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Phylogenetic Relationships among Groupers (Genus *Epinephelus*) Based on Mitochondrial Cytochrome *b* DNA Sequences

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To infer phylogenetic relationships among *Epinephelus* species inhabiting coastal regions of Korean peninsula, mitochondrial cytochrome *b* genes from 9 species belonging to the subfamily Epinephelinae were PCR-amplified, cloned and sequenced. Aligned cytochrome *b* sequences of 10 species containing one additional sequence from GenBank were 1,140 base pairs in length, including 439 variable and 330 parsimony informative sites. The cytochrome *b* genes of 10 species, as other vertebrates studied to date, exhibit unequal base compositions: an entirely low G content (15.2±0.3% on average) and almost equal T, C and A contents (29.3±0.8%, 30.7±1.0%, and 24.8±0.5% on average, respectively). In third codon positions, transitional substitutions especially between *Epinephelus* species and outgroup species are almost certainly saturated or near saturation. Phylogenetic analyses were performed with sequence data from 8 *Epinephelus* species and 2 outgroup species (*Cephalopholis urodela* and *Variola louti*) by using distance-based (neighbor-joining and minimum evolution) and parsimony-based (maximum parsimony) methods. The results showed that the monophyly of the genus *Epinephelus* was supported by relatively high bootstrap values. However, phylogenetic relationships among *E. areolatus*, *E. moara*, *E. septemfasciatus*, and *Epinephelus* sp were poorly resolved. Within the genus *Epinephelus*, three resolved monophyletic groups were found: clade 1 included *E. akaara* and *E. awoara*; clade 2 included *E. fasciatus* and *E. merra*; and clade 3 included *E. akaara*, *E. awoara*, *E. fasciatus*, *E. merra*, *E. areolatus*, *E. moara*, *E. septemfasciatus* and *Epinephelus* sp.

Key words: Phylogenetic relationships, Groupers, *Epinephelus*, Cytochrome *b* DNA sequence

Introduction

The subfamily Epinephelinae of the family Serranidae consists of approximately 159 species of marine fishes in 15 genera, commonly known as groupers, rockcods, hinds, and seabasses (Heemstra and Randall, 1993). The majority of these groupers live in almost all tropical and temperate seas of the world and exhibit a wide variety of reproductive strategies (Heemstra and Randall, 1993; Morris et al., 2000).

Grouper species are identified by their color pattern and (or) morphological characters such as configuration and size of the fins, the number of fin rays, scales and gill rakers (Heemstra and Randall, 1993). However, it is sometimes difficult to identify grouper species visually because of their wide distribution and color variation (Nugroho et al., 1998).

Relatively few studies have been published on the evolution and phylogenetic relationships of the family Serranidae (Gosline, 1966; Leis, 1986; Johnson, 1983, 1988; Kendall, 1984; Baldwin and Johnson, 1993), and these studies have proposed different hypotheses. Until a formal definition of the family was proposed by Gosline (1966), the Serranidae had historically served as a classificatory wastebasket within the percoids (Johnson, 1983).

Gosline (1966) removed a number of genera and subfamilies from the Serranidae on the basis of several shared morphological features and restricted the family to three subfamilies: Serraninae, Anthiinae, and Epinephelinae. Gosline's classification was later supported and refined by Johnson (1983). He further divided the Epinephelinae into five tribes: Niphonini, Epinephelini, Diploprionini, Liopropomini, and Grammistini. On the basis of derived morphological character (absence of an autogenous distal radial on the first dorsal pterygiophore), Johnson (1983) proposed that these five tribes constitute a monophyletic subfamily Epinephelinae. This monophyly of the subfamily Epinephelinae was later supported by Baldwin and Johnson (1993) who studied the phylogeny of the Epinephelinae on the basis of cladistic

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analysis of larval and adult morphology.

Heemstra and Randall (1993) proposed an alternative classification and divided the family Serranidae into five subfamilies such as Serraninae, Anthiinae, Niphoninae, Epinephelinae, and Grammistinae (including the tribe Diploprionini, Liopropomini, and Grammistini). In addition, they further classified the tribe Epinephelini into fifteen genera: *Aethaloperca*, *Alphestes*, *Anyperodon*, *Cephalopholis*, *Cromileptes*, *Dermatolepis*, *Epinephelus*, *Gonioplectrus*, *Gracila*, *Mycteroperca*, *Paranthias*, *Plectropomus*, *Saloptia*, *Triso* (=*Trisotropis*), and *Variola*.

However, these studies focused on relationships at high hierarchical level within the family Serranidae based on morphological characters. Very little is known about its phylogenetic relationships among the *Epinephelus* species. Thus, with mitochondrial cytochrome *b* DNA sequences, we examined phylogenetic relationships among eight *Epinephelus* species by using two representative species within the tribe Epinephelini (*Cephalopholis urodela* and *Variola louti*) as outgroup taxa.

Materials and Methods

Specimens

Details of the species used in this study are presented in Table 1. Specimens were collected from various sources and identified to species level by following Chyung (1977) and Heemstra and Randall (1993). Liver or underlying muscle tissue were removed from each individual avoiding cross-contamination, frozen in liquid nitrogen and stored at -80℃. Four species of groupers were kindly provided by Sigeo Nakamura, technical officer of Tropical Biosphere Research Center, University of the Ryukyus, Japan.

Total DNA extraction

Total DNA preparation was extracted from 25-40 mg of liver or underlying muscle tissue with a QIAamp DNA Mini Kit (Qiagen Inc.) according to the manufacturer's protocol. Total DNA preparation resuspended in Buffer AE (Qiagen Inc.) was directly used for PCR experiments. The concentration of DNA purified by the QIAamp procedure was determined by loading at 0.8% agarose gel or by measuring the absorbance at 260 nm using a Unicam UV/VIS Spectrometer (Heλiosβ, Unicam Ltd., UK). The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm.

PCR amplification

The entire cytochrome *b* genes including parts of tRNA regions (the 3' end of the glutamine transfer RNA, the complete threonine transfer RNA, and the 5' end of the proline transfer RNA) were PCR-amplified with the flanking tRNA primers, Glu-F (GTT GTH RTT CAA CTA CAA RAA) and Pro-R (TAG AAT YYT RGC TTT GGG AG). The PCR reactions were performed in a final volume of 50 μL in 0.5 mL PCR tubes containing 2 μL of genomic DNA (0.1-0.3 μg of DNA), 1 μM each of forward and reverse primers, 2 μL of 100 mM $MgSO_4$, 5 μL of 10× reaction buffer, 1 μL of PCR Nucleotide Mix (containing the sodium salts of dATP, dCTP, dGTP, and dTTP, each at a concentration of 2.5 mM in water, Promega Co.), and 0.5 to 1 unit of *pfu* polymerase (Stratagene Co.). Approximately 2 drops of mineral oil were added prior to the initiation of cycling to serve as an evaporation barrier. The amplifications

Table 1. List of species of the subfamily Epinephelinae to be examined

Genus	Scientific name	Abbreviated species name	Collection locality	GenBank Accession No.
Epinephelus	*Epinephelus akaara*	Eaka	Jeju, Korea	AY786420
	Epinephelus awoara	Eawo	Jeju, Korea	AY786422
	Epinephelus fasciatus	Efas	Jeju, Korea	AY786423
	Epinephelus moara	Emoa	Jeju, Korea	AY786427
	Epinephelus septemfasciatus	Esep	Jeju, Korea	AY786425
	Epinephelus areolatus	Eare	Okinawa, Japan	AY786421
	Epinephelus merra	Emer	Okinawa, Japan	AY786424
	Epinephelus sp.	Esp[1]		AF143193
Cephalopholis	*Cephalopholis urodela*	Curo	Okinawa, Japan	AY786426
Variola	*Variola louti*	Vlou	Okinawa, Japan	AY786428

[1]The cytochrome bsequence of Esp was reported by Allegrucci et al. (1999).

were performed in a Programmable Thermo Controller (PTC-100, MJ Research Inc.). A typical PCR cycle consisted of an initial denaturing step of 94℃ for 2 min, followed by 30 cycles of 45 s at 94℃ for denaturation, 1 min at 43℃ for primer annealing, and 2.5 min at 72℃ for primer extension, and an additional 7 min interval at 72℃ for a final extension. The size of PCR products was checked against a 1 kb DNA ladder (MBI Fermentas) in a 0.8% agarose (Agarose LE, Promega Co.) gel stained with 0.5 μg /mL ethidium bromide in 1× TBE buffer (0.045 M Tris-borate, 0.045 M boric acid, 0.001 M EDTA, pH 8.0). The presence of a single bright band in each lane of the gel was a check for a successful amplification. The initial PCR product was purified by electrophoresis on a low-melt gel (LMP agarose, Bothesda Research Laboratories; stained with ethidium bromide). The gel slice that contained the desired band was cut out as small as possible and purified using the GeneClean II Kit (Bio 101 Inc.) following the manufacturer's instructions. The purified initial PCR product was used for the next cloning step.

Cloning and sequencing of the PCR product

E. coil (DH5) and pBluescript II SK^- were used as a host and a vector for cloning of the PCR products. 1 μg of vector pBluescript II SK^- (Stratagene) was digested with the restriction enzyme, *Hinc* II at 37℃ for 2 hr. The digested vector was purified using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals) according to the supplier's protocol. After purification, the concentration of purified product was determined on an agarose gel. Ligation was carried out in 20 μL reaction mixtures containing 1 μL of pBluescript II SK^- vector, 1.5 to 2 μL of insert DNA, 4 μL of 5× ligation buffer, and 1 unit of T4 DNA ligase (Life Technologies Ltd.). The mixture was incubated at 14℃ for 24 hr. The ligated products mixed with DH5α competent cells were heat-shocked for 1.5 min at 42℃ and then transformed by incubating with shaking the mixtures for 30 min at 37℃. The *E. coli* cells were spread on Luria-Bertani (LB) agar containing ampicillin, X-gal, and IPTG and incubated overnight at 37℃. Completely white colonies were inoculated into 4 mL LB broth containing ampicillin and grown overnight at 37℃ with shaking. For the double-stranded sequencing, plasmid DNA was collected from overnight culture containing potential clones and isolated using a High Pure Plasmid Isolation Kit (Roche Molecular Biochemicals). To verify the success of cloning and transformation, plasmid DNA was run in a 0.8% agarose gel.

DNA sequencing was performed using an SEQ4X4 personal sequencing system (Amersham Pharmacia Biotech) with a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) according to the supplier's guide. T7 (GTA ATA CGA CTC ACT ATA GGG C) and SK (CGC TCT AGA ACT AGT GGA TC) primers were used to obtain first 5' and 3' end of sequence information and 16 internal primers (data not shown) were designed and used for accurate internal sequencing.

Data analysis

Segenerated from individual primers were verified by comparing the sequences obtained automatically by the computer program with the sequences modified with inspection by eye from the profile of fluorescent peaks, and assembled into a continuous sequence using the DNAssist program (shareware, version 2.2, Patterton and Graves, 2000). In addition to sequences investigated for this study, an additional sequence from GenBank was used (see Table 1 for Accession No.).

Base composition and patterns of substitution for pairwise comparisons were computed using MEGA version 2.1 (Kumar et al., 2001). Saturation assessments were performed at each codon position by plotting numbers of transitions (Ts) and transversions (Tv) separately against *p*-distance values (Song et al., 1998; Nei and Kumar, 2000). To recognize saturated data, two outgroups were included in these analyses. Assessments of saturated data in graphs were conducted according to the guide proposed by Griffiths (1997).

Phylogenetic analyses were carried out using both distance-based and parsimony-based methods with the program MEGA and the program PAUP (version 4.0b8, Swofford, 1998), respectively. Distance analyses were conducted according to the tree-building algorithm of neighbor-joining (Saitou and Nei, 1987; NJ) and minimum evolution (ME) methods with Kimura's (1980) two-parameter and Tamura and Nei's (1993) distances. To filter the noise generated from the saturated data, analyses were performed with two data sets: Tv only at all three codon positions, and all substitutions at the first and second codon positions. In the parsimony analysis, the phylogenetic tree was constructed by the maximum-parsimony (Fitch, 1971; MP) method. The heuristic search option of parsimony algorithms was used for finding the

shortest tree. Heuristic searches were carried out using the tree bisection-reconstruction (TBR) method of branch swapping. Firstly, all data were weighted equally. For the next analyses, characters were weighted differentially: all substitutions unweighted, excluding third codon positions, and Ts and Tv weighted in all three codon positions (Tv:Ts=3:1 and 50:1). *Cephalopholis urodela* and Variola louti were used as outgroups to root the trees. Bootstrappings (Felsenstein, 1985) of 1000 replications were performed to evaluate statistically the strength of support for each internal node in resulting trees. Bootstrap analyses were conducted with MEGA for the NJ and ME methods and with PAUP for the MP method.

Results and Discussion

Sequence variation, base compositional bias, and saturation

Aligned cytochrome *b* sequences of 10 species containing an additional sequence were 1,140 base pairs in length, including 439 variable and 330 parsimony informative sites. The partial mitochondrial gene order investigated in this study was identical to that of the common vertebrate: $tRNA^{Glu}$-Cyt*b*-$tRNA^{Thr}$-$tRNA^{Pro}$ (data not shown). As expected, third codon positions were more variable than first or second codon positions. The predicted translation products for the cytochrome *b* genes of nine species studied in this study are 380 amino acids in length, beginning with an initiation codon (ATG) and terminating with a TAA termination codon or an AGA termination codon. Based on the transcriptional processing and polyadenylation model for termination of translation (Anderson et al., 1981), we predicted that transcripts of these cytochrome *b* genes ended in U or AG which became termination codons (UAA or AGA) by polyadenylation.

Base compositions, the number of transitions and transversions in cytochrome *b* sequences of 10 species containing an additional species are shown in Table 2 and Table 3, respectively. Table 2 shows that the cytochrome *b* genes of 10 species, as other vertebrates studied to date (Johns and Avise, 1998), exhibit unequal base compositions: an entirely low G content (15.2±0.3% on average) and almost equal T, C and A contents (29.3±0.8%, 30.7±1.0%, and 24.8±0.5% on average, respectively). As in other reported fishes (Meyer, 1993; Cantatore et al., 1994; Allegrucci et al., 1999), the first codon positions revealed the almost balanced base composition (T, C, A and G contents: 24.3±0.5%, 26.3±0.4%, 23.3±0.6%, and 26.1±0.3% on average, respectively). In contrast, the second and third codon positions exhibited the strong anti-G bias (13.4±0.2% and 6.1±1.0% on average, respectively). Indices of calculated compositional bias for the first

Table 2. Base composition and calculated base compositional bias (Irwin et al., 1991) of the cytochrome *b* gene in Ephinephelinae species examined (See Table 1 for species abbreviations)

Species	Codon position															
	All				1st				2nd				3rd			
	T	C	A	G	T1	C1	A1	G1	T2	C2	A2	G2	T3	C3	A3	G3
Eaka	31.1	29.2	24.2	15.4	24.2	26.6	23.2	26.1	40.0	26.3	20.0	13.7	29.2	34.7	29.5	6.6
Eare	29.1	31.1	24.7	15.1	25.8	25.8	22.9	25.5	40.8	25.3	20.3	13.7	20.8	42.1	31.1	6.1
Eawo	29.8	30.4	24.1	15.7	23.9	26.8	22.9	26.3	40.3	26.1	20.0	13.7	25.3	38.2	29.5	7.1
Efas	28.9	31.8	24.0	15.4	24.5	26.6	22.1	26.8	39.7	26.6	20.3	13.4	22.4	42.1	29.7	5.8
Emer	26.8	33.2	25.0	14.9	24.7	26.3	22.1	26.8	39.7	26.8	20.0	13.4	16.1	46.6	32.9	4.5
Emoa	29.5	30.8	24.9	14.8	23.9	26.6	23.4	26.1	40.3	26.1	20.0	13.7	24.2	39.7	31.3	4.7
Esep	28.2	31.9	23.9	16.0	23.7	27.1	23.2	26.1	40.3	26.1	20.5	13.2	20.5	42.6	28.2	8.7
Esp	29.3	31.0	25.3	14.5	23.2	26.6	24.2	26.1	40.5	26.1	20.3	13.2	24.2	40.3	31.3	4.2
Curo	29.6	29.8	25.6	14.9	24.5	26.1	24.2	25.3	40.8	26.1	19.7	13.4	23.7	37.4	32.9	6.1
Vlou	30.8	27.9	25.8	15.5	24.5	25.0	24.5	26.1	40.5	26.1	20.3	13.2	27.4	32.6	32.6	7.4
Mean	29.3	30.7	24.8	15.2	24.3	26.3	23.3	26.1	40.3	26.1	20.1	13.4	23.4	39.6	30.9	6.1
Bias[a]	0.133				0.032				0.219				0.273			

The frequencies are shown as percentages.
[a]Bias in base composition is calculated as $C=(2/3)\sum_{i=1}^{4}|c_i-0.25|$, where C is the compositional bias and c_i is the frequency of the *i*th base.

Table 3. Observed numbers of transitional and transversional substitutions between 10 pairs of cytochrome *b* DNA sequences from Epinephelinae species

Codon position	Identical pairs					Transitional pairs			Transversional pairs					R^1
	TT	CC	AA	GG	Total	TC	AG	Total	TA	TG	CA	CG	Total	
First	83	92	84	95	354	15	6	21	2	1	1	1	5	4.2
Second	151	97	76	50	374	3	0	3	0	0	1	1	2	2.0
Third	33	92	79	5	209	84	26	110	22	4	28	6	60	1.8
All	267	281	239	150	937	102	32	136	24	5	30	8	67	2.0

All frequencies are averages (rounded) over all taxa.
[1]R indicates the ratios of the number of transitions to the number of transversions.

codon positions were smaller than those for the second or third codon positions. Such patterns were also reported in the percid fishes (Song et al., 1998).

Saturation analyses to recognize noise in sequence data were performed at all three codon positions, first codon positions, second codon positions, third codon positions, and the first and second codon positions (data not shown). In these analyses, transitions were accumulated much faster than transversions. These analyses indicated that both transitions and transversions at the first and second codon positions are accumulated nearly linearly with increasing *p*-distances.

On the other hand, a number of authors (Meyer, 1993; Griffiths, 1997; Takezaki and Gojobori, 1999) suggested that it is important to take into account substitution rate variation across sites in order to obtain a correct tree. Based on the saturation analyses, it was suggested that transitional substitutions (especially in third codon positions) between ten sequences investigated in this study are almost certainly saturated or near saturation (Fig. 1). Since these saturated data will obscure phylogenetic relationships, phylogenetic inference should be carefully performed. Although many researchers (e.g. Brito et al., 1997; Song et al., 1998) have used all three codon positions for constructing phylogenetic trees for distantly related taxa, some authors have used the first and second codon positions only or transversions only at all three codon positions to correct multiple substitutions (e.g. Birolay et al., 1998). In this study, phylogenetic reconstructions were made with different data sets: all substitutions at the first and second codon positions; transversions only at all three codon positions; and all codons with different weighting ratio of Ts/Tv.

Phylogenetic analyses

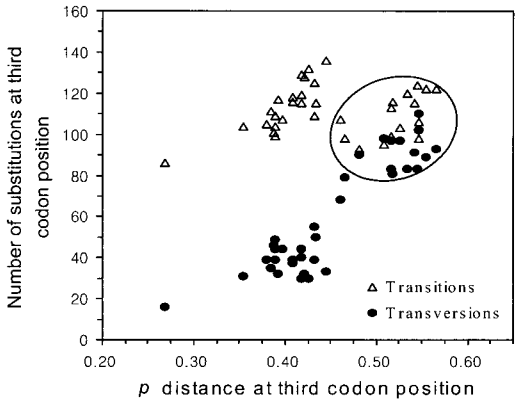

Fig. 1. Plots of the numbers of transitions (open triangles) and transversions (dark circles) at the third codon position against *p* distance values at third codon position. Points enclosed in oval are comparisons between *Epinephelus* species and outgroups.

Phylogenetic trees were constructed according to both distance-based methods and parsimony-based methods. Since the saturated data will obscure phylogenetic relationships (Meyer, 1993), phylogenetic analyses using NJ and ME methods were performed with both all substitutions at the first and second codon positions and Tv only at all codon positions taking into account saturation (i.e., hidden multiple hits on a single site). In addition, when each of *Cephalopholis urodela* and *Variola louti* was used as an outgroup, tree topologies were very similar to that generated by using two outgroups (data not shown). Also, trees inferred without outgroup option showed that outgroup taxa were always distinctively separated from ingroup species. Therefore, the outgroup species used in this study proved to be very reliable for the analyses of the *Epinephelus* in the subfamily Epinephelinae.

Fig. 2-3 illustrate the phylogenetic trees recovered from complete cytochrome *b* sequences of eight Epinephelus species and two outgroups using NJ and ME methods. In these analyses, the monophyly of the genus *Epinephelus* was supported by relatively high bootstrap values of 79-88. However, phylogenetic relationships between Eare, Emoa, Esep, and Esp species were poorly resolved because the tree topology constructed with Tv only at all three codon positions differed from that constructed with substitutions at the first and second codon positions in a few respects. The most striking discrepancy among each trees was the phylogenetic position of *Epinephelus areolatus*.

Fig. 4A shows a 50% majority rule consensus tree (with tree length 152, consistency index of 0.6645, and retention index of 0.4632) inferred by MP method with unweighted data at the first and second codon

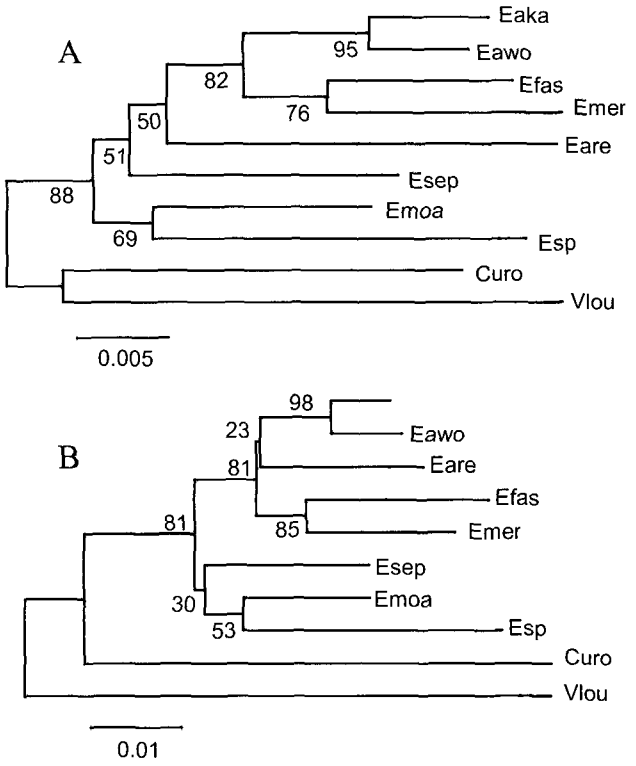

Fig. 2. Phylogenetic tree of 10 Epinephelinae species recovered from cytochrome *b* sequences according to the NJ method-Tamura and Nei's distance. Bootstrap values are displayed overinternal nodes. (A) first and second codon position, transitions and transversions. (B) all three codon position, transversions only. Branch lengths are proportional to the estimated mean number of substitutions per site (See scale bar).

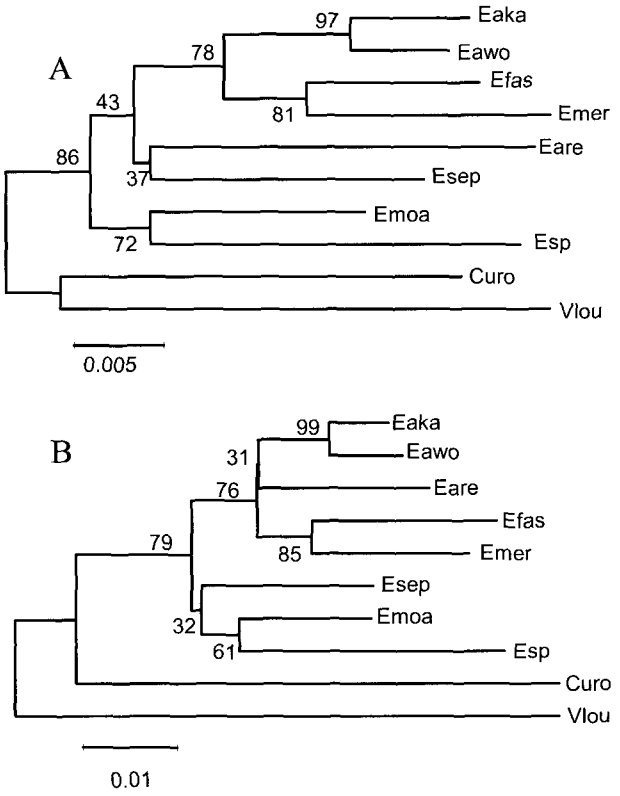

Fig. 3. Phylogenetic tree of 10 Epinephelinae species recovered from cytochrome *b* sequences according to the ME method-Tamura and Nei's distance. Bootstrap values are displayed over internal nodes. (A) first and second codon position,transitions and transversions. (B) all three codon position, transversions only. Branch lengths are proportional to the estimated mean number of substitutions per site (See scale bar).

positions. Considering the potential problem of saturation, analyses were attempted with all three codons by using either equal substitution (Fig. 4B) or unequal substitution rates (Fig. 4C, D) between Ts and Tv. Three different step matrix (Tv:Ts=1:1, 3:1, and 50:1) at all three codon positions were used for this purpose. The tree topologies inferred from these three analyses were similar each other.

All trees constructed by several methods and different data set consistently supported a monophyletic clade of the genus *Epinephelus*, although different methods and data sets provided slightly different topologies within Epinephelus species. On the phylogenetic trees, the species *E. akaara* and *E. awoara* were closely related each other and turned out to be a monophyletic group by high bootstrap value ranging from 92 to 100. Thus, The overall results drawn from this study are shown in Fig. 5. That

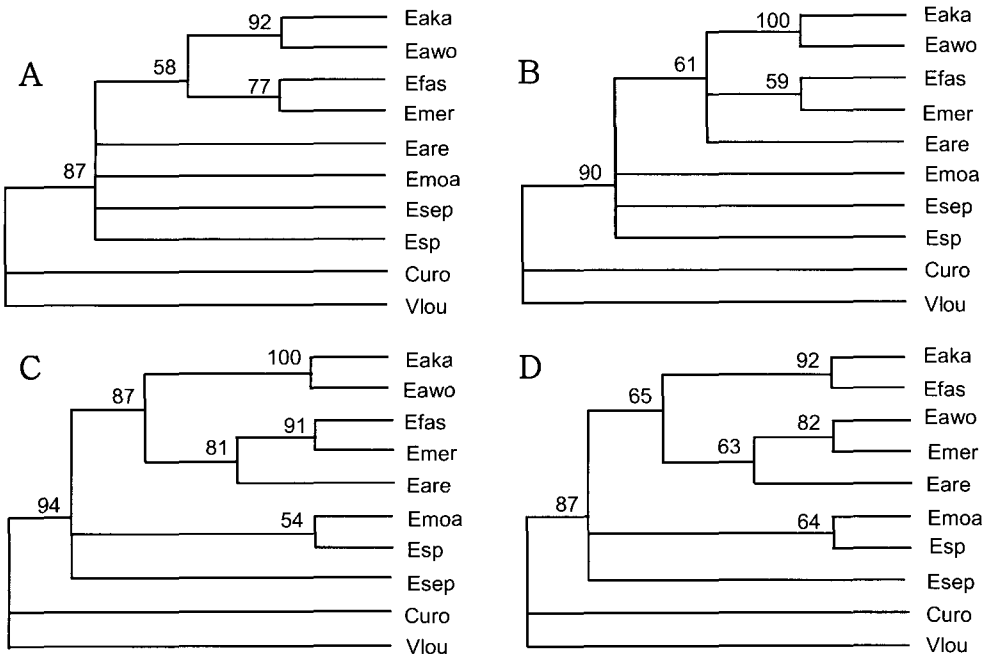

Fig. 4. 50% majority-rule consensus tree obtained from MP analyses using four different data set: A, the first and second codon positions; B, all three codon positions, unweighted; C, all three codon positions, Tv:Ts =3:1 weighted; D, all three codon positions, Tv:Ts=50:1 weighted. The numbers are bootstrap values for the maximum parsimony. Bootstrap values are percentages over 1,000 replicates.

is, within the genus *Epinephelus*, three monophyletic groups were recognized: clade 1 included *E. akaara* and *E. awoara*; clade 2 included *E. fasciatus* and *E. merra*; and clade 3 included *E. akaara*, *E. awoara*, *E. fasciatus*, *E. merra*, *E. areolatus*, *E. moara*, *E. septemfasciatus* and *E.* sp. However, further efforts should be made on sequencing more species or other genes for more resolved results.

Phylogenetic relationships and their usage for genetic improvement by hybridization

Since all groupers are important to the commercial, recreational, and artisanal fisheries in tropical and subtropical seas of the world, considerable efforts have been made on developing culture techniques for several species including Epinephelus coioides, *E. malabaricus*, *E. akaara*, *E. striatus*, *E. septemfasciatus* and *Mycteroperca microlepis*. These species have spawned in captivity, whereas other several species are commonly used in cage-culture operation (Heemstra and Randall, 1993). In recent years, the seed production of *E. akaara* and *E. septemfasciatus* has been attempted in Korea and a few studies on the biological aspects of those species are on-going with particular attention on the reproduction and larval rearing. Once grouper culture techniques are established, people may be interested in genetic improvement of groupers. Hybridization is known as a good way to achieve the genetic improvement with minimizing public concerns about genetically modified organism (GMO). Since improvement by hybridization is hit-or-miss proposition, it is best to stay within family, and success will be improved

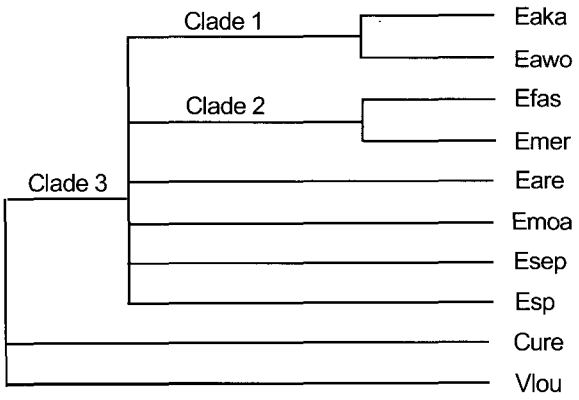

Fig. 5. Phylogenetic relationships referred from trees constructed by different methods and data sets. Clade 1 was composed of Eaka and Eawo. Clade 2 includes Efas and Emer. Clade 3 is composed of Eaka, Eawo, Eare, Efas, Emer, Emoa, Esep and Esp.

in hybridization between the closely related species because hybridization will have little success when the species crossed are so distantly related each other. Tave (1986) mentioned that information of phylogenetic relationships will give us the greatest likelihood of hybridization success. Thus, the result in this study will provide fundamental baseline data for artificial hybridization of Korean grouper species.

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