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

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국문초록

우레기속(*Epinephelus*)에 속하는 어종 간의 계통분류학적 관계를 추정하기 위하여, 능성어아과(Epinephelinae)에 속하는 10 종의 어류로부터 cytochrome *b* 유전자를 클로닝하고, 그 염기서열을 밝혔다. 지금까지 밝혀진 여러 어종의 cytochrome *b* 유전자 주변부의 tRNA 유전자의 보존된 영역에 근거하여, PCR 증폭을 위한 프라이머들을 디자인하였다. 염기서열 분석 결과에 근거하여 이 유전자들이 1140 개의 염기쌍으로 이루어져 있고, cytochrome bc_1 complex 를 암호화하고 있는 380 개의 아미노산으로 이루어진 ORF 를 가지고 있는 것으로 추정되었다

유전적 거리(genetic distance)와 단순성(parsimony)에 근거한 방법을 이용하여 우레기속 어류 8 종과 이들과 근연 관계에 있는 것으로 알려진 2 종의 참조분류군(outgroup)을 위한 계통수를 작성하였다. 그 계통수들은 우레기속이 단진화군(monophyletic group)임을 보여 주었다. 이들 어류들은 크게 3 개의 clade 들로 분류되었다. Clade 1 은 붉바리(Eaka)와 도도바리(Eawo)로 구성되어 있으며, 높은 boostrap 값에 의하여 뒷받침되었다. Clade 2 는 홍바리(Efas)와 Emer 를 포함하고 있다. Clade 3 는 붉바리, 도도바리, 대문바리(Eare), 홍바리, Emer, 자바리(Emoa), 능성어(Esep)와 Esp 로 묶였다. 자바리와 Esp 도 일부 분석에서 clade 로 묶였으나, 상대적으로 낮은 bootstrap 값을 보였다. 결론적으로 말하면, 붉바리(*Epinephelus akaara*)와 도도바리(*E. awoara*)가 계통분류학적으로 가장 밀접한 관계에 있고, 비교적 높은 bootstrap 값에 의하여 단진화군(monophyletic group)인 것으로 밝혀졌다. 그리고, 대문바리(*E. areolatus*), 홍바리(*E. fasciatus*)와 *E. merra* 는 clade 1 (Eaka-Eawo)에 대한 자매종(sister species)들이지만, 자바리(*E. moara*), 능성어(*E. septemfasciatus*)와 *E.* sp 는 조사된 다른 모든 *Epinephelus* 종들에 대한 자매종들인 것으로 밝혀졌다. 또한, 이 연구에서 조사된 *Epinephelus* 속에 속하는 8 종 모두는 비교적 높은 bootstrap 값에 의하여 단진화군인 것으로 밝혀졌다.

I. Introduction

The subfamily Epinephelinae of the 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). Groupers are one of commercially important group of marine fishes, being highly valued at markets and heavily targeted in fisheries (Morris et al., 2000). Due to these reasons, many attempts have been made to establish aquacultural techniques for groupers, especially in several regions of Asia.

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 difficult to identify grouper species visually because of their wide distribution and color variation (Nugroho et al., 1998). Furthermore, some literature on grouper classification had misidentified the species concerned (Leis, 1986).

In spite of their economic importance, 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 analysis of larval and adult morphology.

On the other hand, 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*. On the basis of a cladistic analysis of larval characters, Leis (1986) mentioned about relationships between six genera of the tribe Epinephelini: he led to the hypothesis that *Plectropomus* is the primitive sister group of other studied epinephelines and that *Cephalopholis* is distinct from *Epinephelus*. However, these studies focused on relationships at high hierarchical level within the family Serranidae. Furthermore, very little is known about its phylogenetic relationships in the *Epinephelus* Species.

Nearly all phylogenetic studies of the tribe Epinephelini have based on morphological characters of larvae. These characters, while taxonomically informative, may be problematic in convincing the phylogeny of genera within the tribe Epinephelini on the basis of larval characters because known larvae of members of this tribe are all quite similar.

To overcome these potential limitations, an alternative data set based on nucleotide level characters was used in this study. Since Kocher et al. (1989) published sequences of a primer set for polymerase chain reaction (PCR) amplification of a portion of the cytochrome *b* gene, use of cytochrome *b* sequences

in phylogenetic inference has increased explosively. Because of several advantages of this gene, a number of authors (e.g., Brito et al., 1997; Kocher and Stepien, 1997; Song et al., 1998; Rocha-Olivares et al., 1999; Allegrucci et al., 1999) have used the mitochondrial cytochrome *b* gene to study various problems of fish evolution. This gene is probably most extensively sequenced to date for the vertebrates (Irwin, 1991; Johns and Avise, 1998). In addition, its protein product is probably one of the best characterized molecular systems in terms of its structure, function and evolutionary rate (Esposti et al., 1993).

The purpose of this paper is to examine phylogenetic relationships among eight *Epinephelus* species and two representative species of other genera within the tribe Epinephelini based on mitochondrial cytochrome *b* DNA sequences.

II. Materials and Methods

1. Specimens

Details of the species used in this study are presented in Table 1. Specimens were collected in the field and identified to species level using the information of 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℃.

2. 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 (Heλios β, Unicam Ltd, UK). The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm.

3. 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 amplified with the flanking tRNA primers, Glu-F and Pro-R, by PCR method. The details of primer sequences were reported in Table 2.

The PCR reactions were usually performed in a final volume of 50 $\mu\ell$ in 0.5

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

Genus	Scientific Name	Abbreviated species name	Collection locality
Epinephelus	*Epinephelus akaara*	Eaka	Cheju, Korea
	Epinephelus awoara	Eawo	Cheju, Korea
	Epinephelus fasciatus	Efas	Cheju, Korea
	Epinephelus moara	Emoa	Cheju, Korea
	Epinephelus septemfasciatus	Esep	Cheju, Korea
	Epinephelus areolatus	Eare	Okinawa, Japan
	Epinephelus merra	Emer	Okinawa, Japan
	Epinephelus sp.	Esp*	
Cephalopholis	*Cephalopholis urodela*	Curo	Okinawa, Japan
Variola	*Variola louti*	Vlou	Okinawa, Japan

* The cytochrome *b* sequence of Esp was reported by Allegrucci et al. (1998). The GenBank accession number of Esp cytochrome *b* sequence is AF143193.

Table 2. Primers used for PCR amplification and sequencing of the cytochrome *b* gene

Primer Name	Gene position	Sequence	
Glu-F[a]	$tRNA^{Glu}$	GTT GTH RTT CAA CTA CAA RAA	All taxa
Pro-R[a]	$tRNA^{Pro}$	TAG AAT YYT RGC TTT GGG AG	All taxa
SK[b]		CGC TCT AGA ACT AGT GGA TC	All taxa
T7[b]		GTA ATA CGA CTC ACT ATA GGG C	All taxa
136F	Cytb	CTY ACA GGA CTA TTC CTA GC	Curo, Esep
153F	Cytb	AGC CAT ACA CTA CAC ATC AGA T	Emer, Emoa
205F	Cytb	ATY TGY GGD GAY GTD AAY TA	Vlou
209F	Cytb	GTC GAG ATG TAA ACT ATG GCT G	Eaka, Eare
214F	Cytb	GAC GTA AAC TAC GGC TGA TTA AT	Eawo, Efas
403F	Cytb	TGA GGR CAA ATA TCW TTC TGA GG	Efas, Curo
416F	Cytb	CTT TTT GAG GTG CCA CTG TCA TTA CC	Emoa
416F	Cytb	CCT TCT GAG GAG CCA CTG TCA TCA CC	Emer
449F2	Cytb	TAT CYG CCR TCC CCT ATG TTG G	Eawo, Esep
479F1	Cytb	TAG TYC AAT GAA TTT GAG GTG GCT T	Eare, Vlou
479F2	Cytb	TAG TTC AAT GAA TCT GGG GCG GGT T	Eaka
1037R1	Cytb	GGG TGT TCT ACA GGC ATR CCT CCA AT	Esep, Curo
1037R2	Cytb	GGG TGT TYT ACR GGT ATT CCT CCG AT	Emer
1076R1	Cytb	AAG TAC AGG AYG GAT GCG ATT TGG CC	Eawo, Eaka
1076R2	Cytb	AAG TAY AGR ACG GAT GCA ATT TGG CC	Emoa, Efas
1082R	Cytb	AGT GAG AAG TAC AGA ACG GAT GC	Eare

Numbering is according to the cytochrome *b* sequences of *Danio rerio*, and F and R mean the direction of the mitochondrial DNA strand (F; forward and R; reverse).

a : primers designed for PCR amplification

b : primers designed for the pBluescript phagemid vector

The following symbols were used for sequences: R for A and G; Y for C and T; M for A and C; K for G and T; S for G and C; W for A and T; V for A, C, and G; H for A, T, and C; B for G, T, and C; D for G, A, and T; N for A, G, C, and T.

ml PCR tubes containing 2 ㎕ of genomic DNA (0.1-0.3 ㎍ of DNA), 1 μM each of forward and reverse primers, 2 ㎕ of 100 mM $MgSO_4$, 5 ㎕ of 10× reaction buffer, 1 ㎕ of PCR Nucleotide Mix (containing the sodium salts of dATP, dCTP, dGTP, and dTTP, each at a concentration of 10 mM in water, Promega Co.), and 0.5 to 1 unit of *Vent* polymerase (New England BioLabs Inc.). Approximately 2 drops of mineral oil from a 200 ㎕ micropipette tip was added prior to the initiation of cycling to serve as an evaporation barrier. The amplifications 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 ㎍/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.

To generate sufficient DNA for sequencing, reamplification was performed as follows: 1-2 ㎕ of the purified initial PCR product was used as the DNA template and the reactions were performed under the same PCR-reaction conditions as given above. The successfully reamplified products were again isolated using a LMP agarose gel and then purified using GeneClean II kit (Bio 101 Inc.). The concentrations of second PCR products were measured by a spectrometer. Depending on the efficiency of the PCR reaction, 150 to 250 ㎕ of pooled PCR products were used for the next cloning step.

4. Cloning of the PCR product

E. coli (DH5α) and pBluescript II SK(-) were used as a host and a vector for cloning of the PCR products.

2.5 μg of vector pBluescript II SK(-) (Stratagene) was digested with the restriction enzyme, Hinc II at 37℃ for 2 hrs. 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 ㎕ reaction mixtures containing 1 ㎕ of pBluescript II SK(-) vector (cutted with Hinc II), 1.5 to 2 ㎕ of insert DNA, 4 ㎕ of 5× ligation buffer, and 1 unit of T4 DNA ligase (Life Technologies Ltd). The mixture was incubated at 14℃ for 24 hours. 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 10 ml LB broth containing ampicillin and grown overnight at 37℃ with shaking. For the double-stranded sequencing, plasmid DNA was collected from overnight culture contaning 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.

5. Sequencing

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.

For each sequencing reaction, 27 ㎕ of the master mix (approximately 600 ng

of plasmid DNA; 3.5 $\mu\ell$ of reaction buffer (150 mM Tris-HCl, pH 9.5, 35 mM $MgCl_2$); 2 $\mu\ell$ of 1 μM sequencing-primer; 2 $\mu\ell$ of thermo sequenase DNA polymerase (10 U/$\mu\ell$); and distilled water to adjust total volume to 27 $\mu\ell$) were prepared in a microcentrifuge tube. After the contents of the master mix were mixed thoroughly, 6 $\mu\ell$ of the master mix was aliquoted into each tube (labelled A, C, G, and T) containing 2 $\mu\ell$ of the corresponding Cy5.5 ddNTP termination mix. After each sequencing reaction was mixed thoroughly, one drop of mineral oil from a 200 $\mu\ell$ micropipette tip was added to each reaction mix. The cycle-sequencing conditions were conducted with the following process: (1) an initial danaturation for 2 min at 95 ℃ and (2) 45 cycles of: 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 1.5 min. For some combinations of primers and templates, higher (60℃) or lower (50℃) annealing temperatures were used to optimize the cycle-sequencing reactions.

To remove unincorporated dye terminators, the products were purified by ethanol precipitation method and resuspended in 6 $\mu\ell$ of formamide loading dye. Resulting samples were denatured at 70℃ for 2.5 min and chilled on ice. DNA sequencing was performed on an automated SEQ4×4 sequencer and the profile was analyzed automatically by the software SEQ4×4 Basecaller in the end of each run.

T7 and SK primers were used to obtain first 5' and 3' end of sequence information. For additional sequencing, internal sequencing primers based on the sequence alignment of the preliminary data were designed. The primers (from Bioneer Inc., Korea) used for sequencing were shown in Table 2.

6. Data analysis

Sequences generated 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 1.02, Patterton and Graves, 2000). In addition to sequences investigated for this study, an

additional sequence from GenBank was used (Table 1).

Base composition, patterns of substitution for pairwise comparisons, and codon usage were computed with the program MEGA (version 2b3, programmed by Kumar et al., 2000).

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 4.0d54, Swofford, 1997), 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, all 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.

III. Results

1. Sequencing and base compositional bias

The complete sequences of the cytochrome *b* gene were sequenced from 9 species belonging to the Epinephelinae (Fig. 1). The partial mitochondrial gene order of these 9 species investigated in this study was identical to that of the common vertebrate: $tRNA^{Glu}$ – Cyt*b* - $tRNA^{Thr}$ - $tRNA^{Pro}$ (data not shown).

Base compositions, the number of transitions and transversions, and codon usage in cytochrome *b* sequences of 10 species containing an additional species are shown in Table 3, Table 4, and Table 5, respectively. Table 3 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 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).

2. Sequence evolution

Aligned cytochrome *b* sequences of 10 species containing an additional sequence were 1140 base pairs in length, including 439 variable and 330 parsimony informative sites. As expected, third codon positions were more variable than first or

```
#Eaka ATG GCT AGC CTC CGC AAA ACG CAT CCT CTC CTA AAA ATC GCA AAC [  45]
#Eare --- --C --- --T --- --G --- --C --C --T T-- --- --- --- --- [  45]
#Eawo --- --- --- --T --- --- --- --- --- --- --- --- --- --- --- [  45]
#Efas --- --C --- --T --- --- --C --C --- --- --- --- --- --- --- [  45]
#Emer --- --C --- --T --- --- --- --C --C --- --- --- --- --- --- [  45]
#Emoa --- --- --- --A --T --- --A --- --C --- --- --- --- --- --T [  45]
#Esep --- --- --- --T --- --- --- --C --C --T --- --- --- --- --- [  45]
#E_sp --- --- --- --T --A --- --A --- --C --T --- --- G-T --- --T [  45]
#Curo --- --A --- --- --- --- --A --C --A --A --- --- --T --G --- [  45]
#Vlou --- --A --- T-A --A --- --T --C --C --- --- --- --- --- --T [  45]

#Eaka GAC GCC CTA GTA GAC CTT CCA GCC CCC TCC AAC ATC TCG GTC TGG [  90]
#Eare --- --A T-- --- --- --C --- --T --A --- --- --T --A --- --A [  90]
#Eawo --- --A --- --- --- --C --- --- --T --- --- --T --- --- --- [  90]
#Efas --- --A T-- --- --- --- --- --- --T --- --T --T --- --- --A [  90]
#Emer --- --A --- --- --- --C --- --- --- --- --T --- --- --- --- [  90]
#Emoa --- --A --G --T --- --C --- --- --- --- --- --T --A --- --A [  90]
#Esep --- --A --- --- --T --C --- --- --- --- --- --T --- --- --- [  90]
#E_sp --T --A --- --- --- --C --T --- --- --- --- --T --A --G --A [  90]
#Curo --- --T T-- --C --- --A --C --A --T --- --- --- --A --- --A [  90]
#Vlou --T --T T-- --G --- --C --G --T --A --T --- --T --- --- --- [  90]

#Eaka TGA AAT TTC GGT TCA TTA CTT GGA CTC TGT CTC ATT GCC CAA ATC [ 135]
#Eare --- --- --T --C --- C-G --A --- --- --C --T --C --A --- --- [ 135]
#Eawo --- --- --T --- --- --- --C --- --- --- --A --- --- --- --- [ 135]
#Efas --- --C --T --C --- --- --C --- --- --C --T GCC --A --- --T [ 135]
#Emer --- --C --T --C --- C-- --C --- --- --C --T GCC --A --- --- [ 135]
#Emoa --- --- --T --C --G C-- --C --- --- --C --T --- --T --- --T [ 135]
#Esep --- --C --T --C --- C-C --C --- --- --C --T --- T-- --G --T [ 135]
#E_sp --- --- --T --C --C C-G --A --- --- --C --- --- --A --- --- [ 135]
#Curo --- --C --- --- --G C-T --- --- T-A --- T-A --C T-A --- --- [ 135]
#Vlou --- --C --T --C --C C-T --A --T --A --- T-A --- A-T --- --- [ 135]

#Eaka CTT ACA GGC CTA TTC CTA GCT ATA CAC TAT ACA TCA GAC ATT GCC [ 180]
#Eare --- --- --- --- --T --- --C --G --- --C --- --- --- --C --- [ 180]
#Eawo --- --T --- --G --- --- --C --- --T --C --- --- --T --- --T [ 180]
#Efas --- --- --- --C --T --- --A --- --T --- --- --C --T --- --T [ 180]
#Emer --C --- --T --C --- --- --C --- --- --C --- --- --T --C --- [ 180]
#Emoa --C --- --- --- --T --- --C --- --- --C --- --- --T --- --- [ 180]
#Esep --- --- --A --- --- --- --C --- --- --- --- --- --T --C --- [ 180]
#E_sp --A --G --- --- --T --- --- --- --- --C --G --- --- --C --- [ 180]
#Curo --C --- --A --- --- --- --- --- --- --C --C -TT --T --C --- [ 180]
#Vlou --- --G --A --C --T --G --- --- --T --C --C --- --T --- --T [ 180]
```

Fig. 1. Aligned sequences of cytochrome *b* gene in ten grouper species. The sequences identical to those of *Epinephelus akaara* cytochrome *b* gene were expressed by bars in each alignment position. The termination codon position was indicated by an asterisk.

```
#Eaka ACA GCC TTT TCA TCT GTT GCC CAC ATT TGT CGA GAT GTA AAC TAT [ 225]
#Eare --- --- --C --G --- --C --- --- --- --- --- --- --- --- --- [ 225]
#Eawo --- --- --- --- --C --- --- --- --- --C --- --C --- --- --C [ 225]
#Efas --- --T --- --- --C --A --- --- --- --C --G --C --- --- --C [ 225]
#Emer --- --- --C --- --C --A --- --- --C --- --- --- --- --- --C [ 225]
#Emoa --- --- --- --G --- --- --- --T --C --C --- --- --- --- --C [ 225]
#Esep --- --- --- --- --C --- --- --T --C --- --- --C --G --- --C [ 225]
#E_sp --- --- --- --- --C --C --- --T --C --C --- --- --- --T --- [ 225]
#Curo --- --- --C --- --C --C --T --T --C --- --- --- --T --- --C [ 225]
#Vlou --- --- --C --- --A --- --A --- --- --- --G --C --- --T --C [ 225]

#Eaka GGC TGG CTA ATC CGT AAT ATA CAT GCC AAC GGG GCC TCC TTT TTC [ 270]
#Eare --- --A --- --- --- --C --- --C --T --- --A --- --A --C --- [ 270]
#Eawo --- --A T-- --T --C --C --- --- --- --- --- --- --- --- --- [ 270]
#Efas --- --A T-- --- --C --- --G --- --T --T --C --T --- --- --- [ 270]
#Emer --A --A --- --- --C --C --- --- --- --- --A --T --- --- --- [ 270]
#Emoa --- --A --T --- --C --- --- --- --- --T --- --- --T --C --T [ 270]
#Esep --T --A --T --- --C --- --- --- --- --- --- --- --- --C --- [ 270]
#E_sp --- --A --T --T --- --- --- --C --- --- --- --- --T --C --- [ 270]
#Curo --T --A --C --- --- --C --G --- --T --- --- --- --- --C --- [ 270]
#Vlou --A --A T-- --T --- --- --- --- --T --T --C --A --A --C --T [ 270]

#Eaka TTT ATC TGC ATT TAT GCA CAC ATT GGA CGA GGA CTT TAC TAC GGT [ 315]
#Eare --- --- --- --- --C --C --- --C --- --- --C --C --- --- --C [ 315]
#Eawo --- --- --T --C --- --- --- --- --G --- --G --- --T --- --C [ 315]
#Efas --- --T --- --- --C --C --- --C --- --- --- --- --T --- --- [ 315]
#Emer --- --- --- --C --- --C --- --- --T --C --C --- --- --T --C [ 315]
#Emoa --C --T --- --- --- --C --- --- --- --- --- --- --- --- --C [ 315]
#Esep --C --- --T --C --C --T --- --- --- --- --C --C --T --- --C [ 315]
#E_sp --- --- --T --C --C --C --- --- --- --- --- --- --- --T --C [ 315]
#Curo --- --- --- --C --C --C --- --C --- --G --- --- --- --T --C [ 315]
#Vlou --- --T --T --C --C --- --- --C --T --T --- --G --- --- --C [ 315]

#Eaka TCC TAC CTC TAT AAA GAA ACC TGA AAC ATT GGA GTC ATC CTT CTC [ 360]
#Eare --- --T --- --- --- --- --- --- --T --- --T --A --T --- --T [ 360]
#Eawo --- --- --- --- --- --G --- --- --T --- --- --T --- --C --- [ 360]
#Efas --A --- --- --C --G --- --- --- --T --- --T --T --T --C --T [ 360]
#Emer --T --- --T --C --- --- --- --- --- --C --- --T G-- --C --- [ 360]
#Emoa --T --- --T --C --- --- --- --- --- --C --- --T --T --- --- [ 360]
#Esep --- --T --T --C --- --- --- --- --- --- --G --T --T --- --G [ 360]
#E_sp --A --- --- --C --- --- --A --- --T --- --T --T --- --G --- [ 360]
#Curo --A --T --T --C --- --- --T --- --T --C --C --T --T --- --A [ 360]
#Vlou --T --- T-G --C --G --- --A --- --- --C --T --T G-- T-A --- [ 360]
```

Fig. 1. Continued.

```
#Eaka CTT CTA GTA ATA ATA ACA GCT TTT GTT GGT TAC GTT CTC CCC TGA [ 405]
#Eare --C --G --G --- --G --- --C --C --- --C --- --- --T --- --- [ 405]
#Eawo --C --- --- --G --- --- --C --- --- --- --C --- --C --- --T --- [ 405]
#Efas --C --G --- --G --- --G --- --C --C --C --- --- --- --- --- [ 405]
#Emer --C T-- --- --G --- --- --C --- --- --A --T --A T-A --- --- [ 405]
#Emoa --C --- --- --- --- --- --- --C --- --- --C --T --C --- --- --- [ 405]
#Esep --C --- --- --G --G --- --C --C --G --- --T --C --- --- --- [ 405]
#E_sp --C --- --- --- --- --T --C --C --G --A --T --- --T --- --- [ 405]
#Curo --C T-- --T --G --- --- --C --C --A --C --T --C --T --- --- [ 405]
#Vlou --G --T --C --- --- --T --A --C --C --C --T --C --T --T --- [ 405]

#Eaka GGG CAA ATA TCT TTC TGA GGG GCC ACT GTC ATT ACC AAC CTT TTA [ 450]
#Eare --T --- --G --C --- --- --A --T --C --- --C --T --- --- --- [ 450]
#Eawo --T --G --- --- --- --- --- --T --- --T --- --- --- --- C-- [ 450]
#Efas --- --- --- --- --- --- --C --- --- --T --- --- --- --C C-- [ 450]
#Emer --A --- --- --C --- --- --A --- --- --- --C --- --- --C --- [ 450]
#Emoa --A --- --G --- --T --- --T --- --- --- --- --- --T --- C-- [ 450]
#Esep --A --- --- --- --T --- --- --- --C --- --- --T --- --C C-- [ 450]
#E_sp --A --G --G --C --T --- --- --T --C --T --C --T --- --C C-- [ 450]
#Curo --A --- --- --A --- --- --T --T --A --- --- --- --T --- C-- [ 450]
#Vlou --A --G --G --C --- --- --T --T --- --- --C --- --- T-A C-C [ 450]

#Eaka TCC GCC ATC CCC TAT GTC GGA AAT ACC CTA GTT CAA TGA ATC TGG [ 495]
#Eare --T --T T-- --T --C A-T ---- --C GAT T-- --- --- --- --T --A [ 495]
#Eawo --- --- --- --- --- --T --- --- --- --T --C --- --G --- --A [ 495]
#Efas --T --- G-- --- --C A-T --- --- --- T-- --A --- --- --- --A [ 495]
#Emer --T --- G-- --- --- A-T --- --C --- T-- --C --- --- --- --A [ 495]
#Emoa --T --- G-- --- --- A-T --T --- --T --G --C --- --- --- --A [ 495]
#Esep --T --- G-- --- --- --T --G --C -AT T-- --- --- --- --- --A [ 495]
#E_sp --- --- G-T --- --C A-- --C --C --- --- --C --- --- --- --A [ 495]
#Curo --T --A --- --G --C A-T --T --- --- --- --A --- --- --- --A [ 495]
#Vlou --T --A --T --G --- --T --T --C --- --- --C --- --- --T --A [ 495]

#Eaka GGC GGG TTT TCT GTA GAT AAT GCT ACC CTT ACT CGC TTC TTT GCA [ 540]
#Eare --T --C --- --C --- --- --C --C --- --C --- --- --- --C --- [ 540]
#Eawo --A --A --- --- --- --- --- --C --- --- --- --- --- --- --- [ 540]
#Efas --G --C --C --A --- --- --- --C --- --- --A --T --- --- --C [ 540]
#Emer --A --C --C --C --- --C --C --C --- --C --- --- --T --C --- [ 540]
#Emoa --A --C --C --C --- --- --C --C --- --C --C --T --- --- --- [ 540]
#Esep --G --C --C --C --- --C --C --C --- --- --C --- --- --- --- [ 540]
#E_sp --A --C --- --A --- --C --C --C --- --- --C --- --- --- --- [ 540]
#Curo --A --C --C --G --- --C --C --A --- --A --C --G --- --- --C [ 540]
#Vlou --T --C --C --A --T --- --- --- --- --- --A --- --- --C --C [ 540]
```

Fig. 1. Continued

```
#Eaka TTC CAC TTC TTG TTT CCC TTC GTA ATT GCA GGT GCT ACT CTT CTT [ 585]
#Eare --- --- --- --A --C --- --- --- --- --- -CC --C --A --C --C [ 585]
#Eawo --T --- --- --A --C --- --- --- --- --- --C --- --A --C --- [ 585]
#Efas --- --- --- C-C --- --- --- --- --- --- -CC --G --A --C --A [ 585]
#Emer --- --- --- C-A --- --- --- --- --- --- -C- --- --A --- --A [ 585]
#Emoa --- --- --- --A --C --- --T --C --- --- -CC --C --- G-A --C [ 585]
#Esep --- --- --- --A --- --- --T --- --- --- -CC --C --C --C --C [ 585]
#E_sp --- --T --- C-A --C --- --- --T --- --- -C- --C --C T-A --C [ 585]
#Curo --- --- --- C-C --C --- --T --- --- --G -CC --C --C T-C --C [ 585]
#Vlou --- --- --- C-C --C --A --T --- --C --G -CC A-A --A --- --- [ 585]

#Eaka CAC CTC CTT TTC CTT CAT GAA TCA GGC TCT AAC AAT CCC CTC GGG [ 630]
#Eare --- --- --C --T --C --C --G --G --- --A --T --- --- --G --A [ 630]
#Eawo --- --A --C --- --- --C --- --G --- --A --- --C --- --T --- [ 630]
#Efas --- --T --C --T --C --C --- --- --- --A --- --C --- -AT --C [ 630]
#Emer --- --- --C --T --- --C --- --- --A --A --T --- --- --A --C [ 630]
#Emoa --- --- --G --- --- --C --- A-- --- --A --- --C --- --- --A [ 630]
#Esep --- --T --G --- --C --- --- A-- --- --A --- --C --- --T --A [ 630]
#E_sp --- --- --C --T --C --C --- A-- --- --A --T --C --- A-T --A [ 630]
#Curo --- T-G A-- --- --A --C --- A-- --- --A --- --- --T --A --T [ 630]
#Vlou --- --T --A --T --- --C --- A-C --- --- --- --C --A T-A --A [ 630]

#Eaka CTT AAT TCT GAT GCA GAC AAA ATC TCC TTC CAC CCA TAC TTT TCA [ 675]
#Eare --- --C --- --C --- --T --- --- --T --- --- --G --- --- --- [ 675]
#Eawo --- --C --- --C --- --- --- --- --- --- --- --- --- --C --T [ 675]
#Efas --- --C --- --- --C --- --- --- --A --- --- --- --- --C --- [ 675]
#Emer --- --C --C --C --G --- --- --- --- --- --- --- --T --C --- [ 675]
#Emoa --- --C --C --C --- --T --- --- --- --- --- --- --- --- --T [ 675]
#Esep --C --C --C --- --- --- --- --- --- --- --- --- --- --C --- [ 675]
#E_sp --- --C --C A-C --T --T --- --- --- --- --- --- --T --- --- [ 675]
#Curo --- --C --A --- --- --T --- --T --T --- --- --C --- --C --- [ 675]
#Vlou T-A --C --A --C --- --- --- --- --A --- --- --C --- --C --- [ 675]

#Eaka TAT AAG GAT CTG CTA GGG TTC GCA GCC CTA CTT ATT GCA CTA ACA [ 720]
#Eare --- --A --C --- T-- --A --- --- --- --- --C C-C A-- --C --- [ 720]
#Eawo --- --A --C --- T-- --A --- --- --- --- --- --- --- --C --- [ 720]
#Efas --- --- --- T-A --- --C --- --- --- --C --- C-- --- --C --G [ 720]
#Emer --- --A --C T-A T-- --C --- --- --- --C --C C-- --- --- --- [ 720]
#Emoa --C --A --C --A --- --- --T --- --- T-- --- --- --- --T --- [ 720]
#Esep --C --A --C --C T-- --A --T --- --- --- --- --- --- --T --- [ 720]
#E_sp --- --A --C --A T-- --A --- --- --- --- --C --- A-- --T -TT [ 720]
#Curo --C --A --- --T --- --A --T --- --- T-- --- --C --- --T --- [ 720]
#Vlou --- --A --C --A --- --T --- --- --A --C --A --- --- --T --- [ 720]
```

Fig. 1. Continued

```
#Eaka TCC CTA GCA CTT TTC TCA CCA AAC CTG TTA GGA GAC CCA GAT AAC [ 765]
#Eare -G- T-- --- --- --- --- --T --- --- C-- --- --- --- --C --T [ 765]
#Eawo --- --- --- --- --- --- --C --- --- C-- --- --- --- --- --- [ 765]
#Efas --- --C --- --C --- --- --T --T --T C-- --G --T --- --C --T [ 765]
#Emer --- --T --- --C --- --- --C --- T-- C-- --- --- --- --C --- [ 765]
#Emoa --- --- --- --A --- --C --C --- --A --- --- --- --- --C --T [ 765]
#Esep --A T-- --C --A --T A-- --- --- --A C-- --- --- --- --C --- [ 765]
#E_sp A-- --- --- --A --T --C --T --- --A C-- --T --- --C --- --- [ 765]
#Curo --A T-- --T --C --- A-- --C --- --A C-G --- --- --- --C --- [ 765]
#Vlou A-T T-- --- T-A --T A-- --C --- --A C-- --- --- --C --C --- [ 765]

#Eaka TTC ACC CCG GCC AAT CCT CTT GTT ACA CCA CCC CAC ATT AAA CCC [ 810]
#Eare --T --A --C --- --C --C --C --A --C --T --T --- --- --- --- [ 810]
#Eawo --- --- --- --- --C --C --- --C --G --- --- --T --- --- --- [ 810]
#Efas --T --- --A --- --C --C --G --A --C --T --T --- --- --- --- [ 810]
#Emer --- --- --A --T --C --C --C --G --C --- --T --- --- --G --T [ 810]
#Emoa --- --- --C --- --C --C --C --C --G --T --T --- --- --G --T [ 810]
#Esep --- --- --A --- --C --C --- --C --C --T --- --- --C --- --- [ 810]
#E_sp --- --- --A --- --C --C T-C --- --C -TT --- --- --- --- --- [ 810]
#Curo --- --- --A --- --C --A --C --- --T --C --A --T --- --G --- [ 810]
#Vlou --- --- --T --A --C --C T-A --C --C --- --- --- --C --- --A [ 810]

#Eaka GAA TGA TAC TTC TTA TTT GCT TAT GCT ATT CTT CGA TCA ATC CCA [ 855]
#Eare --G --- --- --- --- --- --- --- --C --C --C --- --- --G --T --- [ 855]
#Eawo --- --- --T --- C-G --C --C --- --- --- --A --- --- --- --- [ 855]
#Efas --- --- --- --- C-G --C --C --C --C --C --C --- --C --- --T --- [ 855]
#Emer --G --- --- --- C-- --- --- --C --C --- --A --- --- --- --T [ 855]
#Emoa --- --- --- --- --- --C --- --C --C --C --A --G --- --T --- [ 855]
#Esep --- --G --- --- --- --C --G --C --C --C --C --- --G --T --- [ 855]
#E_sp --- --- --- --- --- --C --- --C --- --C --C --- --T --- --T [ 855]
#Curo --- --- --T --- C-- --- --C --- --C --- --C --- --- --T --- [ 855]
#Vlou --- --- --- --- C-- --- --A --- --- --- --- --- --T --- --C [ 855]

#Eaka AAC AAA CTT GGA GGA GTC CTT GCA TTA CTA GCC TCA ATC TTA ATC [ 900]
#Eare --- --G --A --C --- --T --C --- C-- --- --- --- --T --- G-- [ 900]
#Eawo --- --- --- --- --C --- --C --- C-G --- --- --- --- --- --- [ 900]
#Efas --- --- --A --- --- --- --C --- --- --- --- --- --- --- G-- [ 900]
#Emer --- --- T-A --- --T --- --- --C C-G --- --- --- --- C-G G-- [ 900]
#Emoa --- --- --G --- --- --- --G --- C-- --- --- --- --T C-- G-- [ 900]
#Esep --T --- --G --C --- --- --A --C C-C --G --- --- --- C-- G-T [ 900]
#E_sp --- --- --A --- --- --- T-G --- C-C T-- --- --T --- C-- G-- [ 900]
#Curo --- --- --A --C --T --A T-A --T C-C --- --T --T --- C-- G-A [ 900]
#Vlou --- --- --G --- --G --T --- --- C-- --T --T --- --- C-T G-- [ 900]
```

Fig. 1. Continued

```
#Eaka CTT ATA TTA GTT CCA ATC CTT CAC ACC TCT AAA CAG CGT GCC CTG [ 945]
#Eare --C --- C-- --- --- --- --C --T --- --- --- --A --A --- T-- [ 945]
#Eawo --- --- --- --- --- --- --C --- --- --- --- --- --A --- --A [ 945]
#Efas --A --G --G --- --- --- --C --- --T --- --- --A --A --- --A [ 945]
#Emer --C --G --- --C --- --- --C --- --- --- --- --A --A --A --A [ 945]
#Emoa T-G --- C-- --- --- T-A --- --- --- --C --- --A --A --- --A [ 945]
#Esep --- --G C-- --C --- --- --A --- --- --- --- --A --A --- --A [ 945]
#E_sp --- --- C-T --A --T --- --C --- --- --- --- --A --G --- --- [ 945]
#Curo --- --G C-T --C --C --T --G --- --- --- --- --A --A --G --A [ 945]
#Vlou --- --- C-- --- --- --- --C --- --A --C --- --A --A --- --C [ 945]

#Eaka ACT TTC CGC CCT TTA ACC CAA TTC CTA TTT TGA GCT CTA ATT GCC [ 990]
#Eare --- --- --- --- --- --- --- --- T-- --- --- ATC --- --C --- [ 990]
#Eawo --C --- --- --- --G --- --- --- T-- --- --- -T- --- --- --T [ 990]
#Efas --C --T --- --- --- --- --- --- --C --C --- --C --- --C --- [ 990]
#Emer --C --- --A --- --- --- --- --- --- --C --- --C --- --C --- [ 990]
#Emoa --C --- --- --C C-T -G- --G --- --T --C --- A-- T-- --- --T [ 990]
#Esep --C --- --T --- --- --- --- --- --- --- --- A-- T-- --C --- [ 990]
#E_sp --- --- --- --A G-T -G- --- --- --- --C --- --C --- --- --T [ 990]
#Curo --C --- --G --C A-C --- --- --- --T --C --- A-C --- --- --A [ 990]
#Vlou --A --T --A --A GCC --- --- --- --G --- --- -TA --T --C --A [ 990]

#Eaka GAC GTG GCA ATT CTC ACC TGA ATC GGG GGC ATA CCC GTA GAA CAT [1035]
#Eare --- --A TT- --C --- --- --- --- --C --G --- --- --- --C [1035]
#Eawo --- --A --- --- --- --- --G --T --A --A --- --T --- --- --- [1035]
#Efas --- --- -T- --- --- --- --- --- --- --A --- --- --- --- --- [1035]
#Emer --- --A -T- --- --- --- --- --- --A --A --- --- --- --- --C [1035]
#Emoa --T --A ATT --C --- --- --- --- --A --T --- --T --- --- --C [1035]
#Esep --T --A ATT --- --G --- --- --T --A --T --G --T --- --- --C [1035]
#E_sp --T --A ATT --- --T --T --- --T --A --- --- --A --- --- --C [1035]
#Curo --- --A CT- --- --T --- --G --T --A --- --G --T --- --- --C [1035]
#Vlou --T --- ATG --- --T --- --- --T --A --A --G --- --- --- --C [1035]

#Eaka CCT TTT ATT ATT ATC GGC CAA ATC GCA TCC ATC CTG TAC TTT TCA [1080]
#Eare --C --- --C --- --- --- --- G-- --- --- G-T --- --- --C --- [1080]
#Eawo --- --- --- --- --T --- --- --- --- --- G-- --- --- --C --- [1080]
#Efas --C --C --C --- --- --- --- --T --- --- G-T --- --- --C --- [1080]
#Emer --G --C --C --C --- --- --- --T --- --T --- --A --- --C --G [1080]
#Emoa --A --- --- --- --T --- --- --T --- --- G-- --A --- --C --G [1080]
#Esep --G --C G-C --- --T --- --- --T --G --- C-- T-- --- --- --- [1080]
#E_sp --A --- --- --- --- --- --- --- --- --- GC- --A --- --- --C [1080]
#Curo --- --C --- G-A --T --A --- G-T --G --- T-G --A --T --C --C [1080]
#Vlou --- --C --- --C --T --A --- --T --- --T G-- --T --T --C --G [1080]
```

Fig. 1. Continued

```
#Eaka CTT TTC CTA TTC CTA ATG CCA GCA GCT GGA TGG GCC GAA AAT AAA [1125]
#Eare --C --- --- --- T-- --A --- A-T --A --- --A -TT --- --- --- [1125]
#Eawo --C --- --- --- --- --A --- --- --- --- --- --- --- --- --- [1125]
#Efas --A --- T-- --- --- --A --- --- --C --C --A --T --- --C --- [1125]
#Emer --C --- --- --T T-- --A --- --- --C --C --A --- --- --- --- [1125]
#Emoa --C --- T-- --T T-- --A --- --- --A --- --A --A --- --C --- [1125]
#Esep --C --- --- --T --G --A --- --G --G --C --A -TA --G --- --- [1125]
#E_sp --- --- --- --- T-- --A --- --- --A -C- --A --A --G --C --- [1125]
#Curo --A --- --- A-T --T --A --T TT- --A --- --- --A --- --C --- [1125]
#Vlou --G --- T-G A-T --G --A --- CTC --A --- --A --A --- --- --- [1125]

#Eaka GTC CTT GAG TGA CGA T [1141]
#Eare A-A --A --A --G --- T [1141]
#Eawo --- --- --- --- --- T [1141]
#Efas --- --C --A --G --- T [1141]
#Emer -C- --C --A --G --G T [1141]
#Emoa A-- --C --A --- --G T [1141]
#Esep -C- --C --A --G -AG T [1141]
#E_sp A-- --C --A --G -A-   [1140]
#Curo A-A T-A -CA --- --C A [1141]
#Vlou --T T-A --- --G AAT T [1141]
                          *
```

Fig. 1. Continued.

Table 3. Base composition and calculated base compositional bias (Irwin et al., 1991) at each codon position in the mitochondrial cytochrome *b* genes of groupers

Species	All				Codon position											
					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 4. Observed numbers of the 10 pairs of nucleotides between the DNA sequences for the mitochondrial cytochrome *b* genes of groupers

Codon Position	Identical pairs					Transitional pairs			Transversional pairs					
	TT	CC	AA	GG	Total	TC	AG	Total	TA	TG	CA	CG	Total	R*
First	83	92	84	95	354	15	6	21	2	1	1	1	5	3.9
Second	151	97	76	50	374	3	0	4	0	0	1	1	2	2.0
Third	33	92	79	5	209	84	26	111	22	4	28	6	60	1.8
All	268	280	239	151	938	102	33	135	25	5	30	8	67	2.0

All frequencies are averages (rounded) over all taxa.

* R indicates the ratios of the number of transitions to the number of transversions.

Table 5. Codon usage observed in the cytochrome *b* genes of groupers

UUU Phe 10.1	UCU Ser 4.7	UAU Tyr 5.1	UGU Cys 1.4
UUC Phe 20.1	UCC Ser 6.9	UAC Tyr 8.9	UGC Cys 1.7
UUA Leu 8.8	UCA Ser 8.6	UAA * 0.0	UGA Trp 11.0
UUG Leu 1.2	UCG Ser 1.8	UAG * 0.0	UGG Trp 2.0
CUU Leu 14.8	CCU Pro 4.4	CAU His 2.9	CGU Arg 1.1
CUC Leu 15.9	CCC Pro 8.7	CAC His 9.2	CGC Arg 2.8
CUA Leu 17.0	CCA Pro 7.0	CAA Gln 5.4	CGA Arg 3.9
CUG Leu 4.5	CCG Pro 0.8	CAG Gln 0.8	CGG Arg 0.9
AUU Ile 15.4	ACU Thr 3.2	AAU Asn 5.9	AGU Ser 0.0
AUC Ile 14.9	ACC Thr 9.3	AAC Asn 12.4	AGC Ser 1.2
AUA Met 6.0	ACA Thr 6.6	AAA Lys 8.1	AGA * 0.0
AUG Met 3.3	ACG Thr 1.2	AAG Lys 0.9	AGG * 0.0
GUU Val 5.7	GCU Ala 6.4	GAU Asp 4.2	GGU Gly 3.5
GUC Val 7.0	GCC Ala 16.5	GAC Asp 6.8	GGC Gly 8.3
GUA Val 7.4	GCA Ala 12.1	GAA Glu 5.0	GGA Gly 10.5
GUG Val 1.0	GCG Ala 1.1	GAG Glu 0.9	GGG Gly 2.8

The number indicates the frequency in which the codons are used. All frequencies are averages over all taxa. The termination codons were indicated by asterisks.

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 (see Table 5). These hypotheses based on the transcriptional processing and polyadenylation model for termination of translation (Anderson, 1981) were supposed by predicting that transcripts of these cytochrome *b* genes end in U or AG which become termination codons (UAA or AGA) by polyadenylation.

3. Saturation analyses

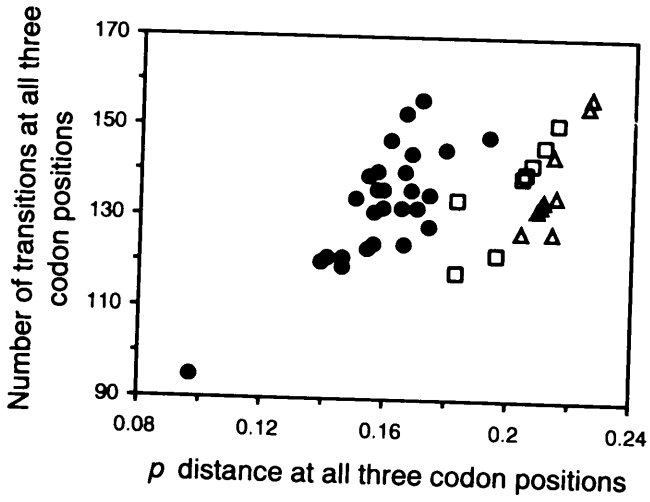
Saturation analyses to recognize noise in sequence data were performed at all three codon positions (Fig. 2), first codon positons (Fig. 3), second codon positions (Fig. 4), third codon positions (Fig. 5), and the first and second codon positions (Fig. 6). In these analyses, transitions were accumulated 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. Based on these results, substitutions at the first and second codon positions and transversions only at all three codon positions were used for phylogenetic analyses.

4. Phylogenetic analyses

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 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).

Figure 7 to 10 illustrate the phylogenetic tree 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

A



B

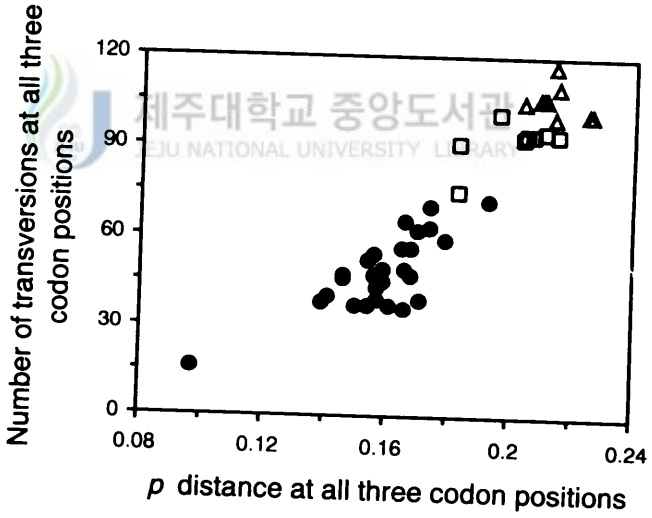


Fig. 2. Relationships of the observed number of transitions (A) and transversions (B) for all pairs of sequences to *p*-distance observed at all three codon positions. The following symbols were used – dark circles, comparisons between *Epinephelus* species; squares, comparisons of *Epinephelus* species with *Cephalopholis urodela*; triangles, comparisons of *Epinephelus* species with *Variola louti.*

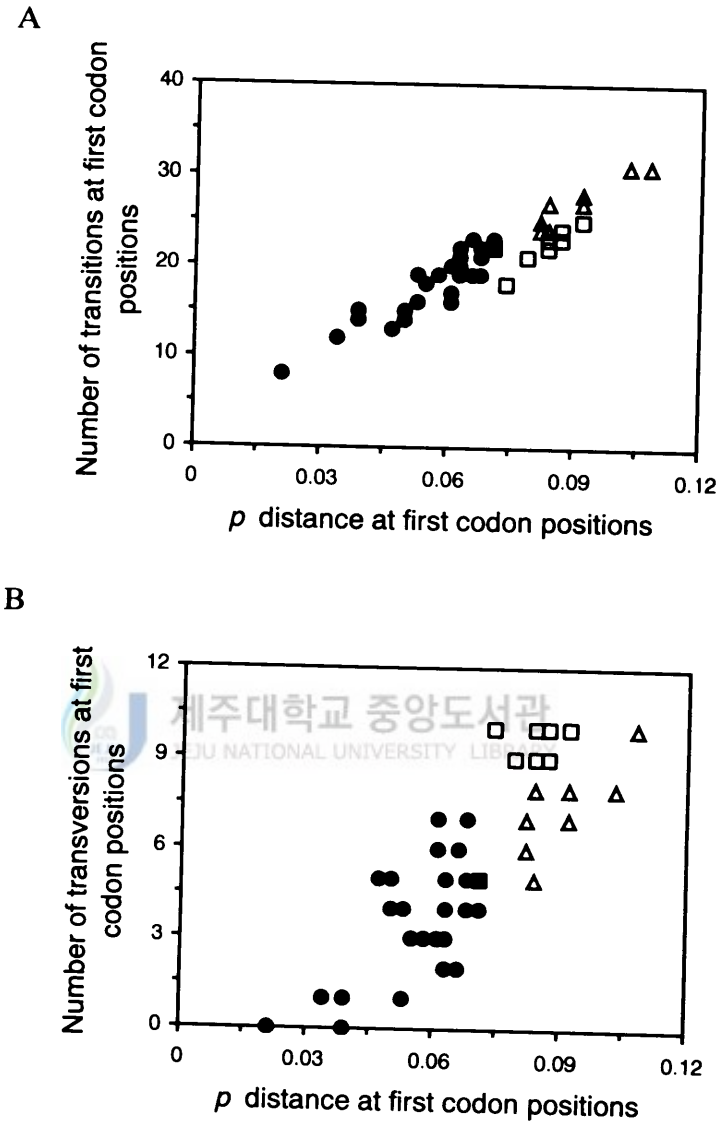


Fig. 3. Relationships of the observed number of transitions (A) and transversions (B) for all pairs of sequences to *p*-distance observed at first codon positions. The following symbols were used – dark circles, comparisons between *Epinephelus* species; squares, comparisons of *Epinephelus* species with *Cephalopholis urodela*; triangles, comparisons of *Epinephelus* species with *Variola louti*.

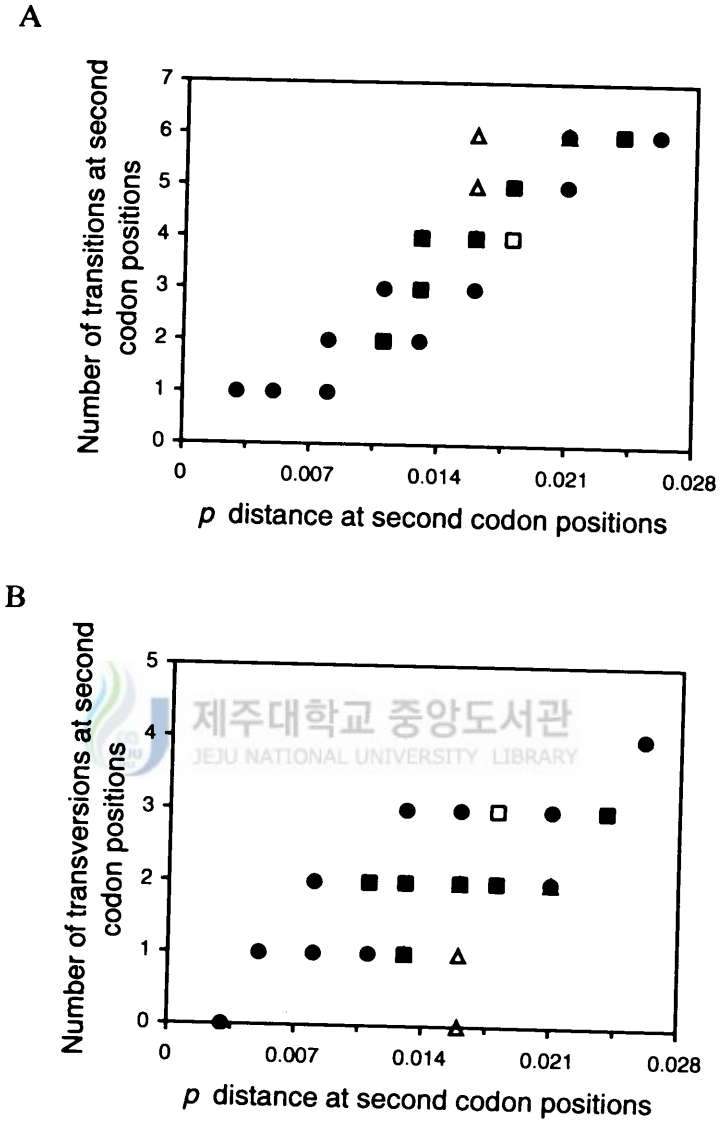


Fig. 4. Relationships of the observed number of transitions (A) and transversions (B) for all pairs of sequences to *p*-distance observed at second codon positions. The following symbols were used – dark circles, comparisons between *Epinephelus* species; squares, comparisons of *Epinephelus* species with *Cephalopholis urodela*; triangles, comparisons of *Epinephelus* species with *Variola louti*.

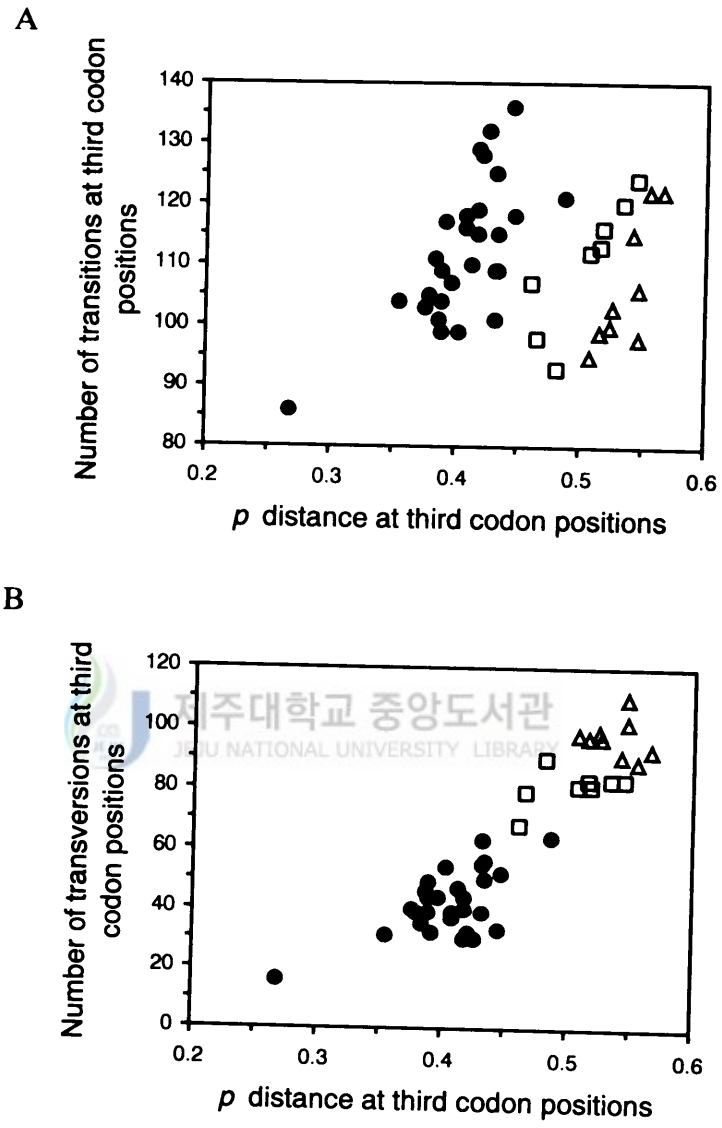


Fig. 5. Relationships of the observed number of transitions (A) and transversions (B) for all pairs of sequences to *p*-distance observed at third codon positions. The following symbols were used – dark circles, comparisons between *Epinephelus* species; squares, comparisons of *Epinephelus* species with *Cephalopholis urodela*; triangles, comparisons of *Epinephelus* species with *Variola louti*.

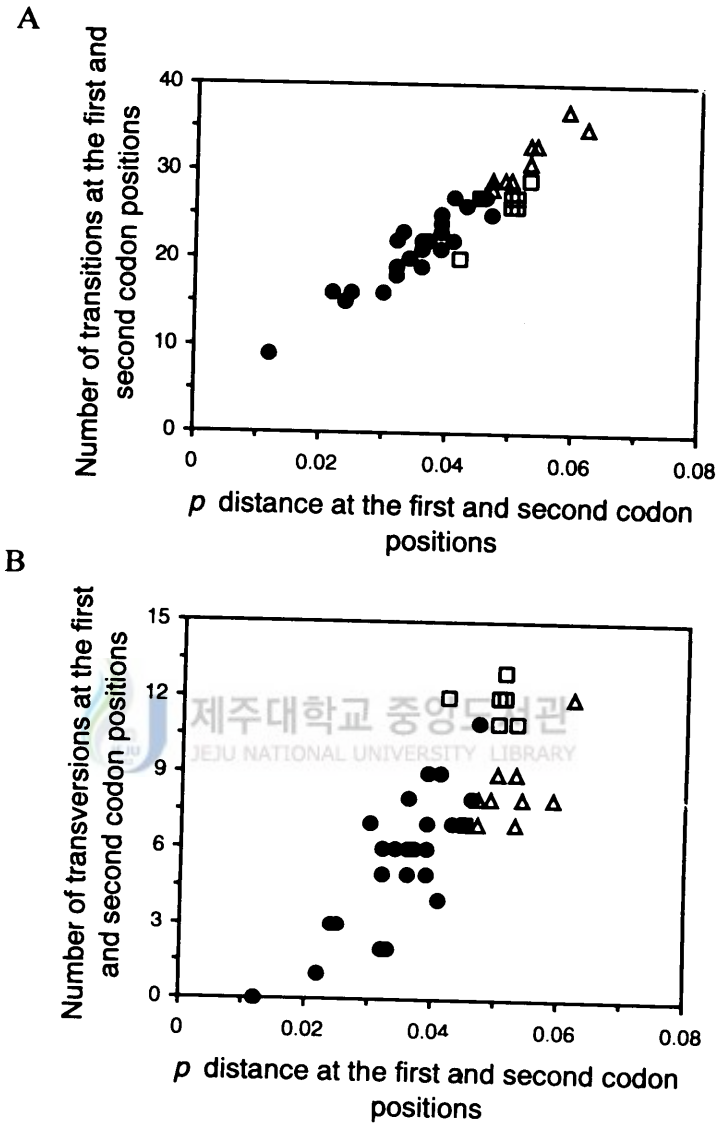


Fig. 6. Relationships of the observed number of transitions (A) and transversions (B) for all pairs of sequences to *p*-distance observed at the first and second codon positions. The following symbols were used – dark circles, comparisons between *Epinephelus* species; squares, comparisons of *Epinephelus* species with *Cephalopholis urodela*; triangles, comparisons of *Epinephelus* species with *Variola louti*.

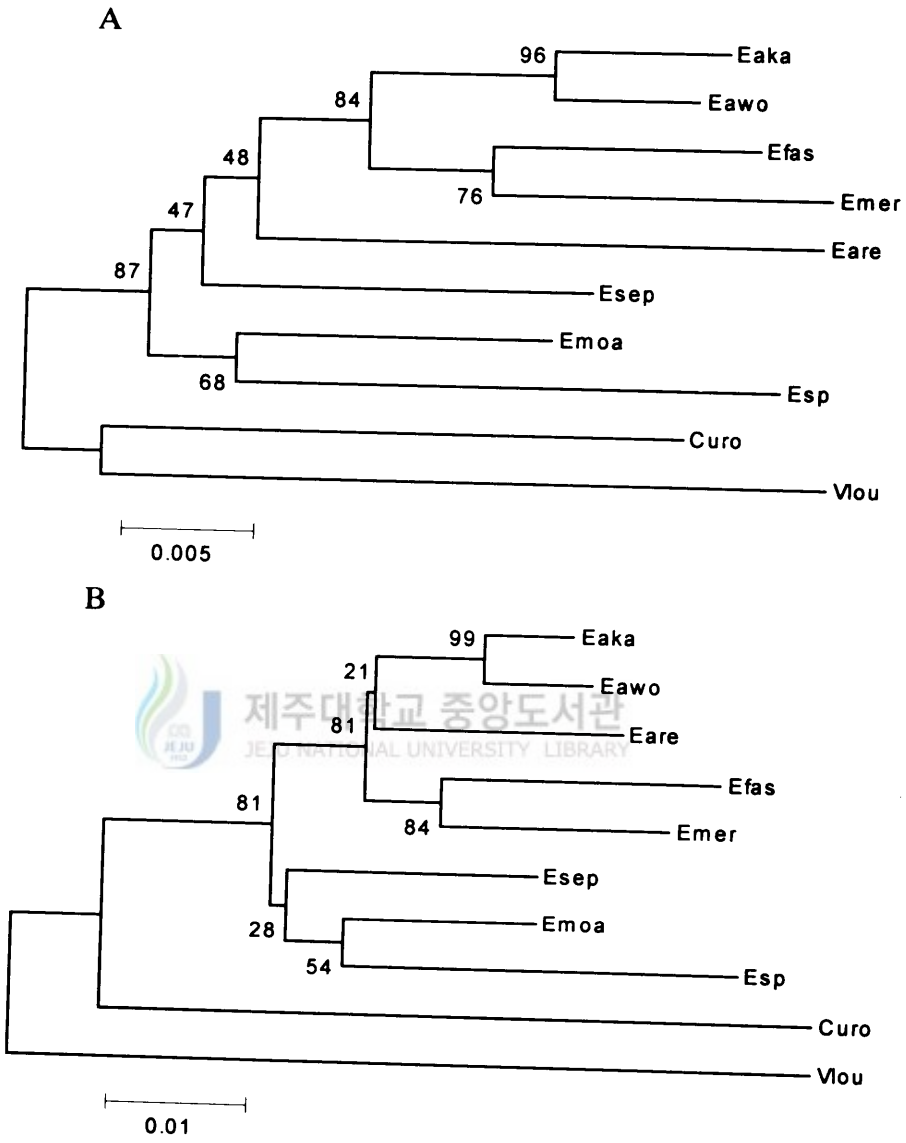


Fig. 7. Phylogenetic tree of 10 Epinephelinae species recovered from cytochrome *b* sequences according to the NJ method – Kimura's distance. Bootstrap values are displayed over internal nodes. (A) first and second codon position, transitions and transversions. (B) all three codon position, transversions only. Branch lengths are proportional to the estimated mean number of substitutions per site (see scale bar).

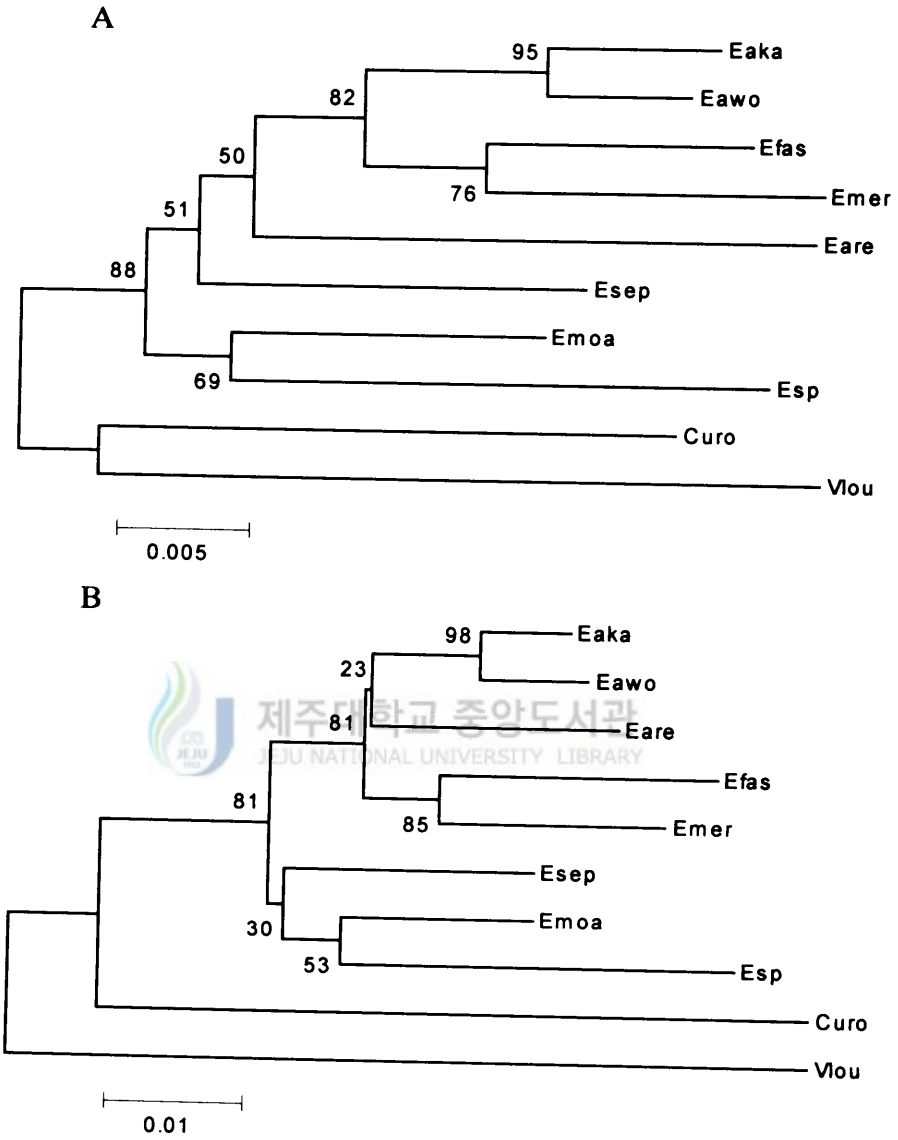


Fig. 8. Phylogenetic tree of 10 Epinephelinae species recovered from cytochrome *b* sequences according to the NJ method – Tamura and Nei's distance. Bootstrap values are displayed over internal nodes. (A) first and second codon position, transitions and transversions. (B) all three codon position, transversions only. Branch lengths are proportional to the estimated mean number of substitutions per site (see scale bar).

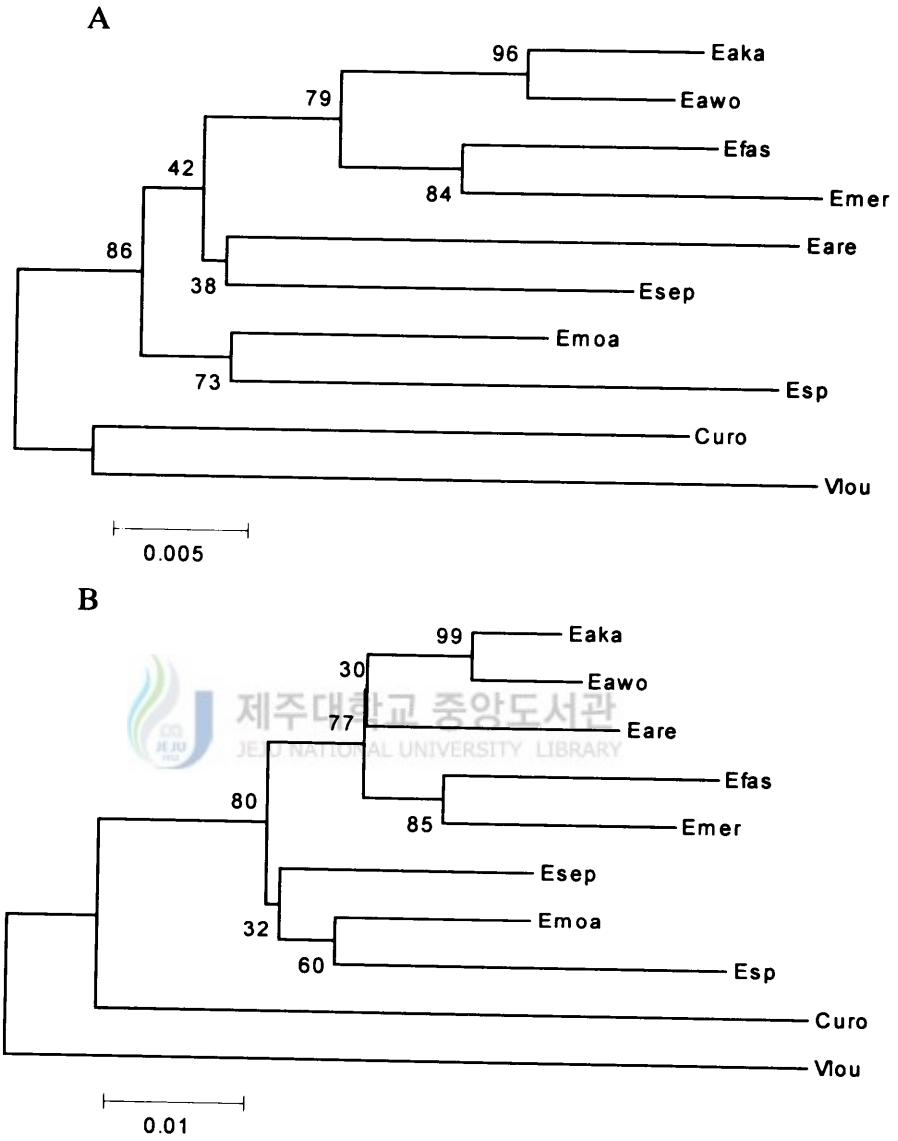


Fig. 9. Phylogenetic tree of 10 Epinephelinae species recovered from cytochrome *b* sequences according to the ME method – Kimura's distance. Bootstrap values are displayed over internal nodes. (A) first and second codon position, transitions and transversions. (B) all three codon position, transversions only. Branch lengths are proportional to the estimated mean number of substitutions per site (see scale bar).

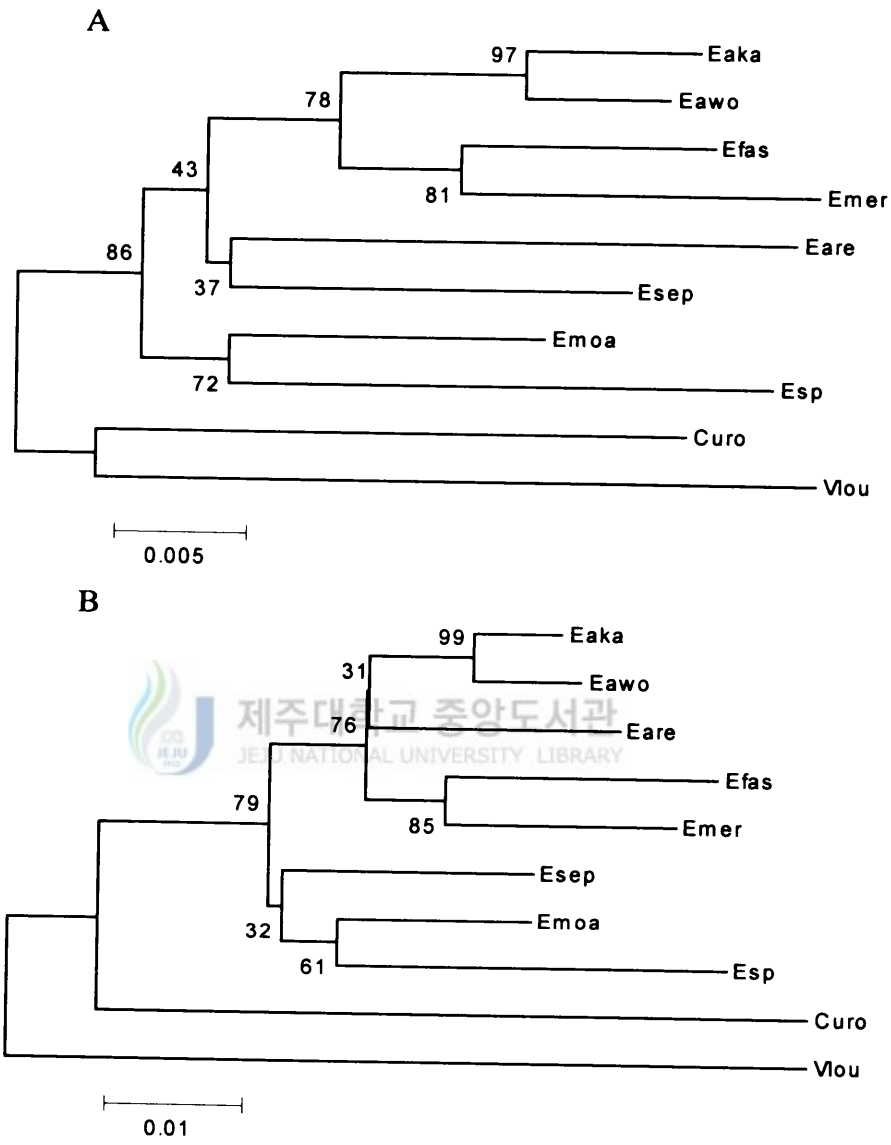


Fig. 10. Phylogenetic tree of 10 Epinephelinae species recovered from cytochrome *b* sequences according to the ME method – Tamura and Nei's distance. Bootstrap values are displayed over internal nodes. (A) first and second codon position, transitions and transversions. (B) all three codon position,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

supported by relatively high bootstrap values. However, phylogenetic relationships between Eare, Emoa, Esep, and Esp species were poorly resolved. Within the genus *Epinephelus*, three major divergence were found: clade 1 included Eaka and Eawo, clade 2 included Efas and Emer, and clade 3 included Eaka, Eawo, Efas, Emer, Eare, Emoa, Esep and Esp.

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). Therefore, these species used in this study proved to be very reliable outgroups for the analyses of the *Epinephelus* in the subfamily Epinephelinae. A 50% majority rule consensus tree (with tree length 152, consistency index of 0.6645, and retention index of 0.4632) inferred using unweighted data at the first and second codon positions was illustrated in Figure 11. Based on the results of saturation analyses, phylogenetic analyses were attempted taking into account the unequal substitution rate 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 topology inferred from these three analyses is the same.

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 (Figure 7 to 14) is the location of species Eare.

5. Phylogenetic relationships inferred from phylogenetic trees

All trees constructed by several methods supported a monophyletic clade of the genus *Epinephelus*, although different methods and data sets provided slightly different topologies within the *Epinephelus*. 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. The overall result of these trees was shown in Figure 15.

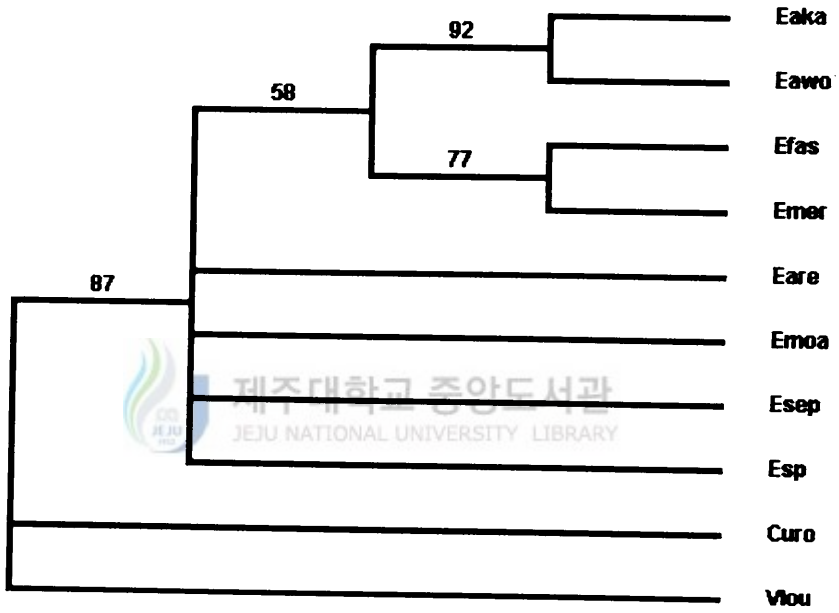


Fig. 11. The 50% majority-rule consensus tree obtained from a MP analysis using the first and second codon positions, unweighted. Bootstrap values above branches are percentages over 1000 replicates. *Cephalopholis urodela* and *Variola louti* were used as outgroups.

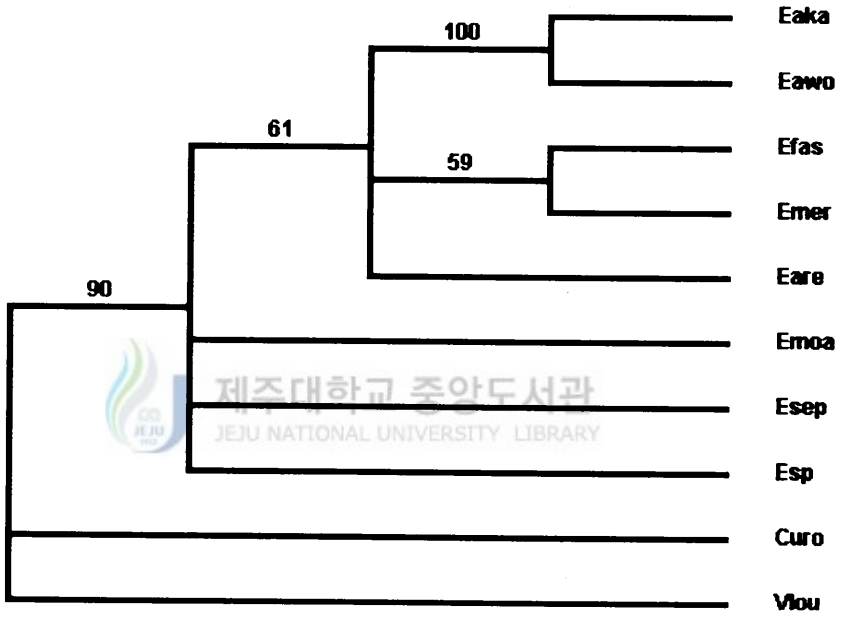


Fig. 12. The 50% majority-rule consensus tree obtained from a MP analysis using all three codon positions, unweighted. Bootstrap values above branches are percentages over 1000 replicates. *Cephalopholis urodela* and *Variola louti* were used as outgroups.

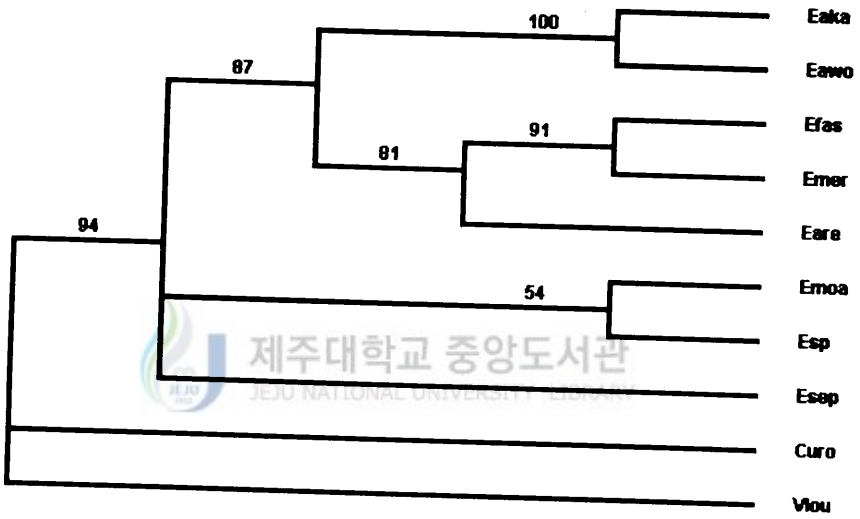


Fig. 13. The 50% majority-rule consensus tree obtained from a MP analysis using all three codon positions, Tv:Ts = 3:1 weighted. Bootstrap values above branches are percentages over 1000 replicates. *Cephalopholis urodela* and *Variola louti* were used as outgroups.

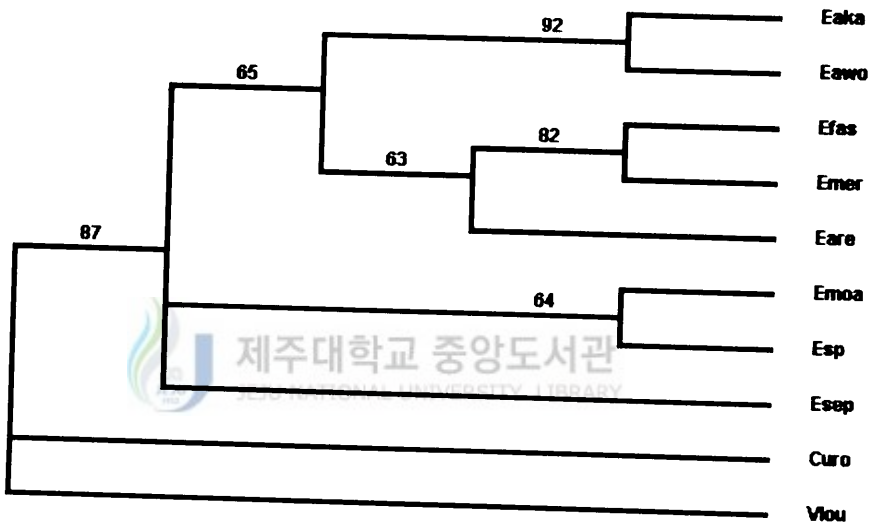


Fig. 14. The 50% majority-rule consensus tree obtained from a MP analysis using all three codon positions, Tv:Ts = 50:1 weighted. Bootstrap values above branches are percentages over 1000 replicates. *Cephalopholis urodela* and *Variola louti* were used as outgroups.

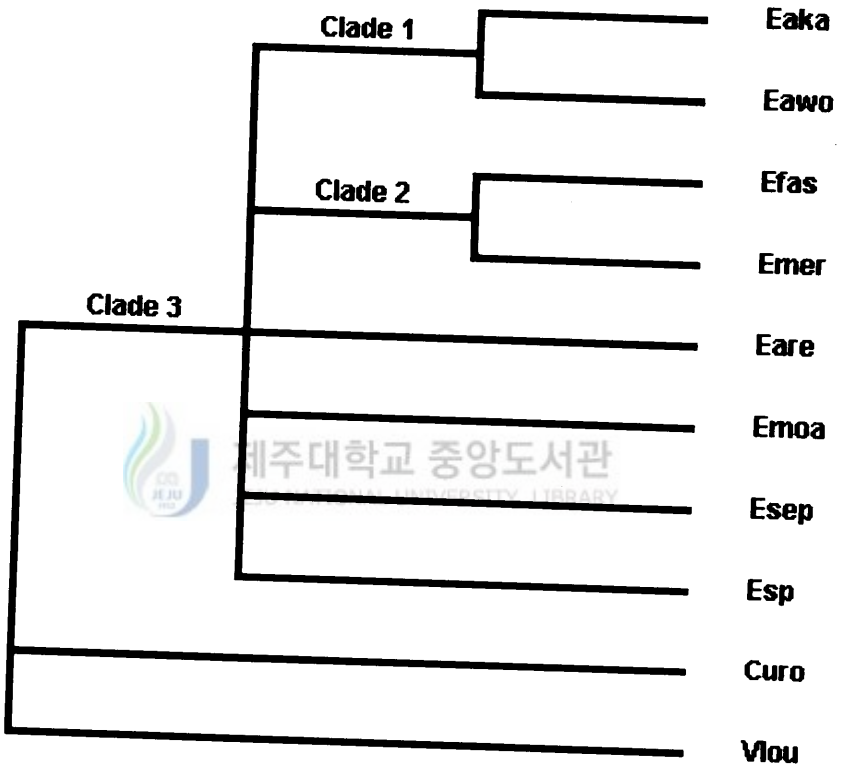


Fig. 15. Phylogenetic relationships referred from trees constructed by different methods and data sets. Clade 1 was composed of *Epinephelus akaara* and *E. awoara*. Clade 2 includes *E. fasciatus* and *E. merra*. Clade 3 is composed of *E. akaara*, *E. awoara*, *E. areolatus*, *E. fasciatus*, *E. merra*, *E. moara*, *E. septemfasciatus* and E. sp.

V. Discussion

Mitochondrial DNA (mtDNA) is known to evolve in a more straightforward manner and contain more sequence diversity compared to nuclear DNA. For these reasons together with recent advances in sequencing techniques, mtDNA has been used to examine a variety of levels of phylogenetic questions.

Furthermore, the cytochrome *b* gene has been used to analyze a variety of levels of relationships ranging from population genetics to higher-level systematics in fishes (Kocher and Stepien, 1997). Song et al. (1998) presented a revised classification of Percidae and discussed the phylogenetic evidence for the independent evolution of small benthic species within Etheostomatinae and Luciopercinae using the cytochrome *b* sequences. Schmidt et al. (1998) used nucleotide sequences of cytochrome *b* gene to infer a hypothesis of phylogenetic relationships among species of the Cyprinid genus *Lythrurus*. In addition, the cytochrome *b* sequences also have been used to detect intraspecific variation in the dusky grouper (Gilles et al., 2000), in the Pacific sockeye salmon (Bickham et al., 1995), and in the Atlantic cod (Carr et al., 1991).

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. Since these data will obscure phylogenetic relationships, phylogenetic inference should be carefully performed. A number of authors (e.g. Brito, 1997; Song et al., 1998) have used all three codon positions for constructing phylogenetic trees for distantly related taxa. Recently, 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 conducted with two data sets: all substitutions at the first and second codon positions and transversions only at all three codon positions.

The variation of the tree topologies obtained from the two different data sets suggests that the cytochrome *b* genes of these fishes here analyzed have different nucleotide substitution rate depending on codon positions.

Members of the widely distributed genus *Epinephelus*, commonly called groupers, are nonschooling sea basses that generally congregate in the same area (Migadalski and Fitcher, 1983). Since all groupers are good to eat, they are important to the commercial, recreational, and artisanal fisheries in tropical and subtropical seas of the world. Recently, a few species including *Epinephelus coioides, E. malabaricus, E. akaara, E. striatus, E. septemfasciatus.* and *Mycteroperca microlepis* are used in aquaculture. These species have been spawned in captivity, whereas other several species are commonly used in cage-culture operation (Heemstra and Randall, 1993). In recent years, considerable efforts for the seed productions of *E. akaara* and *E. septemfasciatus* have been made in Korea. Various studies on the biological aspects of those species are carrying out to understand their reproduction and rearing conditions for larvae and breeders. However, very little studies on the evolutionary intra- or inter-specific relationships of the genus *Epinephelus* have attempted so far.

Hybridization is a good way to improve productivity in a short period. However, its importance for aquaculture in Korea has been recognized in the recent years (Lee et al., 1997). Since improvement by hybridization is hit-or-miss proposition, it is best to stay within family, and success will be improve in intraspecific hybridization 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 success. However, further efforts have to be made on sequencing more species or other genes for more resolved results. The result in this study will provide some insight for improvement by hybridization for Korean grouper aquaculture in near future.

V. Summary

To infer phylogenetic relationships among the species in the genus *Epinephelus*, mitochondrial cytochrome *b* genes in ten fish species belonging to the subfamily Epinephelinae were cloned and sequences were determined. Sequence analyses showed that these genes were 1140 bp long in size and were inferred that it had an ORF of 380 amino acids which encodes a subunit of the cytochrome bc_1 complex. Based on conserved regions of the flanking tRNA genes in several fishes sequenced to date, primers for PCR amplification were designed.

Phylogenetic trees were constructed using distance-based and parsimony-based methods for the sequences of 10 species of the genus *Epinephelus* and the related genera. The trees showed that the *Epinephelus* is a monophyletic group. Trees were characterized by three clades. Clade 1 was composed of Eaka and Eawo and which was supported by a high bootstrap value. Clade 2 includes Efas and Emer. Clade 3 is composed of Eaka, Eawo, Eare, Efas, Emer, Emoa, Esep and Esp. It was supported by bootstrap values but the relationships of Clade 2 species were somwhat doubtful. The results indicated that the species *E. akaara* and *E. awoara* were closely related each other and turned out to be a monophyletic group. *E. areolatus*, *E. fasciatus*, and *E. merra* were sisters to the clade Eaka-Eawo, whereas *E. moara*, *E. septemfasciatus*, and *E.* sp were sisters to all other *Epinephelus* species. Thus, within the genus *Epinephlus*, all eight species examined were strongly supported by bootstrap values as a monophyletic group.

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감사의 글

학위 과정과 이 연구를 통하여 학문의 길로 정진할 수 있도록 배움을 베푸시고 이끌어 주신 송춘복 교수님과 항상 관심을 가지고 조언을 해 주신 정상철 교수님과 학문의 길을 지키어 보시고 인도하여 주신 이제희 교수님께 깊은 감사를 드립니다. 또한, 논문을 자상하게 심사해주신 이정재 교수님, 이기완 교수님, 노섬 교수님, 이영돈 교수님, 최광식 교수님, 허문수 교수님과 여인규 교수님께 깊은 감사를 드립니다.

이 연구를 위하여 귀중한 표본을 제공해 주신 이영돈 교수님과 해양연구소 여러분들에게 특히 감사를 드립니다. 또한, 실험을 하는 동안에 여러 가지 기자재와 시약을 사용하도록 해 주신 유전공학실험실 여러분들에게도 감사를 드립니다.

그리고, 학위 과정 중에 누구보다도 많은 배려와 격려를 해 주신 어류유전육종실험실의 이병문 선배님과 해산식물학실험실의 강세훈 선배님을 비롯하여 어류유전육종실험실의 김지예, 변정선, 강윤석, 김맹진, 고범석, 문영건, 이기정, 김동희 후배님에게 진심어린 고마움을 전합니다.

마지막으로, 어려운 환경에서도 자식들 교육에 평생을 바쳐 일하시고 헌신하셨던 아버님 그리고 어머님께 작은 보답으로 이 논문을 바칩니다.

2000 년 12 월

강 거 영