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A New Species of Hagfish *Eptatretus rubicundus* (Myxinidae: Myxiniformes) from Taiwan, with Reference to Its Phylogenetic Position Based on Its Mitochondrial DNA Sequence

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Chien-Hsien Kuo, Sin-Che Lee, and Hin-Kiu Mok (2010) A new species of hagfish *Eptatretus rubicundus* (Myxinidae: Myxiniformes) from Taiwan, with reference to its phylogenetic position based on its mitochondrial DNA sequence. *Zoological Studies* **49**(6): 855-864. A new species of hagfish, *Eptatretus rubicundus* sp nov., collected from the northeastern coast of Taiwan is described. *Eptatretus rubicundus* sp. nov. was diagnosed by the branchial slime pores and gill apertures arranged in a straight line, the distances from a branchial pore to its immediate preceding and posterior gill apertures were similar; the ventral aorta was not bifurcated; and the combination of the following characters: 5 pairs of gill pouches and gill apertures, gill apertures not crowded, 100-104 total slime pores including those in the branchial region, branchial ducts of approximately the same lengths, the pharyngocutaneous-duct opening fused to the posteriormost left gill aperture, the 1st pair of afferent branchial arteries located between the 1st and 2nd gill pouches, a pair of low, round nasal papillae without supporting cartilage on the roof of the nasal tube, and a pink body. The mitochondrial 16S ribosomal RNA gene fragment sequences confirmed the most basal position of *E. rubicundus* sp. nov. in the Eptatretinae. Gene-sequence data on phylogenetic relationships of the species in the previously recognized genera of *Quadratus*, *Paramyxine*, and *Eptatretus* indicated that these genera are not all monophyletic and suggested that they should be combined into a single genus under the generic name *Eptatretus*. http://zoolstud.sinica.edu.tw/Journals/49.6/855.pdf

Key words: *Eptatretus rubicundus* sp. nov., Myxinidae, Phylogeny, Taiwan.

Hagfishes are cartilaginous and jawless marine craniates with an ambiguous phylogenetic relationship to the lampreys and gnathostomes (Stock and Whitt 1992; Forey and Janvier 1993, Nelson 2006). The order Myxiniformes is thought to be monophyletic, based on molecular evidence from mitochondrial 16S ribosomal (r)RNA (Kuo et al. 2003, Chen et al. 2005). This order is divided into 2 subfamilies, the Myxininae and Eptatretinae, based on morphological features (Fernholm 1998). The subfamily Myxininae consists of 4 genera (*Myxine*, *Neomyxine*, *Nemamyxine*, and *Notomyxine*). In the Eptatretinae, the number of recognized genera is still controversial (Strahan 1975, Fernholm 1998, Kuo et al. 2003, Chen et al. 2005, Kuraku and Kuratani 2006), with various specialists originally recognizing only *Eptatretus* Cloquet, 1819 (Strahan 1975, Fernholm 1998). *Eptatretus* is primarily characterized by all of the efferent gill-pouch ducts being of approximately the same length and connecting to each aperture on each side of the body, and ducts on the left being confluent with the pharyngocutaneous duct (Adam and Strahan 1963, Nelson 2006). A 2nd

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eptatretine genus, *Paramyxine*, was later erected (Dean 1904), and later some species in this genus were moved to a new genus, *Quadratus* (Wisner 1999). However, a consensus is lacking on these taxonomic treatments due to the paucity of phylogenetic studies (Jansson et al. 1995, Kuo et al. 2003, Chen et al. 2005). The Eptatretinae has been extensively revised in the past 25 yr (Fernholm and Hubbs 1981, Kuo et al. 1994, McMillan and Wisner 2004), almost tripling the number of known species from about 16 in 1980 to 46 currently. Among them, 9 species occur in Taiwan, including *Paramyxine yangi* (Teng 1958), *P. cheni*, *P. taiwanae* (Shen and Tao 1975), *P. nelsoni*, *P. sheni*, *P. fernholmi*, *E. okinoseanus*, *E. burgeri* (Kuo et al. 1994), and *E. rubicundus* sp. nov. (this paper). Two other species with 5 gill-pouches assigned to the genus *Myxine* in the Myxininae were also reported from the coastal waters of Taiwan (Mok 2002, Mok and Kuo 2001). A hypothesis of the phylogenetic interrelationships of these 12 myxinid species was constructed by Kuo et al. (2003) and Chen et al. (2005) based on the mitochondrial 16S ribosomal (r)RNA gene sequence; the following species were included: 4 *Eptatretus* species (i.e., *E. burgeri*, *E. cirrhatus*, *E. stoutii*, and *E. chinensis*), 2 *Paramyxine* species (*P. cheni* and *P. sheni*), 3 *Quadratus* [*Paramyxine*] species (*Q. nelsoni*, *Q. taiwanae*, and *Q. yangi*), 3 *Myxine* species (*M. circifrons*, *M. formosana*, and *M. glutinosa*), and 3 undescribed *Myxine* species from Taiwanese waters (*Myxine* sp. 1, sp. 2, and sp. 3). The resulting molecular phylogenetic trees showed that (1) both the Myxininae and Eptatretinae are monophyletic groups; (2) *P. cheni* is the plesiomorphic sister species for the remaining 7 (fig. 5 in Kuo et al. 2003) or 8 (fig. 4 in Chen et al. 2005) eptatretine species which form a closely related group; (3) the genus *Paramyxine* is not monophyletic; (4) the genus *Eptatretus* is paraphyletic; (5) there is a close phylogenetic affinity of *E. chinensis* to *P. sheni*, *Q. nelsoni*, *Q. taiwanae*, and *Q. yangi* instead of to its *Eptatretus* congeners; and (6) among *Eptatretus* species, *E. burgeri* is the sister species to *Paramyxine* and *Quadratus* species. Similar results for the phylogenetic positions of *E. burgeri*, *P. cheni*, *P. sheni*, *Q. taiwanae*, and *Q. yangi* were reached in earlier electrophoretic studies (Kuo 1991, Jansson et al. 1995).

One fresh specimen originally recognized as an *Eptatretus* hagfish was incidentally collected by commercial bottom trawl in Sept. 2000 at a depth of approximately 800 m off northeastern Taiwan. It was later identified as a new species seemingly resembling the East Pacific Rise (38°S) species *E. strickrotti* (Møller and Jones 2007) and the New Zealand species *E. eos* (Fernholm 1991) in having the same pink body coloration but differing in lower counts of slime pores (100-102 vs. 128-130 for *E. strickrotti* and 119 for *E. eos*). In this paper, we compared morphological features of this proposed new species with its congeneric species and attempted to confirm its taxonomic status by comparing its 16S rDNA sequences with those of eptatretine species.

MATERIALS AND METHODS

The single specimen trawled off the northeastern coast of Taiwan (24°56.5'N, 121°53.0'E) was deposited in the Museum of the Research Center of Biodiversity, Academia Sinica, Taipei, Taiwan. Methods of counting and measuring and the terminology generally followed Fernholm and Hubbs (1981), McMillan and Wisner (1984), and Wisner and McMillan (1988). All measurements are in millimeters (mm); body proportions are in percent (%) of total length (TL); body width was measured at the pharyngocutaneous duct (PCD), while the body depth was measured at the deepest place, which is usually about mid-body. Winser and McMillan (1990) adopted the length of the barbel as one of the characters for *Eptatretus* taxonomy, but we considered the degree of sharpness of the barbel instead. Frequently used abbreviations are: BR, branchial; CFF, caudal finfold; DM, dental muscle; EBD, efferent branchial duct; GA, gill aperture; GP, gill-pouch; PBR, prebranchial; TA, tail; TR, trunk; VA, ventral aorta; VFF, ventral finfold; w/VFF, with VFF; w/o VFF, without VFF. We used the term "1st" for the most anterior, "slender" for a body width of less than 1/2 the depth, and "robust" for a specimen the body width of which was greater than 1/2 the body depth. In describing the VFF, "prominent" or "well-developed" described a finfold that was 3 mm or deeper, "low" or "poorly developed" for 1-2 mm, and "vestigial" if < 1 mm.

Tissue preparation, polymerase chain reaction (PCR) amplification, and sequencing

Crude DNA was extracted from a muscle sample according to Kocher et al. (1989) with initial homogenization of 0.4 g of muscle tissue with

1 ml of extraction buffer containing 150 mM NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecylsulfate (SDS), and 0.1 mg/ml proteinase K. DNA was subsequently purified by 2 extractions with saturated phenol, 1 with phenol/chloroform, and 1 with chloroform/isoamyl alcohol. This was followed by procedures of isopropanol precipitation and PCR amplification using the universal oligonucleotide primers: forward (16SAR), 5'-CGCCTGTTTAACAAAAACAT-3'; and reverse (16SBR), 5'-CCGGTTTTGAACTCAGA TCACGT-3' (Palumbi et al. 1991). Amplification conditions were designated as 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 2 min (elongation). The amplified 16S rRNA gene fragment DNA was directly sequenced on an automated DNA sequencer (ABI PRISM 377; Applied Biosystem/Perkin-Elmer,Waltham, MA, USA) using a fluorescence dye terminator cycle sequencing kit (Applied Biosystem/Perkin-Elmer). The sequence was deposited in GenBank with the accession no. AY033088 (Table 1). We also included previously obtained sequences of hagfish and lamprey in this study to confirm the taxonomic status of this proposed new species. GenBank accession numbers for those additional species are listed in table 1. Sequences were edited with the DNAstar program package, MegAlign (DNASTAR) and initially aligned using the default parameters of CLUSTALW (Thompson et al. 1994).

Phylogenetic analysis

Phylogenetic trees were produced using the Neighbor-joining (NJ; Saitou and Nei 1987) and maximum-parsimony (MP) methods with the MEGA (Kumar et al. 1994) and Phylip vers. 3.66 (Felsenstein 2005) programs, respectively. In the case of NJ, evolutionary distances were corrected using the equations of Jukes and Cantor (1969), Kimura (1980), and Tajima and Nei (1984). The HKY model (Hasegawa et al. 1985) was also applied to the data. This model takes into account the transitional bias and among-site

Table 1. List of species used in this study, along with localities, GenBank accession numbers, and references cited

Family	Specimen	Collecting locality	Accession number	Reference
Myxiniformes				
Eptatretidae				
Eptatretinae	*Quadratus nelsoni*	Taiwan	AF364608-9	Kuo et al. 2003
	Paramyxine sheni	Taiwan	AF364610	Kuo et al. 2003
	Paramyxine taiwanae	Taiwan	AF364611	Kuo et al. 2003
	Quadratus yangi	Taiwan	AF364612-15	Kuo et al. 2003
	Paramyxine cheni	Taiwan	AF364620-21	Kuo et al. 2003
	Eptatretus burgeri	Taiwan	AF364616-17	Kuo et al. 2003
			AY619579	Chen et al. 2005
			AJ278504	Gachelin 2000
	Eptatretus chinensis	Taiwan	AY619580	Chen et al. 2005
	Eptatretus cirrhatus	New Zealand	AF364619	Kuo et al. 2003
	Eptatretus deani	California, USA	EF014477	Moller and Jones 2007
	Eptatretus longipinnis	South Australia	EF014476	Moller and Jones 2007
	Eptatretus stoutii	Oregon, USA	AF364618	Kuo et al. 2003
	Eptatretus strickrotti	East Pacific Rise	EF014478	Moller and Jones 2007
	Eptatretus rubicundus	Taiwan	AY033088	This study
Myxinidae				
Myxininae	*Myxine formosana*	Taiwan	AF364625	Kuo et al. 2003
	Myxine circifrons	USA	AF364628	Kuo et al. 2003
	Myxine glutinosa	Norway	AF364629	Kuo et al. 2003
			AJ404477	Delarbre et al. 2001
	Myxine sp. 1	Taiwan	AF364622-24	Kuo et al. 2003
	Myxine sp. 2	Taiwan	AF364626	Kuo et al. 2003
	Myxine sp. 3	Taiwan	AF364627	Kuo et al. 2003
Petromyzontiformes				
Petromyzontidae	*Petromyzon marinus*		U11880	Lee and Kocher 1995

heterogeneity observed for the 16S fragment (Ortì and Meyer 1997). Comparisons of mean distance measures for each major clade were based on this model. Bayesian analyses were performed with software MRBAYES 3.1 (Ronquist and Huelsenbeck 2003). The Markov-chain Monte-Carlo (MCMC) search was run with 4 chains for 3×10^6 generations, with trees being sampled every 100 generations, and the 1st 5000 trees (i.e., 5×10^5 generations) were discarded as burn-in. In order to check the number of simultaneous MCMC searches, it was necessary to avoid being trapped in local optima, and we conducted initial analyses with 200,000 generations and 4, 6, 8, and 10 chains, respectively. The number of chains is considered sufficiently when the separate analyses converge on a similar likelihood value. All Bayesian analyses were run for the same number of generations (3 million), to allow direct comparison of the convergence rate for different partitioning schemes, as indicated by the average standard deviation of the split frequencies among 2 MCMC runs, printed by MrBayes, (Huelsenbeck and Ronquist 2001) at the end of each run. Percentages for the branches in the consensus trees represent Bayesian posterior probabilities, which are the rough equivalent of a maximum likelihood (ML) search with bootstrapping. The resultant Bayesian cladograms were evaluated with the software MODELTEST 3.7 under the hierarchical likelihood ratio test (hLRT) criterion (Posada and Crandall 1998). For the ML analysis, the TRN+Γ+I substitution model with invariable sites and among-site rate heterogeneity was selected using hLRTs implemented in Modeltest 3.7 (Posada and Crandall 1998). A heuristic search with 100 random sequence additions was used to find the most-likely tree.

Eptatretus rubicundus sp. nov.

(Figs. 1-3; Table 2)

Holotype: ASIZP 60660, ♀, 464 mm TL, north-eastern Taiwan (24°56.5'N, 121°53.0'E); 30 Sept. 2000; commercial beam-trawl; 800 m depth.

Diagnosis: *Eptatretus rubicundus* sp. nov. is distinguishable from all other congeners by having the branchial slime pores and the gill apertures arranged in a straight line; distances from a branchial slime pore to its immediately preceding and posterior gill apertures similar; and the VA not bifurcated. It was further characterized by the combination of the following characters: a pink body; 5 gill apertures, not crowded; the presence of a pair of small nasal papillae on the roof of the nasal tube; a robust body; 100-102 total slime pores (prebranchial 16 or 17, branchial 3 or 4, trunk 62, tail 19); 3 multicusps in outer row, 7 unicusps, 2 multicusps in inner row, 7 unicusps; eyespot absent.

Description: Body robust, oval in cross-section, slightly deeper than wide, the latter 4% of TL. Rostrum rather long, rounded (Fig. 1). A

Table 2. Morphometric measurements and meristic counts of *Eptatretus rubicundus* sp. nov., *E. eos*, and *E. strickrotti*

	E. rubicundus	*E. eos*	*E. strickrotti*
Length (mm)			
Total length	464	665	314
Probranchial	93 (20% TL)	156 (23.5% TL)	(19.7%TL)
Branchial	25 (5.3% TL)	31 (4.7% TL)	(9.3%TL)
Trunk	257 (55.4% TL)	358 (54% TL)	(58.9%TL)
Tail	89 (19.2% TL)	120 (18% TL)	(12.1%TL)
Depth (mm)			
Over mouth	18(3.8%TL)	-	-
Branchial region	28(6.0%TL)	-	(2.9%TL)
Over caudal	24(5.2%TL)	-	(2.6%TL)
Width (mm)	22(4.7%TL)	-	-
Slime pores (total)	100-102	128-130	119
Probranchial	16 or 17	26	18
Branchial	3 or 4	4 or 5	12
Trunk	62	75-77	70
Tail	19	26 or 27	19
Dental formula (outer/inner)	3+6/2+7	3+6/2+5	3+9/2+9

pair of small and symmetrical papillae present on dorsal surface of nasal tube (nasal sinus), without supporting cartilage. Ventral finfold vestigial, beginning approximately at middle of body and extending posteriorly to cloaca. Caudal finfold thin and low, extending around tail to dorsal surface, ending nearly above cloaca. Three pairs of barbels on head, tapering, 1st and 3rd almost equal in length (1.4% and 1.5% TL, respectively), 2nd a bit shorter (1.1% TL) (Figs. 2A, B). Prebranchial length measured from snout to 1st gill apertures 20% of TL; branchial length from front of 1st gill aperture to pharygocutaneous opening 5.3% of TL; trunk length from rear of pharygocutaneous opening to cloacal slit about 54% of TL; tail narrow and slender, its length from cloacal slit to end of caudal fin and depth 19.2% and 4% of TL; cloacal aperture a longitudinal slit 8 mm long.

Dental muscle short, length 18% of TL and width 2.5% of TL, slightly overlapping with anteriormost GP (Fig. 2D). Five GPs and GAs on either side of branchial region, left posteriormost one larger for efferent branchial duct and pharygocutaneous duct; last left efferent branchial duct confluent with pharyngocutaneous duct; GAs arranged in a straight line; all efferent branchial ducts of similar lengths. VA not bifurcated, all 5 pairs of afferent branchial arteries on sides of VA, 1st pair of afferent branchial arteries (which cannot be treated as branches of VA) located between 1st and 2nd GPs (counted from snout toward heart; Fig. 2E), bases of last 2 afferent branchial arteries merged.

Lingual teeth comblike, with sharp tips slightly curved rearward, placed close together; 7 unicuspids on either side in outer row and 7 unicuspids in inner row, last one smaller. Outer rows starting with a multicusp each consisting of 3 fused unicusps; inner rows starting with multicusps with 2 fused unicusps; bases of multicusp teeth slightly bulbous; total cusps 38; palatine tooth triangular with broad a base (Fig. 3).

Total slime pores 100 (right) and 102 (left) in ASIZP60660 (holotype), arranged in association with segments and extending from behind head to beyond cloaca. Prebranchial pores 16 (right side) and 17 (left side); branchial pores 3 (right side) and 4 (left side) (those between 2nd and 3rd GAs about size of GA); trunk pores 62; tail pores 19 overlying cloaca to tip of tail. Slime pores in branchial region

Fig. 1. Lateral view of *Eptatretus rubicundus* sp. nov., holotype ASIZP 60660, 464 mm total length.

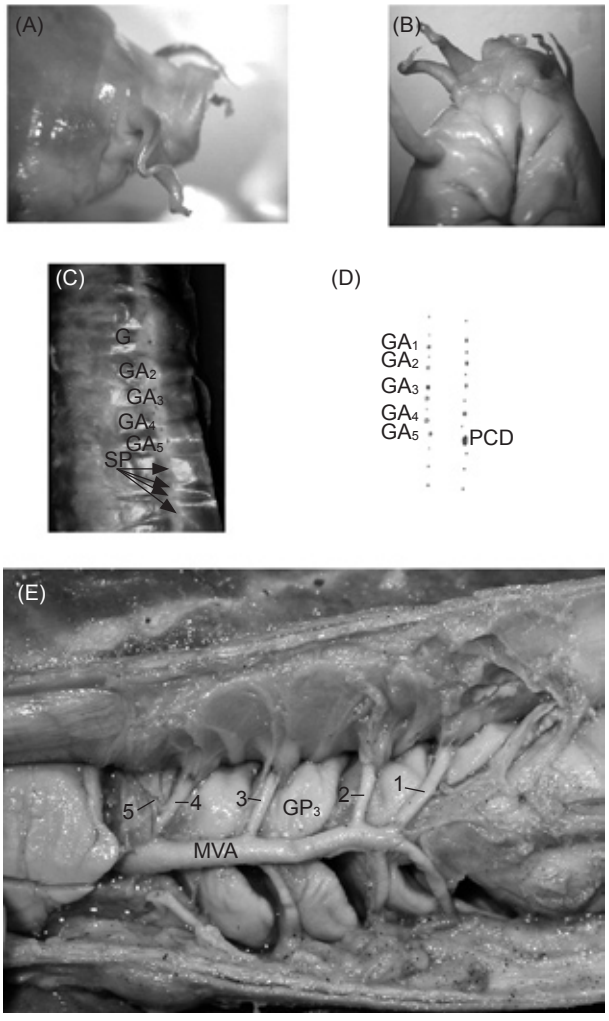

Fig. 2. *Eptatretus rubicundus* sp. nov., holotype ASIZP 60660, 464 mm total length (TL). (A) Head, lateral view. (B) Head, ventral view. (C, D) Gill apertures (GAs) and slime pores (SPs); note that the branchial, prebranchial, and trunk slime pores are arranged in a straight line. (E) Gill region dissected. GP3, 3rd right gill pouch; MVA, median ventral aorta; 1-5: 1st (anteriormost) to 5th afferent branchial arteries.

Fig. 3. Dentition (palatine tooth and 2 cuspid rows) of *Eptatretus rubicundus* sp. nov., holotype ASIZP 60660, 464 mm total length. Scale = 1 mm.

arranged in a straight line with GAs (Fig. 3D).

Color: Body color of live specimen uniformly pink with no pale area around GAs, slime pores, or cloaca; no eyespots. Color of preserved specimen pale, light-orange with pale area around GAs and slime pores.

Distribution: Known only from holotype, caught off northeastern Taiwan (24°56.5'N, 121°53.0'E) by trawl, at 800 m depth.

Etymology: The name refers to the pink body color of the animal.

Phylogenetic analysis: The lengths of the 16S rDNA fragments sequenced were 563 (*E. stoutii*) to 570 (*E. rubicundus* sp. nov) nucleotides (excluding the primers), with their base compositions, indicating similar proportions among these species (Table 1). The similarity and divergence matrices obtained from pair-wise comparisons showed that *E. rubicundus* sp. nov. is far separated from other species by high divergences of 10.3%-11.9% (Table 3). Pair-wise divergence values observed between *P. cheni* and other taxa (i.e., 6.9%-11.9%) were much higher than those from comparisons

Table 3. Pairwise distance matrix for the 16S ribosomal RNA gene, with percentage similarities in the upper diagonal and percentage divergences in the lower diagonal

	E. burgeri	*E. stoutii*	*E. cirrhatus*	*P. cheni*	*Q. nelsoni*	*Q. yangi*
E. burgeri	***	92.5	96.5	87.7	94.9	96.6
E. stoutii	2.4	***	91.1	85.4	89.0	92.2
E. cirrhatus	2.7	2.9	***	87.6	94.3	94.0
P. cheni	7.4	6.9	7.3	***	86.3	88.8
P. nelsoni	1.6	2.2	2.7	7.9	***	95.2
Q. yangi	1.6	2.6	3.1	7.3	1.1	***
P. taiwanae	1.4	2.4	2.7	7.9	0.7	0.9
P. sheni	1.1	2.0	2.7	7.7	0.5	0.5
P. fernholmi	2.0	2.9	3.1	7.9	1.4	1.1
E. rubicunduss	11.1	11.2	10.3	11.9	10.5	11.3
M. glutinosa	10.5	10.0	9.7	12.4	10.4	11.0

	P. taiwanae	*P. sheni*	*P. fernholmi*	*E.rubicundus*	*M. glutinosa*
E. burgeri	95.8	98.1	97.0	86.1	84.6
E. stoutii	89.9	92.2	91.1	81.9	81.7
E. cirrhatus	93.3	95.2	94.9	86.4	85.3
P. cheni	86.6	87.8	87.5	82.2	82.9
P. nelsoni	97.5	96.8	95.9	85.3	82.3
Q. yangi	96.1	98.6	98.1	84.2	82.5
P. taiwanae	***	97.5	96.6	85.7	82.5
P. sheni	0.4	***	99.1	85.5	83.6
P. fernholmi	1.3	0.9	***	84.8	83.4
E. rubicunduss	10.7	10.7	11.7	***	83.2
M. glutinosa	10.6	10.5	11.0	12.8	***

among *Paramyxine* species. Most pairwise comparisons mentioned above had divergence values of < 12.8%. Since the 16S fragment reaches a saturation level at approximately 22% (Ortí and Meyer 1997), divergence values among the hagfish species studied appeared to be below the saturation level for this molecular fragment (Table 3).

When using the lamprey as the outgroup, the phylogenetic tree resolved from the Bayesian analyses (Fig. 4) was divided into 2 monophyletic clusters, the Myxininae and Eptatretinae. Looking at the latter cluster alone, the current new species, *E. rubicundus* sp. nov., was placed as the most basal species to the other eptatretine members that formed another clade (Fig. 4). In the latter clade, *P. cheni* was strongly recognized by the NJ tree as the plesiomorphic sister species to another clade including the remaining *Eptatretus*, *Quadratus*, and *Paramyxine* species analyzed (Fig. 4A). However, *P. cheni*'s most basal position in this clade received less support from the consensus tree produced from the other phylogenetic analytical methods; this tree only indicated that *P. cheni*, *E. strickrotti*, and *E. cirrhatus* were all basal species relative to the other remaining eptatretine species (Fig. 4B). Under the paradigm of cladistics, further subdivision of these complex clusters did not group well in terms of the designated genera which are required to be monophyletic.

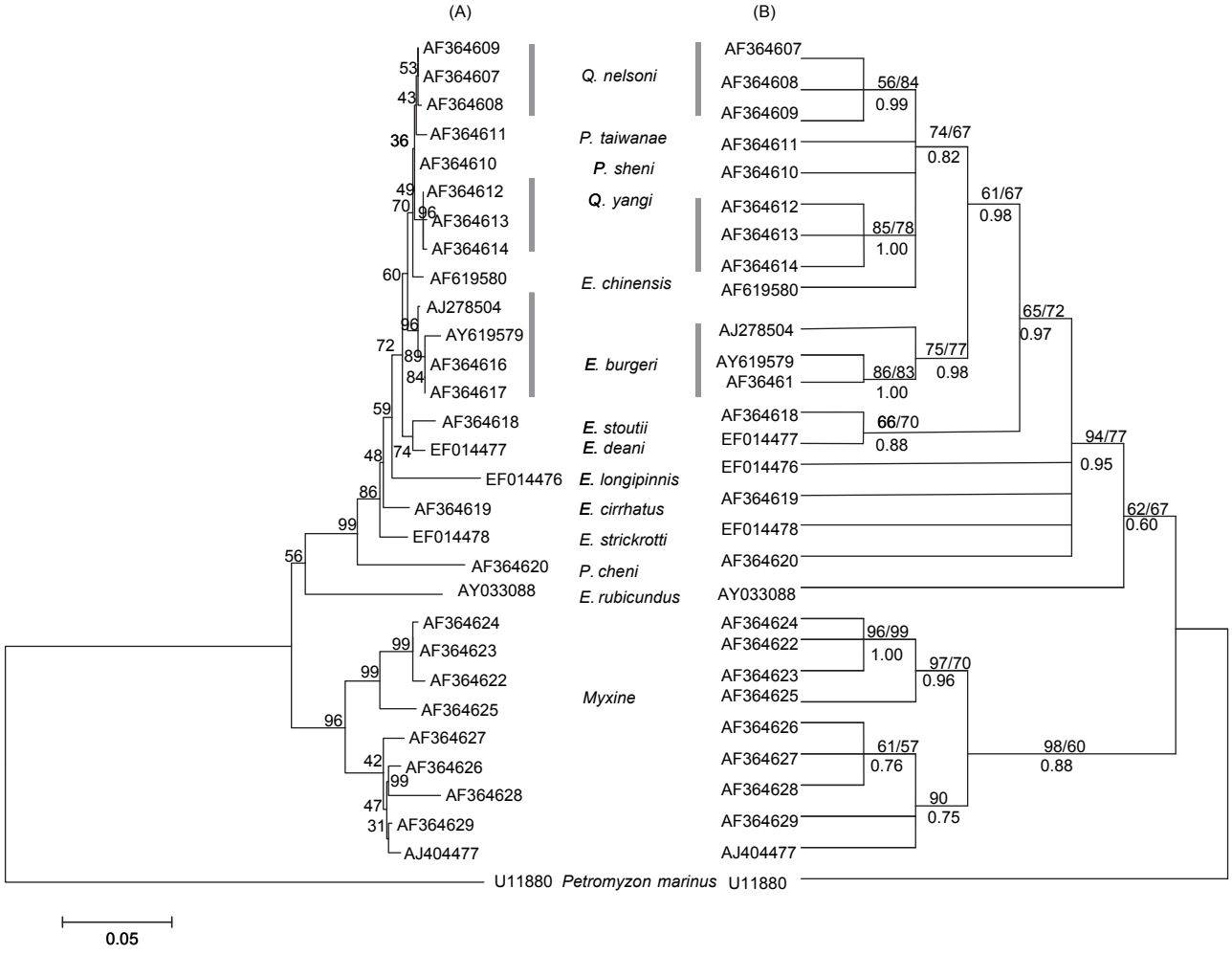

Fig. 4. Phylogenetic trees of hagfishes based on mitochondrial 16S rRNA sequence data, with trees rooted with the lamprey. (A) Phylogenetic tree of hagfish based on mitochondrial 16S rRNA sequence data, constructed with the Neighbor-joining (NJ) method using Kimura 2-parameter distances. Numbers above the branches are bootstrap values based on 1000 replicates. (B) Consensus maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian analysis trees. Numbers above the branches are bootstrap values based on 100 replicates in the MP (1st number) and ML (2nd number) analyses. Numbers below the branches are posterior probabilities recovered by the Bayesian analysis.

The DNA sequence in GenBank used by Kuraku and Kuratani (2006) for the hagfish genus *Rubicundus* which was initially considered to be a new genus by the present authors (CHK and SCL; unpubl. data) should be included under *Eptatretus*.

DISCUSSION

The 5-gilled, deep-sea *E. eos* (Fernholm, 1991) and *E. rubicundus* sp. nov., and the 12-gilled *E. strickrotti* from the East Pacific Rise as a whole differ from all other previously described hagfishes in having a pink body color. However, *E. rubicundus* sp. nov. and *E. strickrotti* can easily be distinguished by the numbers of GP and GA (5 vs. 12). *Eptatretus rubicundus* sp. nov. and *E. eos* can be distinguished from one another by the number of slime pores, with 100-102 and 128-130, respectively. Between them, *E. rubicundus* sp. nov. has 16 or 17 and 19 pores in the prebranchial and tail regions, respectively, compared to 26 and 26 or 27 in *E. eos*. Another distinctive character between the 2 species is the body shape, being slender in *E. eos* and robust in *E. rubicundus* sp. nov. (Table 2).

Speciation occurring among *E. rubicundus* sp. nov., *E. strickrotti*, and *E. eos* is an interesting event, since they live in such deep-sea environments at 800, 2211, and 1000 m, respectively. However, they share several common characters such as a pink body color and 3-cusp multicusps on the anterior row of teeth. Biogeographically; however, their far-distant habitats present a natural isolation barrier favoring speciation. Hagfishes spend most of their lives either within burrows or hiding while emerged, and they have weak mobility when feeding or avoiding unpleasant stimuli (Strahan 1963). The weak mobility may confine their migration distances. The large size of their demersal eggs may limit their dispersal and thus genetic flow. Different habitats, environments, and life histories enable the above 2 allopatric hagfishes to enhance their speciation compared to the 12 *Eptatretus* species reviewed by Fernholm (1998).

Eptatretus rubicundus sp. nov. can also be separated from 3 other Western Pacific congeners, *E. burgeri*, *E. chinensis*, and *E. okinoseanus*, by the number of gill apertures (5 vs. 6, 6, and 8 pairs, respectively). *Eptatretus rubicundus* sp. nov. differs from species under the previously recognized genus *Paramyxine* in the same region by its 6 gill apertures and their arrangement pattern, and the absence of slime pores in the branchial region together with the body color in the latter species.

The phylogenetic positions of *P. cheni*, *E. strickrotti*, *E. cirrhatus*, and *E. longipinnis* shown in figure 4 deserve special attention when the above-mentioned tree is compared to the one proposed by Møller and Jones (2007), who erected a new species, *E. strickrotti*, in that paper. Most of the 13 eptatretine and 5 *Myxine* species compared in their paper were also analyzed in the present paper. The profile of the phylogenetic trees given in the present paper differs from that by Møller and Jones (2007) who placed *E. strickrotti* at the most-basal position in the eptatretine clade (i.e., more primitive than *P. cheni*). Our results support *E. rubicundus* sp. nov. being the plesiomorphic sister species to a clade including all of the remaining eptatretine species analyzed in this paper. Within the latter clade, the possibility exists that *P. cheni* is the most-plesiomorphic sister species to the remaining species (including *E. strickrotti*) that form a monophyletic group (Fig. 4A). However, our consensus tree indicates that within this clade, *P. cheni*, *E. stickrotti*, *E. cirrhatus*, *E. longipinnis*, and a new clade comprising the remaining eptatretines are the 5 lineages (Fig. 4B) the detailed phylogenetic relationships of which are to be resolved. Møller and Jones (2007) also reached a different conclusion regarding the phylogenetic position of *E. longipinnis*; it was more-closely related to *Q. yangi* than the latter to other *Quadratus* and *Paramyxine* species; their conclusion does not seem very likely to us. Crucial differences between the conclusions of these 2 studies concern the relative phylogenetic positions of *P. cheni* and *E. strickrotti*; more data are needed to resolve this phylogenetic issue.

Kuo et al. (1994) reported that *Eptatretus* and *Paramyxine* differ in the number of slime pores in the branchial region. In *Eptatretus*, each branchial aperture is usually associated with a slime pore close to its medial side; the absence of 2 slime pores was noted on the right side of an *E. burgeri* specimen. However, 50% of all of the branchial apertures in the latter genus lack an accompanying slime pore. In *Myxine*, there is a slime pore on the external side of the common branchial aperture. Kuo et al. (1994) hypothesized that the absence of slime pores in the branchial region is an apomorphic character state for myxinids. The most-basal phylogenetic position of *E. rubicundus* sp. nov. was indicated by 2 characters. In 1 character, the relative positions

of the branchial slime pores and gill aperture, the slime pore(s) are neither medial nor external to the gill aperture(s) but alternatively placed in a straight line. The character state in *E. rubicundus* sp. nov. can be considered an intermediate one between the conditions found in myxinines (slime pore external to the common branchial aperture) and other eptatretines (slime pores medial to the branchial apertures). The other character concerns the bifurcation of the ventral aorta. Mok and McMillan (2004) supported the monophyly of the Eptatretinae by a synapomorphic character of having the ventral aorta bifurcating at a more-posterior site, such that at least the anterior 2 pairs of afferent branchial arteries are associated with the side branches of the ventral aorta (i.e., the remaining afferent branchial arteries are on the medial ventral aorta). Within the subfamily Eptatretinae, the transformation series has evolved toward the bifurcation site being closer to the heart, leaving fewer or no afferent branchial arteries on the medial section of the ventral aorta (Mok and McMillan 2004). For example, in *P. cheni* and *E. strickrotti*, 2 basal eptatretine species as indicated by our genetic evidence (Kuo at al. 2003, Møller and Jones 2007; Fig. 4), only the anteriormost and 2nd afferent branchial arteries are associated with the side branch of the ventral aorta as a result of the site of bifurcation being placed further anteriorly, leaving the remaining afferent branchial arteries in the medial section of the ventral aorta. Unexpectedly, the ventral aorta of *E. rubicundus* sp. nov., like *Myxine* species, does not bifurcate: a condition not occurring in any eptatretine and which was treated as a plesiomorphic character state for the Myxinidae by Mok and McMillan (2004). As such, *E. rubicundus* sp. nov. shares a symplesiomorphic character state with *Myxine* species. Despite this similarity, which is a primitive character state, its sister-group relationship with *Myxine* cannot be supported. In this regard, *E. strickrotti* is more closely related to the remaining epatretines than to *E. rubicundus* sp. nov. by having a bifurcated ventral aorta. The molecular evidence shown in figure 4 indicates a similar result.

Wisner (1999) erected a new genus, *Quadratus*, and moved 4 *Paramyxine* species into the new genus. *Quadratus* is characterized by having a clump-like arrangement of gill apertures. The phylogenetic trees constructed in the present study include *Quadratus* species and some *Paramyxine* ones as a derived cluster within the Eptatretinae (Fig. 4). The group with a much-longer 1st efferent duct than the last and the absence of slime pores in the branchial region can be designated the subfamily Paramyxininae which may include *Quadratus* and *Paramyxine*. As evidenced from molecular data and the slime pore alignment, the above 2 genera would be better combined into 1 group. As the monophylies of *Paramyxine* and *Quadratus* are not supported by the present dataset, we tend to favor the notion of Fernholm (1998) that only 1 genus, *Eptatretus*, should be retained in the subfamily Eptatretinae.

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REFERENCES

Adam H, R Strahan. 1963. Systematics and geographical distribution of myxinoids. *In* A Brodal, F Fange, eds. The biology of *Myxine*. Oslo: Univeritetforlaget, pp. 1-8.

Chen YW, HW Chang, HK Mok. 2005. Phylogenetic position of *Eptatretus chinesis* (Myxinidae: Myxiniformes) inferred by 16S rRNA gene sequence and morphology. Zool. Stud. **44:** 111-118.

Dean B. 1904. Notes on Japanese myxinoids. A new *Paramyxine* and a new species *Homea okinoseana*. Reference also to their eggs. J. Coll. Sci. Imp. Univ. Tokyo **19:** 23 pp.

Felsenstein J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Fernholm B. 1991. *Eptatretus eos*: a new species of hagfish (Myxinidae) from the Tasman Sea. Jpn. J. Ichthyol. **38:** 115-118.

Fernholm B. 1998. Hagfish systematics. *In* JM Jørgensen, JP Lomholt, RE Weber, H Malte, eds. The biology of Hagfish. London: Chapman and Hall, p. 578.

Fernholm B, C Hubbs. 1981. Western Atlantic hagfishes of the genus *Eptatretus* (Myxinidae) with description of two new species. Fish. Bull. **79:** 69-83.

Forey PL, P Janvier. 1993. Agnathans and the origin of jaw vertebrates. Nature **361:** 129-134.

Hasegawa M, H Kishino, T Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22:** 160-174

Huelsenbeck JP, F Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics **17:** 754-755.

Jansson H, PI Wyoni, B Fernholm, M Bredwad, A Mierzykowaka, H Tegelstrom. 1995. Genetic relationships among species of hagfish revealed by protein electrophoresis. J. Fish. Biol. **47:** 599-608.

Jukes TH, CR Cantor. 1969. Evolution of protein molecules. In Munro HN, eds, Mammalian Protein Metabolism, Academic Press, New York, pp. 21-132.

Kocher TD, WK Thomas, A Meyer, SV Edwards, S Paddo, FX Villablanca, AC Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA **86:** 6196-6200.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16:** 111-120.

Kumar S, K Tamura, M Nei. 1994. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. Comput Appl Biosci. **10:**189-91.

Kuo CH. 1991. Morphological and genetic variations in hagfishes on the coastal water of Taiwan. Master's thesis, National Sun Yat-sen Univ., Kaohsiung, Taiwan, 55 pp.

Kuo CH, H Huang, SC Lee. 2003. Phylogeny of hagfish based on the mitochondrial 16S rRNA gene. Mol. Phylogenet. Evol. **28:** 448-457.

Kuo CH, KF Huang, HK Mok. 1994. Hagfishes of Taiwan. I-A taxonomic revision with a description of four new *Paramyxine* species. Zool. Stud. **33:** 126-139.

Kuraku S, S Kuratani. 2006. Time scale for cyclostome evolution inferred with a phylogenetic diagnosis of hagfish and lamprey cDNA sequences. Zool. Sci. **23:** 1053-1064.

McMillan CB, RL Wisner. 1984. Three new species of seven-gilled hagfishes (Myxinidae, *Eptatretus*) from the Pacific Ocean. CA Acad. Sci. **43:** 249-267.

McMillan CB, RL Wisner. 2004. Review of the hagfishes (Myxinidae, Myxiniformes) of the northwestern Pacific Ocean, with descriptions of three species *Eptatretus fernholmi*, *Paramyxine moki* and *P. walkeri*. Zool. Stud. **43:** 51-73.

Mok HK. 2002. *Myxine koui*, a new species of hagfish from southwestern Taiwan. Zool. Stud. **41:** 59-62.

Mok HK, CH Kuo. 2001. *Myxine formosana*, a new species of hagfish (Myxiniformes: Myxinidae) from the southwestern waters of Taiwan. Jpn. J. Ichthyol. **48:** 295-297.

Mok HK, CB McMillan. 2004. Bifurcating pattern of the ventral aorta and distribution of the branchial arteries of hagfishes (Myxiniformes), with notes on the taxonomic implications. Zool. Stud. **43:** 737-748.

Møller PR, WJ Jones. 2007. *Eptatretus strickrotti* n. sp. (Myxinidae): first hagfish captured from a hydrothermal vent. Biol. Bull. **212:** 55-66.

Nelson JS. 2006. Fishes of the world, 4th ed. New York: J. Wiley, p. 22.

Ortí G, A Meyer. 1997. The radiation of characiform fishes and the limits of resolution of mitochondrial ribosomal DNA sequences. Syst. Biol. **46:** 75-100.

Palumbi S, A Martin, S Romano, WO McMillan, L Stice, G Grabowski. 1991. The simple fool's guide to PCR, vers. 2. Honolulu, HI: Department of Zoology and Kewalo Marine Laboratory, Univ. of Hawaii.

Posada D, KA Crandall. 1998. MODEL TEST: testing the model of DNA substitution. Bioinformatics **14:** 817-818.

Roquist PR, JP Huelsenbeck. 2003. Mr. Bayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572-1574.

Saitou N, M Nei. 1987. The Neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.

Shen SC, HJ Tao. 1975. Systematic studies on the hagfish (Eptatretidae) in the adjacent waters around Taiwan with description of two new species. Chin. Biosci. **2:** 65-79.

Stock DW, GS Whitt. 1992. Evidence from 18S ribosomal RNA sequences that lampreys and hagfishes form a natural group. Science **257:** 787-789.

Strahan R. 1963. The behaviour of myxinoids. Acta Zool. **44:** 1-30.

Strahan R. 1975. *Eptatretus longipinnis*, n. sp., a new hagfish (Family Eptatretidae) from south Australia, with a key to the 5-7 gilled Eptatretidae. Aust. Zool. **18:** 137-148.

Teng HT. 1958. A new cyclostome from Taiwan. Chin. Fish. Month. **66:** 3-6.

Tajima F, M Nei. 1984. Estimation of evolutionary distance between nucleotide sequences. Mol. Biol. Evol. **1:** 269-285.

Thompson JD, DG Higgins, TJ Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:** 4673-4680.

Wisner RL. 1999. Descriptions of two new subfamilies and a new genus of hagfishes (Cyclostomata: Myxinidae). Zool. Stud. **38:** 307-313.

Wisner RL, CB McMillan. 1988. A new species of hagfish, genus *Eptatretus* (Cyclostoma, Myxinidae) from the Pacific Ocean near Valparaiso, Chile, with new data on *E. bischoffii* and *E. polytrema*. Trans. San Diego Soc. Nat. Hist. **21:** 227-244.