Molecular Phylogenetic Analyses of *Scyliorhinus torazame* (Carcharhiniformes) Inferred from Cu,Zn Superoxide Dismutase

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Copper,zinc superoxide dismutase (Cu,Zn-SOD) plays a key role to the first antioxidant defense system against oxidative stress in diverse aerobic organisms. Due to the housekeeping action of Cu,Zn-SOD, it was reported that the structure and function have been conserved during evolution. In this study Cu,Zn-SOD from cloudy catshark *Scyliorhinus torazame* was subjected to phylogenetic analyses to know its evolutionary relationship in the vertebrate lineage. Molecular phylogenetic trees inferred by NJ, MP, ML and/or Bayesian analyses showed two shark species, *Prionace glauca* and *S. torazame* grouped together with high statistical supports. In general, they placed at the separated position from bony vertebrates. Thereafter, bony vertebrates composed of teleosts and birds/mammals (amniotes) formed a monophyletic group. Each teleost and amniote clade was also supported by relatively high statistical values. These phylogenetic relationships are well congruent with the phylogenetic hypothesis of the ancestral position of cartilaginous fishes to bony vertebrates.

Key words : Cu,Zn-SOD, *Scyliorhinus torazame*, cloudy catshark, cartilaginous fish, phylogeny

Introduction

Copper,zinc superoxide dismutase (Cu,Zn-SOD or SOD1; EC 1.15.1.1) is a key antioxidant enzyme that catalyzes the dismutation of the toxic superoxide ion (O^{2-}) to hydrogen peroxide (H_2O_2) and molecular oxygen, which is the first step in antioxidant defense system against oxidative stress (den Hartog *et al.*, 2003). This enzyme has been given much attention as a potential indicator for metabolic stress and physiological disorders in which oxidative stress plays a role (McIntyre *et al.*, 1999; Johnson, 2002).

Due to the housekeeping action of Cu,Zn-SOD, the structure and function of this enzyme have been reported to be conserved in diverse aerobic organisms, although a puzzle on the irregular evolutionary change of Cu,Zn-SOD gene in the mammalian lineage has been remained to be further resolved (Lee *et al.*, 1985; Ayala, 2000; Fukuhara *et al.*, 2002). Genetic determinants on vertebrate Cu,Zn-SODs have been well studied mainly in mammals and to a lesser extent in teleosts. However, there are few reports on Cu, Zn-SOD genes of cartilaginous fish species: only one SOD sequence has been determined at the amino acid level in blue shark *Prionace glauca* (Calabrese *et al.*, 1989).

Cartilaginous fishes (Chondrichthyes), which had acquired a cartilaginous endoskeleton during evolution, are one of the most successful and ancient group of fishes (Branches on the Tree of Life: Chordates; http://www.ebiomedia.com/prod/BOintro.html). They also show distinctive anatomical and physiological features different from

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bony vertebrates (Martin and Palumbi, 1993; Moyle and Cech, 1996). These evolutionary and biological properties of cartilaginous fishes in comparison to "true" vertebrate groups make them useful model systems for studying the molecular evolution of vertebrate genes.

Cloudy catshark *Scyliorhinus torazame* (Carcharhiniformes, Scyliorhinidae) is found in temperate and tropical waters of the East Asia. Its habitat ranges from coastal area to continental shelf. This shark species is benthic and oviparous. Recently, extensive attentions have been taken to this species because of its biochemical molecules with high economical values such as tumor metastasis (or angiogenesis) suppressors (Cho *et al.*, 1999; Kang *et al.*, 2003) and L-gulono-γ-lactone oxidase (GLO), a key enzyme for L-ascorbic acid biosynthesis (Nam *et al.*, 2002). In the present study, we conducted molecular phylogenetic analyses of *S. torazame* Cu,Zn-SOD gene with various phylogenetic tree-construction methods.

Materials and Methods

1. Sequence isolation

Cloudy catshark (*S. torazame*) Cu,Zn SOD cDNA was isolated from the UniZap XR cDNA library (Stratagene, USA) constructed from liver using degenerate vectorette PCR involving two vector primers (SK: 5′-CGCTCTAGAACTAGTG-GATC-3′ and T7: 5′-TAATACGACTCACTATAG-GG-3′) and a degenerate primer (UniSOD Reverse: 5′-ATBCCAATTACSCCACAGGC-3′) of which sequence was designed based on other vertebrate SOD sequences. The structure of full-length SOD cDNA was characterized and its sequence was available under the GenBank accession number DQ988331 (see Nam *et al.*, 2006).

2. Multiple sequence alignment

Multiple alignment of cloudy catshark Cu,Zn-SOD along with previously known orthologues from teleosts and bony vertebrates were carried out at both nucleotide (ORF sequence only) and amino acid levels. Twenty representative nucleotide sequences from gnathostomes (jawed vertebrates; 10 from teleosts, 2 from birds and 8 from mammals including 4 primates) were compiled from GenBank (Table 1). The amino acid sequence of *P. glauca* was added in the final nucleotide

Table 1. List of gnathostomes (jawed vertebrates) used in molecular phylogenetic analyses of Cu,Zn-SODs with their common names and GenBank accession numbers

Species	Common name	GenBank accession number
Acanthopagrus schlegelii	Black sea bream	AJ000249
Bos taurus	Cow	M81129
Callithrix jacchus	White-tufted-ear marmoset	AB087273
Cebus apella	Brown capuchin	AB087272
Cervus elaphus	Red deer	U93269
Danio rerio	Zebrafish	BC055516
Epinephelus coioides	Orange-spotted grouper	AY735008
Epinephelus malabaricus	Malabar grouper	AY035854
Gallus gallus	Chicken	NM_205064
Homo sapiens	Human	AY049787
Hylobates lar	Common gibbon	AB087268
Melopsittacus undulatus	Budgerigar	AY241393
Mus musculus	House mouse	M35725
Oncorhynchus mykiss	Rainbow trout	AF469663
Oplegnathus fasciatus	Rock bream	AY613390
Oreochromis mossambicus	Mozambique tilapia	AY491056
Pagrus major	Red sea bream	AF329278
*Prionace glauca**	Blue shark	P11418
Rattus norvegicus	Norway rat	NM_017050
Salmo salar	Atlantic salmon	AY736282
Scyliorhinus torazame	Cloudy catshark	DQ988331 (present study)
Tetraodon nigroviridis	Spotted green pufferfish	CAAE01014601

*Only amino acid sequence available

matrix after converting to the amino acid matrix. Multiple sequences were aligned using Clustal W in BioEdit (Hall, 1999) and edited manually to generate optimal alignment.

3. Molecular phylogenetic analyses

For phylogenetic analyses, a molluscan Cu,Zn-SOD sequence (*Haliotis diversicolor supertexta*; GenBank accession no. DQ000610) was used for an outgroup. In all analyses, nucleotide sequences of Cu,Zn-SODs were used after excluding the third codon positions in the reading frame. The nucleotide sequence data were subjected to neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) analyses in PAUP*

4.0b10 (Swofford, 2002). NJ tree was reconstructed using the Kimura 2-parameter model. MP analysis was performed using the heuristic search option with random addition of sequences (100 replicates) and tree-bisection-reconnection (TBR) branch swapping. Model selection strategy of Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall, 1998) was used to determine the best-fit model of evolution for ML analysis. ML tree was reconstructed with the GTR+I+Γ model with the following likelihood settings, determined from Modeltest 3.7: base frequencies of π_A=0.3253, π_C=0.1765, π_G=0.3513, π_T=0.1469; base substitution rates of $\varphi_{A\text{-}C}$=11.8678, $\varphi_{A\text{-}G}$=7.6103, $\varphi_{A\text{-}T}$=7.2211, $\varphi_{C\text{-}G}$=6.4771, $\varphi_{C\text{-}T}$=14.5369, $\varphi_{G\text{-}T}$=1.0000; assumed proportion of invariable sites (P_{INVAR})=0.367; and gamma distribution shape parameter (α)=1.3002. ML analysis was performed using the heuristic search option with random addition of sequences (100 replicates) and TBR branch swapping. Amino acid sequence data were also analyzed with MP method using the heuristic search option with random addition of sequences (100 replicates) and TBR branch swapping. Robustness of tree topologies of NJ, MP and ML analyses was evaluated by bootstrap analyses with 1,000 replicates (Felsenstein, 1985).

Cu,Zn-SODs were analyzed using the GTR+I+Γ (lset nst=6 rates=invgamma) and Dayhoff+I+Γ [lset rates=invgamma; prset aamodelpr= fixed (Dayhoff)] models for nucleotide and amino acid sequence data, respectively, in MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001). Bayesian inference of phylogeny was performed with random starting trees and run for 1,000,000 generations, sampling the Markov chains at intervals of 100 generations (the default setting). A total of 2,500 out of the 10,000 resulting trees were discarded as "burn-in." Statistical supports for tree topologies were determined on the basis of posterior probabilities obtained from a 50% majority-rule consensus tree.

ML analysis was also carried out in Tree-Puzzle 5.2 (Strimmer and von Haeseler, 1996) with 10,000 puzzling steps. The GTR model with rate heterogeneity of 1 invariable+eight Γ-distributed rates was applied for nucleotide sequence data, and the Dayhoff matrix with rate heterogeneity of 1 invariable+eight Γ-distributed rates for amino acid sequence data. Quartet-Puzzling values were also calculated.

Results and Discussion

1. Phylogenetic analyses of Cu,Zn-SOD

Fig. 1 shows molecular phylogenetic trees inferred from the deduced amino acid sequences with various tree-reconstruction algorithms. In all the phylogenetic trees, two cartilaginous fishes, *P. glauca* and *S. torazame* grouped together with high statistical supports. They placed at the separated position from bony vertebrates in MP and QP analyses without sufficient statistical supports (Fig. 1A). Thereafter, bony vertebrates composed of teleosts and birds/mammals (amniotes) formed a monophyly with a relatively low Quartet-Puzzling value (66%). On the other hand, Bayesian tree showed the phylogenetic affiliation of cartilaginous fishes to teleosts with a relatively low posterior probability (0.72), when the Dayhoff+I+Γ model was applied (Fig. 1B).

In consistency with the amino acid trees using QP and MP methods (Fig. 1A), NJ and QP trees inferred from the nucleotide sequences of Cu,Zn-SODs showed the separated position of *S. torazame* without statistical supports, giving rise to a monophyly of bony vertebrates supported by 53% bootstrap and 89% Quartet-Puzzling values (Fig. 2A, B). However, this phylogenetic relationship was not supported in Bayesian, ML and MP trees (Fig. 2C).

The other internal tree topologies except polytomies within Tetraodontiformes/Perciformes clade were identical among different tree-reconstruction algorithms (Figs. 1, 2). For example, in all the phylogenetic trees, teleost and amniote clades were supported by relatively high statistical values. Within the teleost clade, cyprinid *Danio rerio* emerged first followed by salmonoid *Oncorhynchus mykiss* and *Salmo salar*, and then fish species belonging to Tetraodontiformes and Perciformes formed a terminal branch. Within the amniote clade, *Gallus gallus* and *Melopsittacus undulatus* (birds) formed a relatively long branch clearly separated from the mammalian clade, which showed the hierarchical phylogenetic placement of ruminants, rodents and primates.

Multiple alignment using the deduced amino acid sequences showed that shark Cu,Zn-SODs not only shared all the conserved key features, but also contained intermediate and unique features in comparison with teleost and amniote SODs (Nam *et al.*, 2006). These molecular fea-
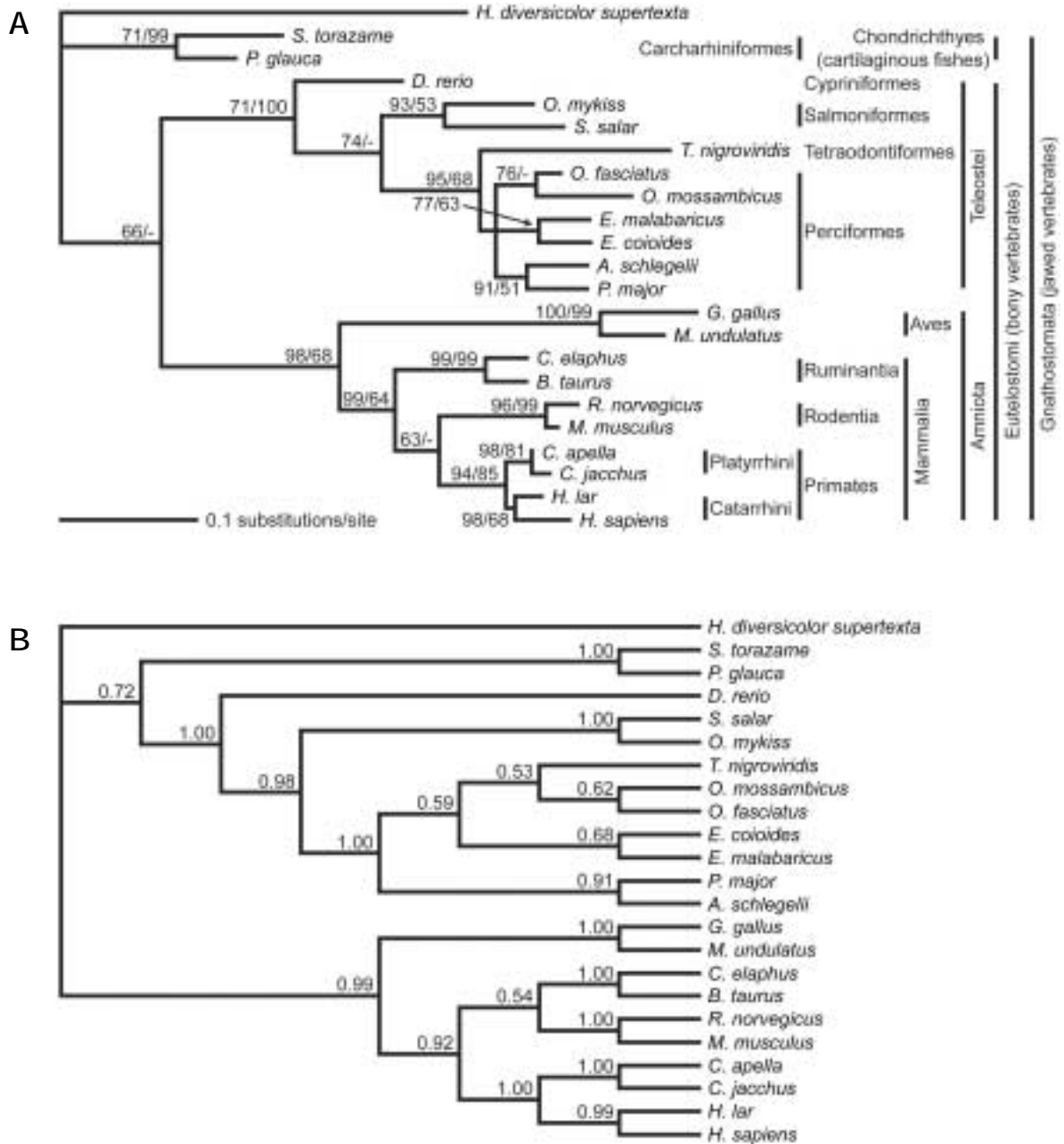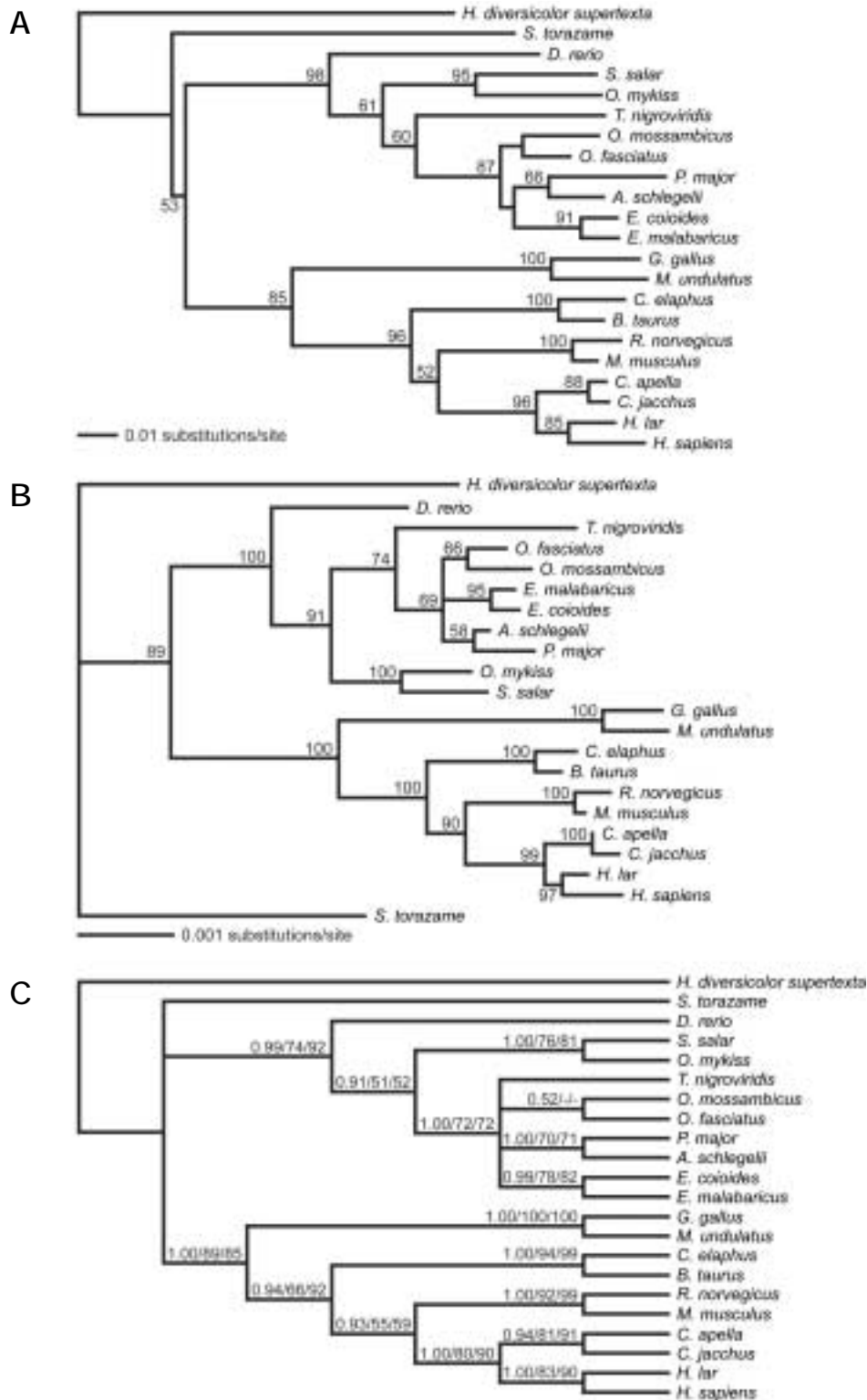
Fig. 1. Molecular phylogenetic trees of gnathostomes (jawed vertebrates) inferred from the deduced amino acid sequences of Cu,Zn-SODs. Molluscan *Haliotis diversicolor supertexta* was used as an outgroup. Trees were reconstructed by Quartet-Puzzling method (A) and Bayesian inference of phylogeny (B). Numbers at each branch node indicate Quartet-Puzzle value/maximum parsimony bootstrap value (A) and Bayesian posterior probability (B). Values above 50% are only shown. Taxonomic placements according to the classification scheme of Metazoa are indicated for each taxon. GenBank accession number for each sequence can be referred to Table 1.

tures of shark Cu,Zn-SODs were well congruent with their monophyletic position relative to teleosts and amniotes shown in Figs. 1 and 2.

Furthermore, our phylogenetic trees are consistent with the traditional phylogenetic hypothesis of the ancestral position of cartilaginous fishes to bony vertebrates (Venkatesh *et al.*, 2001; Takezaki *et al.*, 2003; Kikugawa *et al.*, 2004). However, the low statistical supports for their phylogenetic position are probably due to limited

Fig. 2. Molecular phylogenetic trees of gnathostomes (jawed vertebrates) inferred from the nucleotide sequences of Cu,Zn-SODs. Molluscan *Haliotis diversicolor supertexta* was used as an outgroup. Trees were reconstructed based on neighbor-joining (A), Quartet-Puzzling (B) and Bayesian (C) methods. Numbers at each branch node indicate neighbor-joining bootstrap value (A), Quartet-Puzzle value (B) and Bayesian posterior probability/maximum likelihood bootstrap value/maximum parsimony bootstrap value (C). Values above 50% are only shown. GenBank accession number for each sequence can be referred to Table 1.

phylogenetic information held in a single gene (e.g., 28S rRNA gene tree in Zardoya and Meyer, 1996). In practice, Takezaki *et al.* (2003) and Kikugawa *et al.* (2004) demonstrated the ancestral position of cartilaginous fishes to the monophyletic bony vertebrates using the concatenated sequence matrix of multiple nuclear protein-coding genes. Meanwhile, cartilaginous fishes formed a group with teleosts in Bayesian tree based on amino acid sequence data. This alternative branching pattern was also observed in Takezaki *et al.* (2003). This discrepancy among tree-reconstruction algorithms and the poor resolution among chondrichthyan, teleosts and amniotes appears to be due to saturation of both amino acid and nucleotide substitutions of Cu,Zn-SODs during evolution and loss of the phylogenetic signal during explosive radiation of the Devonian period (Meyer, 1995; Moyle and Cech, 1996). Extended taxon sampling including lancelet, hagfish and lamprey, which are considered to be the most primitive members of chordates, would be valuable to reveal finer-scale phylogenetic resolution of Cu,Zn-SODs in the vertebrate lineage.

Acknowledgments

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두툽상어 (*Scyliorhinus torazame*) Cu,Zn-SOD의 분자 계통학적 분석

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두툽상어 (*Scyliorhinus torazame*)부터 분리된 항산화 효소 Cu,Zn-superoxide dismutase (Cu,Zn-SOD 또는 SOD1)의 핵산 염기서열 및 추정 아미노산 서열을 대상으로 분자 계통학적 분석을 실시하였다. 종래 알려져 있는 척추동물의 Cu,Zn-SOD 서열들을 포함하여 neighbor-joining (NJ), maximum parsimony (MP), maximum likelihood (ML) 및 Bayesian 분석 등을 포함한 다양한 계통 분석을 수행하였으며, 이를 통해 연골어류인 본 어종의 척추동물 분류군 내에서의 계통적 위치를 추정하고자 하였다. 다양한 분자 계통수로부터 얻어진 대부분의 consensus tree들에서 분석에 사용한 분류군들은 종래 알려진 분류학적 위치와 비교적 잘 일치하였고, 이중 두툽상어는 같은 연골어류종인 blue shark와 높은 유연관계를 나타내면서 보다 진화한 경골어류들과는 확연히 구분되는 분지를 형성하였다. 특히 핵산 염기서열을 바탕으로 한 neighbor-joining 분석에서 두툽상어는 경골어류와 양막동물에 비해 보다 원시형태의 척추동물 Cu,Zn-SOD 유전자의 한 형태를 보유하고 있는 것으로 나타났다.