 million years ago (late Miocene), and this result thus did not support Cichar's hypothesis regarding to the divergence time of two *Perca* species. This estimate is similar to the estimate by Billington et al. (1991) for the separation of Eurasian and North American species of *Stizostedion*. They reported that divergence times were consistent from the data obtained from allozyme and from mtDNA restriction endonucleases in showing that the North American *Stizostedion* was separated from their European relatives approximately 10 million years ago. If this estimate is accepted, Eurasian *Perca* and *Stizostedion* might have invaded North America during the similar time period, as suggested by Collette and Banarescu (1977).

The base-compositional heterogeneity of vertebrate DNA, particularly in the third codon position, has been recognized in different species and in different genes (Sueoka, 1988). However, as the closely related species has been examined in this study, such heterogeneity was not detected between two *Perca* species. In comparing data from the fishes examined to those from the cytochrome *b* gene in mammals (Irwin et al., 1991), some major differences were found in the compositional heterogeneity. In mammals, the third position has the strongest bias showing 0.401, whereas those in the first and second codon position were 0.096 and 0.221, respectively. However, in the fishes examined the bias at the third position was not much different from that for the second position (Table 2). Comparing mean base composition in the third codon position of the two *Perca* species with that of mammals, there were some differences such as C (39.2%) >A (29.6%)>T (25.8%)>G (5.4%) in the two *Perca* species, and A (42.7%)>C (37.4%)>T (16.3%)>G (3.6%) in mammals.

According to the eight domain structural model of the cytochrome *b* (adapted from Howell 1989), most of the variable positions were located within the transmembrane segments, or at the amino acid and carboxy ends of the protein when the sequences of less than 5 to about 80 million year divergence time in mammals were compared (Irwin et al., 1991). The similar result was obtained in this study except that

no differences were observed at the amino acid end of the protein. There were 2 differences in the outer surface, 3 in the inner surface, 8 within the transmembrane segments, and 2 at the carboxy end of the protein. Regions that have been defined as Q_o and Q_i redox center by mutational studies (Howell and Gilbert, 1988) or by distant evolutionary comparisons (Howell, 1989) were very conservative in the cytochrome *b* of the two *Perca* species. No amino acid changes were detected at the Q_i redox center and the first and third outer surface segments of Q_o redox center, whereas two changes (residues 159 and 164) were found at the second outer surface segment of the Q_o redox center and this region thus appeared to be relatively less conservative. The region of residue 150 to 170 was also recognized as intermediately conservative in the mammalian cytochrome *b* protein (Irwin et al., 1991).

In the PEW region (residue 270 through 279) of cytochrome *b* of the two *Perca* species, which had known to function in electron transport to the low potential b566 heme group (Howell, 1989), there were amino acid triplet (proline-glutamic acid-tryptophan), five aromatic residues, and hydrophobic amino acids as found in other organisms. As this region was consisted of a highly conservative stretch of amino acids across the different taxomonic groups of organisms and also existed in the fishes examined, it might serve such an important function mentioned above in the teleost.

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