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A New *Anguilla* Species and a Reanalysis of the Phylogeny of Freshwater Eels

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Hui-Yu Teng, Yeong-Shin Lin, and Chyng-Shyan Tzeng (2009) A new *Anguilla* species and a reanalysis of the phylogeny of freshwater eels. *Zoological Studies* **48**(6): 808-822. Evidence of a new species of Anguillid eel, *Anguilla huangi* sp. nov., was obtained from examining eel specimens collected from the Cagayan River estuary, northern Luzon I., the Philippines. Both glass eels and cultured eels reared in Taiwan for 5 yr were examined. The morphological measurements of *A. huangi* sp. nov. overlap with those of *A. celebesensis* Kaup, 1856 and *A. interioris* Whitley, 1938, so that this new species cannot be distinguished using morphological characteristics alone. We sequenced its complete mitochondrial genome and constructed a phylogeny of all currently recognized freshwater eel species and subspecies based on both molecular and morphological data. Although *A. huangi* sp. nov. seemed to be morphologically more similar to *A. celebesensis*, the molecular phylogeny showed a strongly supported clustering of *A. huangi* sp. nov. and *A. interioris*. The genetic distance between *A. huangi* sp. nov. and *A. interioris* was similar to that between *A. anguilla* (Linnaeus, 1758) and *A. rostrata* (Le Sueur, 1817). The distinct molecular phylogeny ensures the specific status of this new freshwater eel. On the other hand, based on the likelihood analyses, the phylogeny of freshwater eels seems to be better represented by polytomies. These polytomies are likely derived from multiple radiation (rapid speciation) events. We further inferred that each radiation event may also have included a large-scale expansion in distribution. The morphological phylogenetic tree generated using the minimum network (MinNet) method was generally consistent with the molecular phylogeny. We also propose a possible evolutionary history for freshwater eels. The Central American Isthmus hypothesis for the dispersal route of the Atlantic eels was supported by various lines of evidence. http://zoolstud.sinica.edu.tw/Journals/48.6/808.pdf

Key words: New species, *Anguilla huangi*, The Philippines, Morphology, Mitochondrial genome.

Freshwater eels are catadromous, spawn in tropical ocean waters, and have a peculiar leptocephalus larval stage that is unique to elopomorph fish. They constitute a single genus, *Anguilla* Schrank 1798. As a whole, different species of freshwater eels are extremely similar in morphology. With 25,265 available *Anguilla* specimens collected worldwide, Ege (1939) was able to name 16 species (and 3 subspecies) by systematic analyses. Castle and Williamson (1974) suggested that *A. ancestralis* Ege, 1939 was a synonym of *A. celebesensis* Kaup, 1856, and therefore reduced the genus to 15 species. Three new species, *A. breviceps* Chu and Jin, *A. foochowensis* Chu and Jin, and *A. nigricans* Chu and Wu were described (Chu 1984) from China. However, their identifications were only based on the external morphology and body proportions of single individuals, so a reexamination of these doubtful species was suggested (Tabeta 1994). Recently, Watanabe et al. (2004) reexamined 1713 specimens that consisted of 1497 collected

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specimens and 216 museum specimens. They identified 15 species, basically consistent with Ege's (1939) milestone study.

In addition, based on 12 morphological characters, Ege (1939) constructed a phylogenetic synopsis for the genus *Anguilla*. Species with a short dorsal fin and those without variegated markings were thought to have been derived from a common ancestor. Although that milestone study has been widely accepted for 60 yr (e.g., Aoyama et al. 1996, Aoyama and Tsukamoto 1997, Tsukamoto and Aoyama 1998, Bastrop et al. 2000, Watanabe et al. 2004), recent molecular phylogenetic analyses showed conflicting results (Aoyama et al. 2001, Lin et al. 2001, Minegishi et al. 2005), and suggested that some morphological characteristics that Ege (1939) used to subdivide eel groups (i.e., without variegated markings, with a short dorsal fin, or with a toothless longitudinal groove in the maxillary and mandibular bands of the teeth) might not be synapomorphic as he proposed but symplesiomorphic or convergent (Lin et al. 2001 2005). Lin et al. (2005) further indicated that when appropriate methods are employed, morphological data can actually give similar phylogenetic trees for freshwater eels as do molecular data.

In an earlier phylogenetic analysis, we examined the molecular and morphological characters of a collection of 9 freshwater glass eels from Luzon, the Philippines (courtesy of Prof. W.N. Tzeng, National Taiwan Univ., Taipei, Taiwan) (Lin 1998). Five species were recorded in a nearby area (around the Philippines and Borneo), i.e., *A. celebesensis*; *A. marmorata* Quoy and Gaimard; 1824, *A. bicolor* (including *A. bicolor bicolor* McClelland, 1844 and *A. bicolor pacifica* Schmidt, 1928); *A. japonica* Temminck and Schlegel, 1846; and *A. malgumora* Kaup, 1856 (a synonym of *A. borneensis* Popta, 1924) (Herre 1923 1958, Ege 1939, Tabeta et al. 1975 1976, Briones et al. 2007, Jamandre et al. 2007). The mtDNA sequences of the first 4 species (Aoyama et al. 1996, Lin 1998) differed from those of the 9 glass eels (Lin 1998). On the other hand, these glass eels were close to *A. malgumora* on the basis of the limited available morphological characters (the number of vertebrae and the distance between the verticals through the anus and origin of the dorsal fin) (Lin 1998). This identification was adopted in the following phylogenetic studies (Lin et al. 2001 2002 2005). However, Aoyama et al. (2001) also reported the mtDNA sequence for *A. malgumora*, which differed from what we reported for the 9 Luzon glass eels described above (Lin 1998, Lin et al. 2001), suggesting that eel specimens studied by these 2 groups belong to different species (Lin et al. 2002, Aoyama 2003).

In this study, we examined more specimens from both a cultured yellow eel stock and a school of glass eels, and report the first identification of a new *Anguilla* species in 70 yr. The complete mitochondrial genome of the holotype of this new species was determined. We constructed a phylogeny for all currently recognized *Anguilla* species, and propose a possible evolutionary history for freshwater eels.

MATERIALS AND METHODS

Sample collection

In 2003, a Taiwanese fish farmer imported a school of glass eels from the Cagayan River estuary, northern Luzon, the Philippines (Fig. 1) for experiments in aquaculture. Many of these eels could be reared to more than 1200 mm from head to tail, weighing over 7 kg, in 5 yr, in contrast to most *Anguilla* species which only reach about 600 mm in nature. Upon a rapid fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified mtDNA fragments following Lin et al. (2002), 3 species were identified among these cultured eels. Two of them were *A. marmorata* and *A. bicolor pacifica*. The 3rd one was a group of specimens with variegated markings. Surprisingly, these specimens showed the RFLP pattern identical to that of the 9 putative glass eels that we examined in 1998. Apparently they cannot be *A. malgumora* because *A. malgumora* does not have variegated markings. We therefore collected 73 specimens, both young and immature, of cultured eels for further analyses. Among them, 10 specimens identified as the new *Anguilla* sp. were deposited in the Biodiversity Research Museum, Academia Sinica, Taipei, Taiwan, with specimen IDs of ASIZP0069360-9. Moreover, 388 glass eel specimens collected by Prof. W.N. Tzeng's group from the estuary of the Cagayan River in Aug. 2008 were also examined.

Morphological character measurements

The morphological characters of these cultured yellow eel specimens were measured following Watanabe et al. (2004): L_T, total length;

L_{PA}, preanal length; L_H, head length; L_{PD}, predorsal length; L_{TR}, length of the trunk, calculated as L_{PA} minus L_H; L_{AD}, distance between the verticals through the anus and origin of the dorsal fin; L_{PDH}, L_{PD} minus L_H; L_{EG}, distance from the perpendicular through the eye-center on the border of the upper jaw to the angle or corner of the gape; L_G, distance from the tip of the lower jaw to the corner of the mouth itself; L_M, length of the left maxillary band; L_V, length of the intermaxillary-vomerine band; L_{WMM}: width of the mid-part of the maxillary band; N_{MM}, number of teeth of the mid-part of the maxillary band; N_{TV}, total number of vertebrae; and N_{PV}, number of prehaemal vertebrae.

Sequencing of the mitochondrial genes

In total, 31 fish-versatile and 7 *Anguilla* species-specific primers, which were newly designed, were used in various combinations to

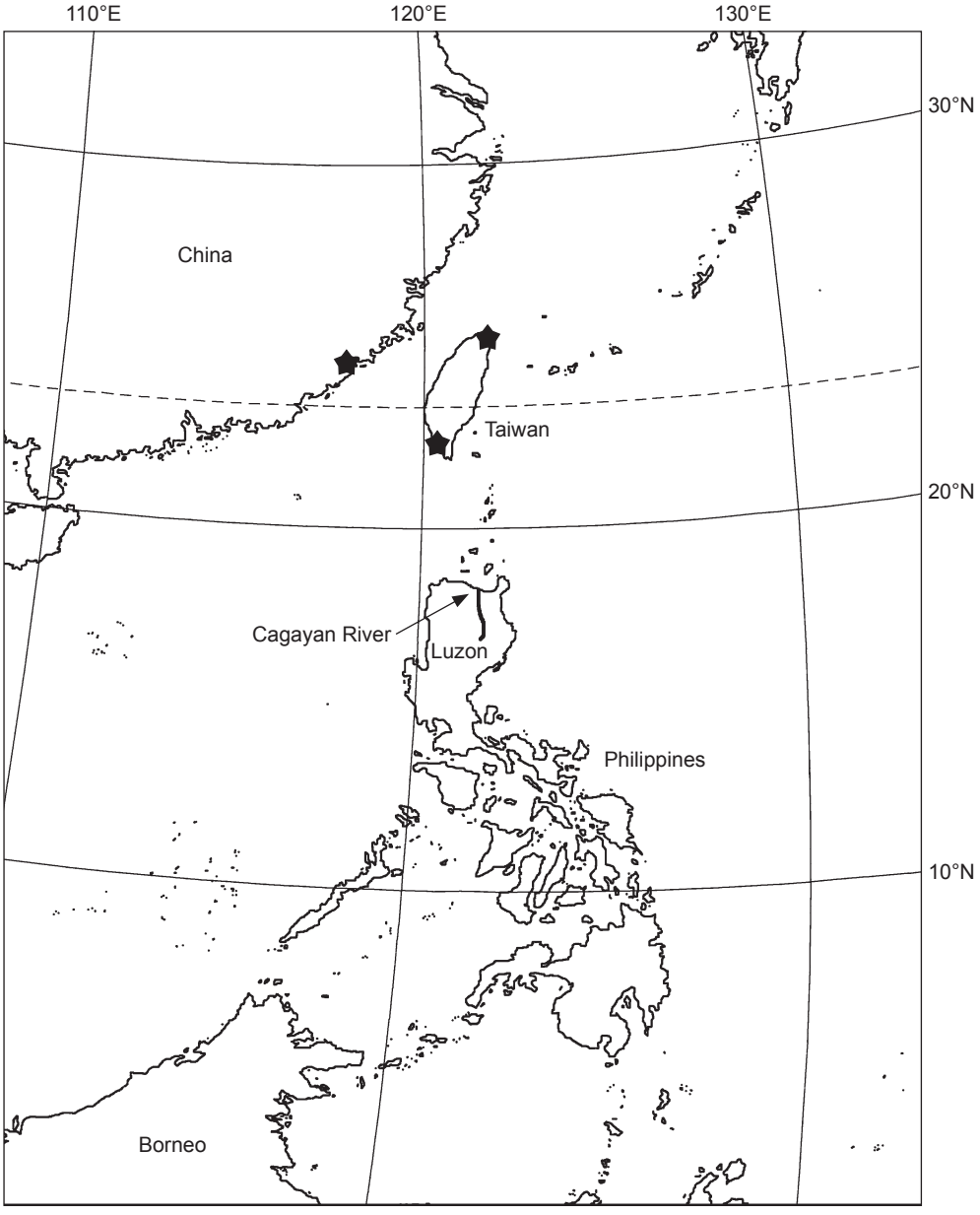

Fig. 1. Cultured *Anguilla huangi* sp. nov. specimens originally collected from the Cagayan River estuary, the Philippines as indicated by the arrow. The 388 glass eel specimens obtained from Prof. W.N. Tzeng's group were collected from the same place. The localities in Taiwan and China where *A. celebesensis* elver specimens were reported (Tzeng 1982, Lue et al. 1999) are marked by stars.

amplify fragments covering the entire mitochondrial genome of the holotype of the new *Anguilla* sp. (specimen ID ASIZP0069360). The genomic sequence was submitted to GenBank with accession number EU917054. Amplifications of the mitochondrial cytochrome *b* gene of the other specimens were performed using primers L15239 and H16468 of Lin et al. (2001). The accession numbers of cytochrome *b* sequences for specimens ASIZP0069360-9 are FJ170069-FJ170073 and FJ170064-8, respectively. The primer sequences used and the conditions for the PCR and sequencing reactions are available upon request.

Construction of the molecular phylogeny

The complete mitochondrial genome sequences of the other 18 *Anguilla* species and subspecies were obtained from Minegishi et al. (2005), including *A. anguilla* (Linnaeus, 1758); *A. australis australis* Richardson, 1841; *A. australis schmidtii* Phillipps, 1925; *A. bengalensis bengalensis* (Gray, 1830) (a synonym of *A. nebulosa* McClelland, 1844); *A. bengalensis labiata* Peters, 1852; *A. bicolor bicolor* McClelland, 1844; *A. bicolor pacifica* Schmidt, 1928; *A. celebesensis* Kaup, 1856; *A. dieffenbachii* Gray, 1842; *A. interioris* Whitley, 1938; *A. japonica* Temminck and Schlegel, 1846; *A. malgumora* Kaup, 1856; *A. marmorata* Quoy and Gaimard, 1824; *A. megastoma* Kaup, 1856; *A. mossambica* Peters, 1868; *A. obscura* Günther, 1871; *A. reinhardtii* Steindachner, 1867; and *A. rostrata* (Le Sueur, 1817). Each of the 13 mitochondrial protein coding sequences had identical lengths among all *Anguilla* species and could therefore readily be aligned. Termination codons were excluded. Synonymous (K_S) and nonsynonymous (K_A) substitutions were estimated using the method of Li (1993) and Pamilo and Bianchi (1993) as implemented in MEGA 4 (Tamura et al. 2007). The good linear correlation between K_S and K_A in the genus *Anguilla* (data not shown) and the small K_S values (all < 0.35) suggest that synonymous substitutions are far from saturated. We therefore used synonymous distances to construct the Neighbor-joining (NJ) tree (Saitou and Nei 1987). We also used the PHYML online web server (Guindon and Gascuel 2003, Guindon et al. 2005) to build the maximum-likelihood (ML) tree (Felsenstein 1981) based on the GTR + I + Γ4 substitution model (Rodriguez et al. 1990, Yang 1994). The bootstrap test (Felsenstein 1985) was performed for both methods. We did not include mitochondrial rRNA or tRNA genes in our analyses. The loop regions were sometimes too divergent to be properly aligned. The control region was excluded for the same reason.

In the NJ and ML trees generated, we found 3 potential polytomies (with 4, 5, and 5 branches, respectively). A set of all possible bifurcating trees (3 × 15 × 15 = 675) were created from these 3 polytomies. We used CODEML implemented in PAML 4 (Yang 2007a) to calculate and compare the log-likelihood values for this set of trees under the M0, M1a, M2a, M3, M7, and M8 models. As suggested by Goldman et al. (2000), the K-H test (Kishino and Hasegawa 1989) was originally devised and applied to trees that were specified as being a priori. Using the K-H test to compare the ML tree derived from the data to either a priori-specified trees or a posteriori-specified trees is inappropriate. We therefore used the S-H test (Shimodaira and Hasegawa 1999) to simultaneously compare these 675 possible bifurcating trees instead.

Tree reconstruction using morphological data

Morphological character measurements were obtained from Ege (1939) except for specimens of the new *Anguilla* sp. Although the induced characters were designed to eliminate the effects of body growth and make the characters stable, the proportions of some characters still significantly vary with sexual maturation (Lin et al. 2005). Following Lin et al. (2005), we used only character measures for eels of identical body length, LT, which implies a similar age. In order to include all possible information, we used a linear regression to obtain interpolated values. We considered all characters for L_T = 300 mm (misstated as 200 mm in Lin et al. 2005); henceforth, all characters referred to are those with L_T = 300 mm. Eels of this length have passed the elver stage but have not matured sexually. The measurements for each species and character were standardized following Lin et al. (2005). Note that all specimens of *A. bengalensis labiata* in Ege (1939) were larger than 500 mm. In this circumstance, for characters varying significantly with sexual maturation, a reasonable linear regression might not easily be obtained. We therefore excluded the measurement of head length, L_H/L_T, for *A. bengalensis labiata* from this study.

During evolution, morphological transformation can be expressed as a trajectory in the

feature space. Therefore, we constructed a network with all operational taxonomic units (OTUs) at external nodes to represent the phylogenetic tree. The conventional strategy to find a tree that may reflect the actual evolutionary history is to find one with the shortest tree length. The minimum-network (MinNet) method based on evolutionary computations (Foster 2001) was designed for this purpose (Lin et al. 2005). We modified the MinNet method by a simple alternative step in this study; i.e., for a species with character deficiencies, we let its deficient character have a flexible value instead of removing this character from the analysis. In other words, a value that can minimize the total tree length was assigned to that deficient character of that species. The purpose was to include all species and as much information as possible when constructing the morphological phylogenetic tree. This modification is only applicable to MinNet and not to the distance matrix method (Lin et al. 2005).

RESULTS

Anguilla huangi sp. nov.

Holotype: ASIZP0069360, 1000 mm in L_T, the Philippines, Luzon I., Cagayan River (Figs. 2, 3).

Paratypes: ASIZP0069361-9, 9 specimens, 219.0-507.7 mm in L_T, same data as for holotype.

Diagnosis: As indicated by Watanabe et al. (2004), most morphological characters overlap in most or all *Anguilla* species. They even found that 5 species pairs in the 15 currently confirmed *Anguilla* species had overlapping morphological character ranges and geographic distributions. Generally, *A. huangi* sp. nov. can be distinguished from the other species by the following combination of characters: (1) variegated markings on the skin; (2) undivided maxillary bands of the teeth; (3) the width of the mid-part of the maxillary band to the length of the maxillary band

Fig. 2. Holotype of *Anguilla huangi* sp. nov. (ASIZP0069360, 1000 mm in total length). (A) Aquarium photograph. (B) A preserved specimen. (C) An x-ray picture, which was pieced together from 3 x-ray films.

is > 14%; (4) the number of teeth of the mid-part of the maxillary band is ≤ 4; (5) the total number of vertebrae is between 103 and 106; and (6) the proportion of the distance between the dorsal fin and anus to the total length is usually < 12.5% and > 10%. However, it is still difficult to distinguish among *A. huangi* sp. nov., *A. celebesensis*, and *A. interioris* on the basis of these morphological characters (see also Silfvergrip 2009). On the contrary, the sequence homology test showed that the 13 mitochondrial protein coding sequences of *A. huangi* sp. nov. differ from those of the 15 known species of freshwater eels (Aoyama et al. 2001, Lin et al. 2001 2002, Minegishi et al. 2005), thus it represents a distinct taxon (Fig. 4). The synonymous distance between *A. huangi* sp. nov. and *A. interioris* (10.7%) was similar to that between *A. anguilla* and *A. rostrata* (10.1%), 2 distinct species. All of the examined *A. huangi* sp. nov. specimens clustered together in the phylogeny constructed using cytochrome *b* sequences (data not shown). Using the restriction enzyme, *Dpn*II, to digest a 1230 bp PCR product (amplified using primers L15239 and H16468 following Lin et al. 2001) produced an 1132 bp fragment for *A. huangi* sp. nov. that was unique in the genus *Anguilla* (Lin et al. 2002).

Description: Figure 2 displays photographs and x-ray films of the holotype, ASIZP0069360. Variegated markings on the skin are present. No toothless longitudinal groove is present in the maxillary or mandibular bands of teeth (Fig. 3). Measurements for both holotype and paratypes are listed in table 1. Proportions of preanal length to total length (L_{PA}/L_T): 36%-43% (39.4% ± 1.7%); head length to total length (L_H/L_T): 11.5%-13.5% (12.3% ± 0.6%); length of the intermaxillary-vomerine band of teeth to the length of the maxillary band (L_V/L_M): 76%-110% (92.5%

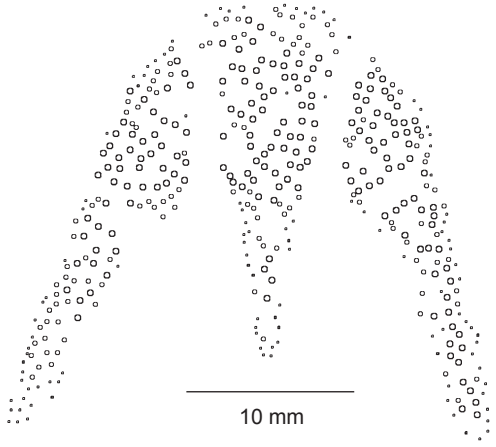

Fig. 3. Upper-jaw teeth-bands of the holotype of *Anguilla huangi* sp. nov.

Table 1. Morphological measurements for the holotype and 9 paratypes of *Anguilla huangi* sp. nov.

Specimen	L_T	L_{PA}	L_H	L_{PD}	L_{TR}	L_{AD}	L_{PDH}	L_{EG}	L_G	L_M	L_V	L_{WMM}	N_{MM}	N_{TV}	N_{PV}
Holotype															
ASIZP0069360	1000.0	425.0	130.0	302.0	295.0	123.0	172.0	12.5	35.0	26.1	22.0	3.9	4	106	40
Paratypes															
ASIZP0069361	507.7	213.3	68.0	156.0	145.3	57.3	88.0	6.5	20.3	14.2	11.5	2.1	4	106	41
ASIZP0069362	385.0	153.5	47.0	108.7	106.5	44.8	61.7	3.5	15.3	9.4	7.2	1.8	4	106	41
ASIZP0069363	360.6	139.3	42.2	98.0	97.1	41.3	55.8	3.3	11.2	8.3	9.1	1.9	4	104	40
ASIZP0069364	325.7	128.8	41.0	89.0	87.8	39.8	48.0	2.4	10.0	7.0	7.0	1.2	4	103	41
ASIZP0069365	303.2	120.0	36.4	83.8	83.6	36.2	47.4	1.9	9.2	6.8	5.8	1.4	4	104	41
ASIZP0069366	233.9	89.0	27.8	67.1	61.2	21.9	39.3	1.2	7.7	5.1	4.6	1.1	4	106	41
ASIZP0069367	219.0	80.8	25.5	60.0	55.3	20.8	34.5	1.1	10.0	4.5	4.7	1.0	4	105	40
ASIZP0069368	254.0	98.6	30.5	70.0	68.1	28.6	39.5	2.6	9.8	6.1	6.1	1.1	3	104	40
ASIZP0069369	219.0	84.0	27.0	61.9	57.0	22.1	34.9	2.4	9.1	4.4	4.1	0.9	4	105	41

L_T, total length; L_{PA}, preanal length; L_H, head length; L_{PD}, predorsal length; L_{TR}, length of the trunk, calculated as L_{PA} minus L_H; L_{AD}, distance between the verticals through the anus and origin of the dorsal fin; L_{PDH}, L_{PD} minus L_H; L_{EG}, distance from the perpendicular through the eye-center on the border of the upper jaw to the angle or corner of the gape; L_G, distance from the tip of the lower jaw to the corner of the mouth itself; L_M, length of the left maxillary band; L_V, length of the intermaxillary-vomerine band; L_{WMM}: width of the mid-part of the maxillary band; N_{MM}, number of teeth of the mid-part of the maxillary band; N_{TV}, total number of vertebrae; and N_{PV}, number of prehaemal vertebrae. * The unit of length is mm.

± 10.8%); length of the trunk to total length (L_{TR}/L_T): 25%-30% (27.1% ± 1.3%); distance from a perpendicular through the eye-center on the margin of the upper jaw and the angle of the gape to the length of the gape (L_{EG}/L_G): 11%-36% (24.4% ± 7.4%); distance between verticals through the anus and origin of the dorsal fin to the total length (L_{AD}/L_T): 9%-12.5% (11.1% ± 1.1%); predorsal length without the head length to total length (LPDH/LT): 14.5%-17.5% (16.0% ± 0.8%); length of the gape to head length (L_G/L_H): 24%-40% (29.8% ± 4.6%); and width of the mid-part of the maxillary band to the length of the maxillary band (L_{WMM}/L_M): 14.5%-23% (19.2% ± 2.9%). The number of teeth of the mid-part of the maxillary band (N_{MM}): 3 or 4 (3.9 ± 0.3). Total number of vertebrae (N_{TV}): 103-106 (104.9 ± 1.1). The number of prehaemal vertebrae (N_{PV}): 40 or 41 (40.6 ± 0.5).

In Ege's (1939) taxonomic system, *A. huangi* sp. nov. should belong to the same group as *A. celebesensis*, *A. interioris*, and *A. megastoma*, i.e., variegated species with broad, undivided maxillary and mandibular bands of teeth. Among them, *A. huangi* sp. nov. seems to have the fewest number of teeth of the mid-part of the maxillary band (Fig. 5). Watanabe et al. (2004) showed that many morphological characters were not always useful for taxonomic analyses due to their overlap in most or all species. In contrast, molecular techniques may provide more-reliable information (Lin et al. 2002).

Distribution and habitat: Although adult *A. huangi* sp. nov. specimens are currently unavailable from the wild, we found abundant glass eels in the Cagayan River, northern Luzon I., the Philippines (Fig. 1). It is possible that some *A. celebesensis* specimens previously obtained from northern Luzon (Tabeta et al. 1976, Arai et al. 1999 2003), Taiwan (Tzeng 1982), and southeastern China (Lue et al. 1999) are actually *A. huangi* sp. nov. specimens (see "Discussion").

Etymology: The new species is named *huangi* in recognition of P.C. Huang for guiding determination of the first complete sequence of a fish mitochondrial genome, which has contributed to pioneering molecular phylogenetic studies in ichthyology. Moreover, he initiated a project monitoring the active trade in freshwater elvers in Taiwan based on molecular techniques, which not only has helped to abolish the trade of fake *A. japonica* elvers in Taiwan but also played a crucial role in identifying this new cryptic species: the first *Anguilla* species discovered through molecular analyses. We also propose the common English name of the Luzon mottled eel after its site of origin and distinct markings.

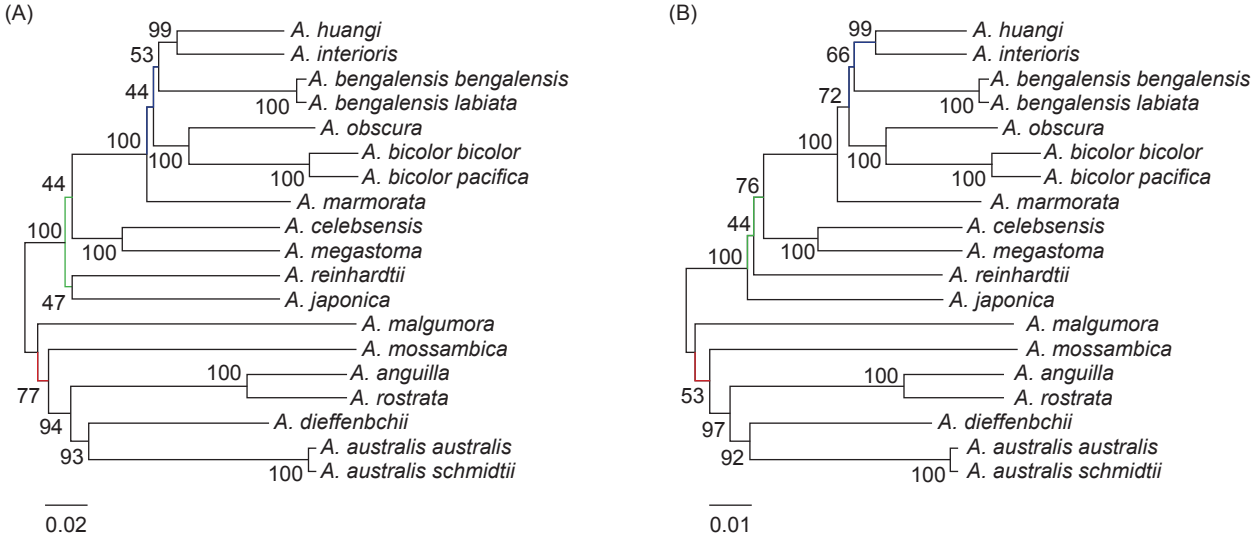

Fig. 4. Phylogenetic trees of the genus *Anguilla* constructed using 13 mitochondrial protein coding sequences based on (A) synonymous distances (Li 1993, Pamilo and Bianchi 1993) and the Neighbor-joining (NJ) method (Saitou and Nei 1987), and (B) the maximum-likelihood (ML) method (Felsenstein 1981) with the GTR + I + Γ4 substitution model (Rodriguez et al. 1990, Yang 1994). The numbers at the nodes are bootstrap values (Felsenstein 1985) from 5000 replicates for the NJ tree and 500 replicates for the ML tree. The scale bar represents the branch length. Three potential polytomies (indicated as red, green, and blue) were revealed in both trees.

Molecular phylogeny

Figure 4 displays the molecular phylogenetic trees of the genus *Anguilla* constructed using 13 mitochondrial protein-coding sequences. In general, the 2 tree topologies are congruent with those of Lin et al. (2001) and Minegishi et al. (2005). In both the NJ and ML trees, *A. huangi* sp. nov. and *A. interioris* form a monophyletic clade with strong bootstrap support. The Atlantic species *A. anguilla* and *A. rostrata* are clustered with the South Pacific species *A. australis* and *A. dieffenbachii*. Three potential polytomies (indicated as red, green, and blue) with bootstrap support values of < 80 were revealed in both trees. The 1st one may connect 4 branches, i.e., *A. mossambica*, *A. malgumora*, Atlantic + South Pacific species, and the branch including all other species. The 2nd one may connect 5 branches, i.e., *A. celebesensis* + *A. megastoma*, *A. reinhardtii*, *A. japonica*, *A. mossambica* + *A. malgumora* + Atlantic species + South Pacific species, and the branch including all other species. The last one may also connect 5 branches, i.e., *A. huangi* sp. nov. + *A. interioris*, *A. bengalensis*, *A. obscura* + *A. bicolor*, *A. marmorata*, and the branch including all other species.

We therefore created a set containing 675 possible bifurcating trees (3 × 15 × 15) from these 3 polytomies. We first calculated the log-likelihood values for the ML tree displayed in figure 4B under models M0, M1a, M2a, M3, M7, and

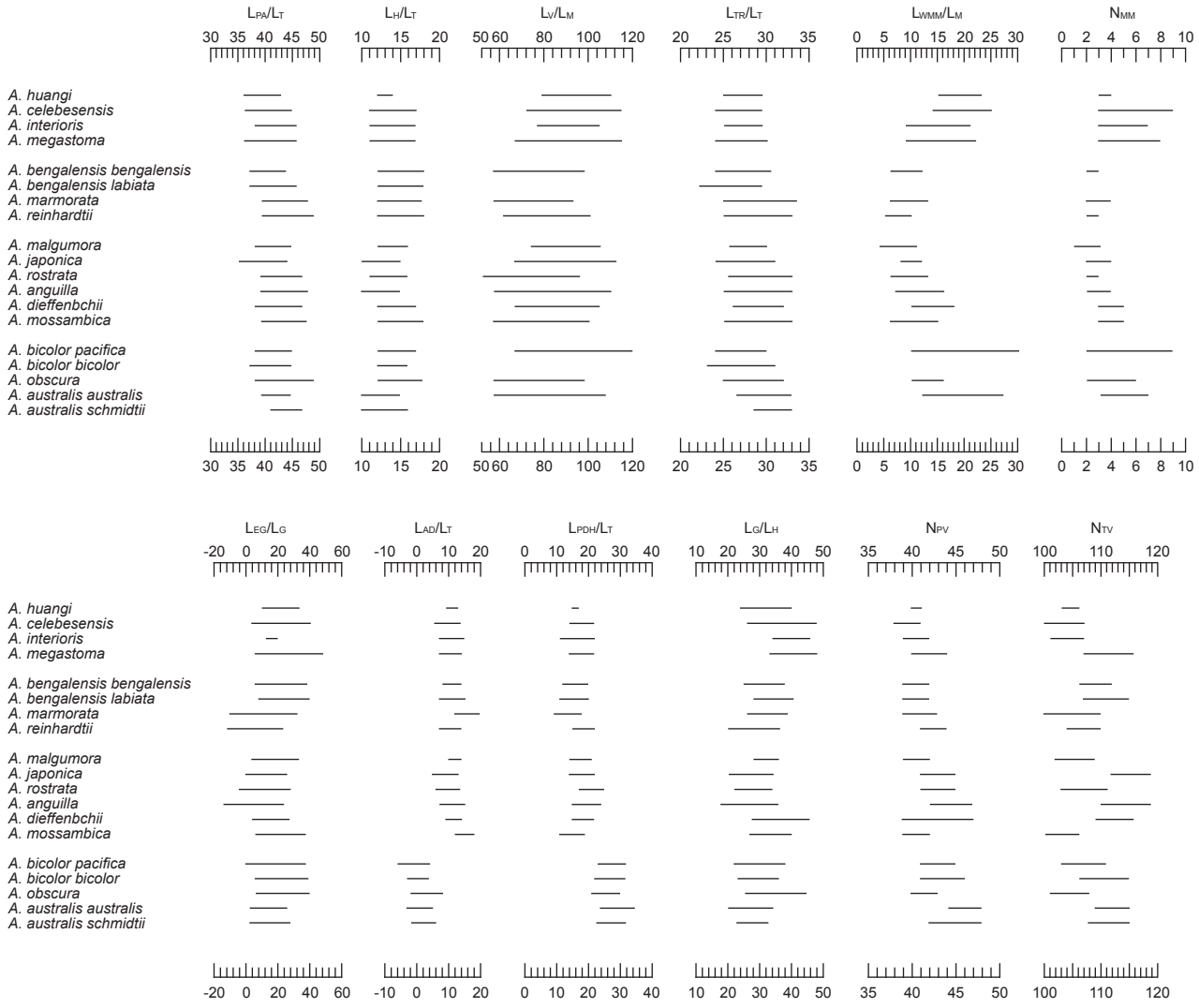

Fig. 5. Comparison of the morphological characters among *Anguilla* species. Measurements are from Ege (1939) and Watanabe et al. (2004) except for *A. huangi* sp. nov. The lines marked with asterisks display combined ranges of both subspecies. Abbreviations of morphological characters followed Watanabe et al. (2004).

M8 (Table 2). Similar to hominoids (Yang et al. 2000), the likelihood ratio test (LRT) identified a small proportion of positively selected sites. Model M3 fit the data better than all the others, and was therefore used to describe the substitution patterns for freshwater eels. Under the M3 model, the S-H test (Shimodaira and Hasegawa 1999) indicated that of the 675 possible trees, 614 trees (91%) were as good as the best tree ($p \geq 0.05$) and therefore could not be rejected. Similar results were obtained under the M0, M1a, M2a, M7, and M8 models (with 633, 633, 602, 618, and 613 trees as good as the best tree, respectively). Rejected trees ($p < 0.05$) were those with certain combinations of binary subtrees from the 3 polytomies. No single binary subtree from each polytomy (3 + 5 + 5) could be entirely rejected.

The results of the S-H test described above imply that the phylogeny of freshwater eels should be better represented by polytomies. Lin et al. (2001) suggested that these polytomies are likely derived from multiple radiation (rapid speciation) events. Figure 6A marks the 3 polytomies as red, green, and blue corresponding to 3 possible radiation events. The current distribution ranges for the freshwater eels are shown in figure 6B.

Morphological phylogenetic tree

The morphological phylogenetic tree for freshwater eels using the 12 characters and the MinNet method following Lin et al. (2005) is shown in figure 6C. Similar to Lin et al. (2005), Atlantic eels and *A. australis* are clustered together, and are neighbor to *A. dieffenbachii*. In this tree, *A. huangi* sp. nov. clusters with *A. celebesensis* rather than *A. interioris* as suggested by the molecular phylogeny.

DISCUSSION

Identification of the new *Anguilla* species

The reason that *A. huangi* sp. nov. was not included in Ege's (1939) monumental survey may have been due to its cryptic morphological differences from other species and its confined distribution. Several previous studies reported *A. celebesensis* elvers from the Cagayan River in the Philippines (Tabeta et al. 1976, Arai et al. 1999 2003). In Tabeta et al.'s treatment (1976), 58.4% of the 5228 elvers were identified as *A. celebesensis*, while 34.6% and 7% were attributed to *A. marmorata* and *A. bicolor pacifica*, respectively. This classification was based on the fact that *A. celebesensis* has fewer ano-dorsal vertebrae than *A. marmorata* (the number of ano-dorsal vertebrae reflects the distance between the verticals through the anus and origin of the dorsal fin). However, no *A. celebesensis* specimens were found using molecular techniques among the 73 cultured eels and 388 glass eels collected by Prof. W.N. Tzeng's group. Cultured eels were mainly composed of *A. huangi* sp. nov., *A. marmorata*, and *A. bicolor pacifica*, while the glass eel samples largely consisted of *A. huangi* sp. nov. (94%, W.N. Tzeng et al. unpubl. data). It is likely that in the past, *A. huangi* sp. nov. was frequently misidentified as *A. celebesensis*, because *A. huangi* sp. nov. also has fewer ano-dorsal vertebrae than *A. marmorata*.

In that case, the northern Philippine population of *A. celebesensis* studied by Arai et al. (2003) might have been *A. huangi* sp. nov. since its larval duration time significantly differed from that of the Indonesian population (Arai et al. 2003), and the 2 populations were suggested to have different spawning areas. Nonetheless, the anguillid leptocephali collected in waters east of Luzon (which were identified as *A. marmorata* or *A. celebesensis* by Ozawa et al. 1989), *A. celebesensis* elver specimens collected in northeastern and southwestern Taiwan (Tzeng 1982), and *A. celebesensis* elver specimens collected from the estuary of Jiulongjiang River in China (Lue et al. 1999) might also be *A. huangi* sp. nov. (Fig. 1).

Molecular phylogeny

In this study, we used no outgroup to root the freshwater eel phylogeny. The main reason is

Table 2. Log-likelihood values and estimated K_A/K_S ratios under various models

Model code	Log-likelihood value	K_A/K_S
M0 (1-ratio)	-42627.279	0.028
M1a (neutral)	-42221.879	0.043
M2a (selection)	-42221.879	0.043
M3 (discrete)	-42127.948	0.031
M7 (beta)	-42157.496	0.033
M8 (beta & ω)	-42132.053	0.034

K_A, nonsynonymous substitutions; K_S, synonymous substitutions.

that the closest outgroup to the genus *Anguilla* is still quite distant (Minegishi et al. 2005). Previous studies showed that it is difficult to accurately root a star-like tree (with rapidly diverging lineages) because there is a tendency for the outgroup to join the tree on one of the longer branches (the long-branch attraction effect, Bergsten 2005), even if its correct position is on a short central internal branch (Holland et al. 2003, Whitfield and Lockhart 2007). It might be even worse, in that rooting a star-like tree by including a distant outgroup often disrupts the ingroup tree (Holland et al. 2003, Shavit et al. 2007). In Shavit et al.'s study (2007), the ingroup was most accurately recovered when no outgroup was used. The 3 arrows in figure 6A indicate the 3 possible roots suggested by Lin et al. (2001), Aoyama et al. (2001), and Minegishi et al. (2005). It is likely that the root of freshwater eel phylogeny is located close to the red polytomy, which corresponds to the 1st radiation event (Fig. 6A). In other words, the 4 lineages (*A. mossambica*, *A. malgumora*, the Atlantic species + the south Pacific species, and the branch including all other species) might have almost simultaneously radiated in the beginning. The long-branch attraction effect (Bergsten 2005) might have played a role in the analyses by Aoyama et al. (2001) and Minegishi et al. (2005).

On the other hand, a star-like tree topology (with short internal branches) would probably lead to discordance between gene trees and species trees. In theory, to achieve a 95% chance that a gene tree is congruent with a species tree, the internode span of time needs to be at least 5 times the effective population size (Nichols 2001). Sometimes the most probable individual gene trees can be incongruent with the underlying species tree (Degnan and Rosenberg 2006). In this circumstance, looking for the most ancestral species (the earliest diverged species) in a star-like tree may be meaningless. Given that the population sizes of most *Anguilla* species are

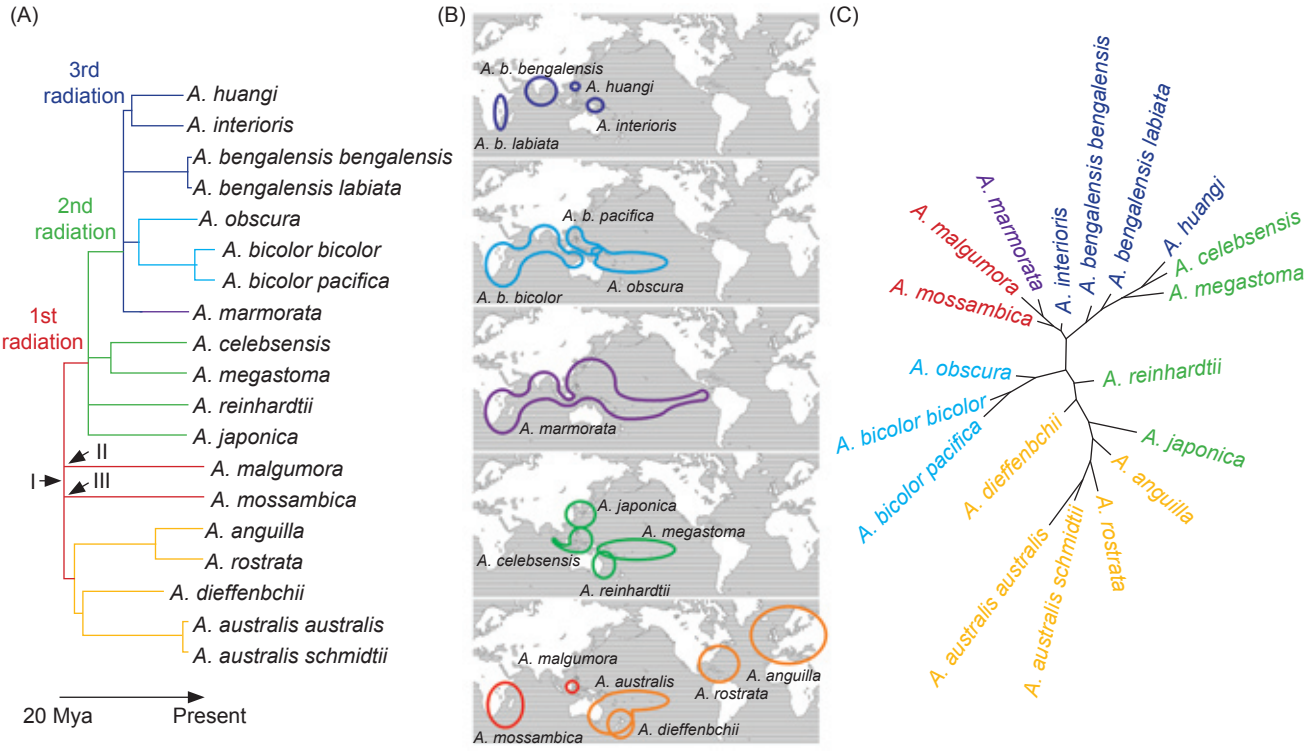

Fig. 6. (A) Inferred molecular phylogeny of the genus *Anguilla* based on 13 mitochondrial protein coding sequences. Three polytomies imply 3 possible radiation events. The roots suggested in Lin et al. (2001), Aoyama et al. (2001), and Minegishi et al. (2005) are indicated by arrows I, II, and III, respectively. (B) The current distribution of each *Anguilla* species according to Ege (1939), McCosker et al. (2003), Watanabe et al. (2004), and Minegishi et al. (2008). The ranges of the 2 subspecies of *A. australis* were merged as suggested by Jellyman (1987). (C) The morphological phylogenetic tree of the genus *Anguilla* constructed using MinNet (Lin et al. 2005). Although the available morphological characters might not be sufficient to clearly resolve their relationships, this tree still shows a highly consistent topology with the molecular phylogeny.

enormous, and that the proposed radiation events are likely to have occurred, the phylogeny of freshwater eels might be more appropriately represented by polytomies (Fig. 6A) than by a bifurcating tree.

However, it should be noted that each node of the Bayesian tree in Minegishi et al. (2005) was supported by high posterior probabilities. It was previously suggested that posterior probabilities are regularly overestimated and produce high rates of false inferences (e.g., Suzuki et al. 2002, Kolaczkowski and Thornton 2007), especially when the true tree has 0- or near-0-length internal branches (e.g., Suzuki et al. 2002, Steel and Matsen 2007, Yang 2007b). Simmons et al. (2004) therefore suggested that Bayesian support values should not be interpreted as probabilities for clades that are correctly resolved. Our results revealed that at least 90% of the trees generated from the 3 polytomies were as good as the best ML tree, which strongly supports the 3 radiation events.

Morphological phylogenetic tree

It should be emphasized that MinNet is a geometric tree construction method (Lin et al. 2005). Sufficient dimensions of the available feature space are necessary to reveal relationships among numerous OTUs. Insufficient dimensions can cause some of the separate branches to combine in the tree-searching procedures (e.g., the clustering of *A. mossambica*, *A. malgumora*, and *A. marmorata* in Fig. 6C), especially when lineages had rapidly diverged (a star-like tree). MinNet does not excel in resolving polytomies. This is comparable to the long-branch attraction effect (Bergsten 2005), although the combined branches here might not necessarily be long, but with similar trajectories in the feature space. Given that only 12 characters are available for freshwater eels (which may be simplified as a 7-dimensional space, see Lin et al. 2005) and that these characters are, in fact, not entirely neutral, it is unlikely that the phylogenetic relationships of the 19 species/subspecies can be fully verified (Lin et al. 2005). The clustering of *A. huangi* sp. nov. and *A. celebesensis* might therefore be attributed to insufficient information, because only 9 characters are available for *A. interioris*. On the other hand, the possibility that morphological measurements of *A. celebesensis* specimens used in this study (adapted from Ege 1939) were mixed with some *A. huangi* sp. nov. specimens cannot be ruled out either. Despite this, most clusters represented in the molecular phylogeny (Fig. 6A) were still approximately reproduced using morphological data in our results (Fig. 6C).

Ege (1939) proposed that a lack of variegated markings is a synapomorphic characteristic, so he used it to subdivide the eel groups. In contrast, Lin et al. (2001) suggested that variegated markings might have resulted from convergent evolution. Although we propose that the green branches were derived from a 2nd radiation event (Fig. 6A) based on the entire mitochondrial genome, there is the possibility that *A. japonica* was the 1st-diverged species in the underlying species tree. In that case, the variegated markings would be a derived character (in the 2nd radiation event except for *A. japonica*) which was eventually lost in the cyan branch (*A. obscura* and *A. bicolor* subspecies, Fig. 6A). Accordingly, variegated markings might not necessarily have resulted from convergent evolution. Further analysis of nuclear DNA sequences may help clarify this issue.

The possible evolutionary history of the genus *Anguilla*

Based on the proposed phylogeny, we suggest a possible evolutionary history for freshwater eels (Fig. 6). It is likely that each radiation event included rapid speciation and also a large-scale expansion in distribution. The 1st radiation event (red branches) might have occurred 20 million yrs ago (Mya) (Lin et al. 2001, Minegishi et al. 2005). One lineage (*A. mossambica*) dispersed westward to South Africa, 1 lineage (*A. malgumora*) remained in Borneo, around the center of their possible origin, and after dispersing eastward, the 3rd lineage (orange branches) further divided into 2 groups. While 1 group migrated to the South Pacific (*A. australis* and *A. dieffenbachii*), the other entered the Atlantic through the Central American Isthmus (*A. anguilla* and *A. rostrata*). The 2nd possible radiation event occurred on the 4th lineage afterward and generated 4 new sub-lineages (green branches). One moved northward (*A. japonica*), one moved southward (*A. reinhardtii*), and the 3rd one divided into 2 sister species (*A. celebesensis* and *A. megastoma*). Later, a 3rd possible radiation event occurred on the 4th lineage, and the other 4 new sub-lineages (blue branches) were thus created. The 1st one further divided into *A. huangi* sp. nov. and *A. interioris*, while the 2nd one (*A. bengalensis*) dispersed to India and Africa. The

3rd lineage (cyan branches) spread widely from the Pacific to Africa and thus subdivided into 3 groups (*A. obscura* and 2 *A. bicolor* subspecies). The 4th lineage (purple branch) has the widest range of distribution. It consists of only 1 species, *A. marmorata*, even though 4 genetically different populations are recognized (Minegishi et al. 2008). We therefore speculate that the 4th radiation event might have occurred on the cyan or purple branches as time went on.

Dispersal route of Atlantic eels

The geographic distribution of the genus *Anguilla* has drawn much attention in recent years. The area near the equator of the Indo-West Pacific Ocean accommodates most *Anguilla* species and is therefore considered to be the center of origin of freshwater eels (Aoyama et al. 1996 2001, Aoyama and Tsukamoto 1997, Tsukamoto and Aoyama 1998, Lin et al. 2001, Minegishi et al. 2005). On the other hand, the Atlantic eels, *A. anguilla* and *A. rostrata*, are geographically separated from other species in the Pacific and Indian Oceans (Avise 2003). Aoyama and Tsukamoto (1997) addressed 4 dispersal routes for Atlantic eels, i.e., the Tethys corridor route, the Cape of Good Hope route, the Central American Isthmus route, and the Arctic route. Tsukamoto and Aoyama (1998) further proposed a mechanism for the speciation of freshwater eels, i.e., a shift in the migration loop, and suggested that the accidental drift of leptocephali by a global westward-trending circum-equatorial current played a critical role. Based on this hypothesis, they preferred the Tethys corridor route for the invasion by the Atlantic species and suggested that the dispersal event must have preceded the closure of the Tethys Sea at around 30 Mya (Haq 1984). However, this hypothesis leaves 1 serious question unresolved: given that the center of origin of freshwater eels is likely in the Indo-West Pacific Ocean, how did *A. obscura*, *A. marmorata*, *A. megastoma*, and *A. australis* extend their distribution ranges eastward into the South Pacific Ocean (Fig. 6B), when the surface current there was westward? Apparently, for some *Anguilla* species, the shift in the migration loop might have been achieved by extending their spawning sites. Following that, their growth habitats were consequently extended or moved. In fact, the extant Pacific *Anguilla* species could have even extended their distribution range eastward to the Galápagos (McCosker et al. 2003).

We hypothesize that the ancestral species of Atlantic eels moved across the Central American Isthmus for spawning in the adult stage through the same process (Lin et al. 2001). A strong easterly current 1500 m below the surface in the tropical Pacific Ocean about 20 Mya (Barron and Peterson 1991) would have made the migration more efficient. The lack of *Anguilla* species along the west coast of the American continents currently is simply due to the westward-flowing circum-equatorial surface current in the Pacific Ocean. Leptocephali can only be transported westerly from spawning sites.

The Tethys corridor route scenario hypothesizes that the common ancestor of Atlantic and South Pacific eels (with *A. mossambica* as their sister species) inhabited the east coast of the African continent, which was close to the opening of the Tethys Sea corridor (Aoyama and Tsukamoto 1997, Tsukamoto and Aoyama 1998). However, the existence of a westerly current in the Tethys Sea assumed in this hypothesis was questioned (Barron and Peterson 1989 1991), and this hypothesis cannot explain how *A. australis* and *A. dieffenbachii* dispersed across the entire Indian Ocean to the east coast of Australia and New Zealand. No existent current could transport their larvae from the western Indian Ocean to the South Pacific Ocean.

Minegishi et al. (2005) rejected all 4 dispersal route scenarios addressed in Tsukamoto and Aoyama (1998), although their molecular phylogeny of the genus *Anguilla* has a similar topology to ours. The key is that they made a logical fallacy, i.e., incorrectly hypothesizing that species with geographically close ranges would have close phylogenetic relationships. Actually, phylogenetically distinct species can spread to the same area through multiple dispersal events; for example, *A. obscura*, *A. marmorata*, *A. megastoma*, and *A. australis* all extended their distribution ranges eastward in the South Pacific Ocean (Fig. 6B). The proper hypothesis should be that species with close phylogenetic relationships would have geographically close ranges, because they shared a common ancestor. The latter is the more-widely accepted hypothesis, while the former is not. The examples listed in Minegishi et al. (2005) can only be used to reject the former, not the latter. In other words, to test the Central American Isthmus hypothesis, one should determine the habitat of the sister species of Atlantic eels (*A. australis* and *A. dieffenbachii*), instead of relying on whether Atlantic eels and the easternmost species in the Pacific Ocean are

clustered together as Minegishi et al. (2005) did. Apparently, the molecular phylogeny of the genus *Anguilla* constructed based on entire mitochondrial genomes and 19 species/subspecies supports the Central American Isthmus hypothesis.

Other evidence supporting the Central American Isthmus hypothesis is that the origin of the speciation of extant *Anguilla* species (the 1st radiation event) was estimated to be 20 Mya by both Lin et al. (2001) and Minegishi et al. (2005). The Tethys seaway was already closed at that time while the Central American gateway still remained opened. Although Minegishi et al. (2005) themselves argued that the metabolic rate of freshwater eels might be slow and their evolutionary rate might therefore be slow, they performed the relative rate test (Robinson-Rechavi and Huchon 2000), and no significant difference was detected among all lineages. On the other hand, the method Lin et al. (2001) used to estimate the divergence time does not rely on the assumption of a constant evolutionary rate. Similar estimates were obtained using both nucleotide and amino acid sequences (Lin et al. 2001). These results suggest that the estimate of 20 Mya for the 1st radiation event of extant *Anguilla* species should be reliable.

The 1st *Anguilla* fossil records discovered in Europe are from 50-55 Mya (Patterson 1993). Parenti (2008) extended Minegishi et al.'s (2005) discussion and argued that the estimate of 20 Mya for the origin of *Anguilla* speciation was rejected by the fossil evidence. However, the existence of *Anguilla* species 55 Mya and the 1st radiation of extant *Anguilla* species 20 Mya do not conflict with each other. The fossil records of 50-55 Mya probably belonged to some ancient species which predated the divergence of all extant freshwater eels (Lin et al. 2001). It is possible that the radiation events occurred several times during 20-55 Mya; however, these ancient *Anguilla* species went extinct afterward and left no modern descendents except for 1 lineage, which led to the last common ancestor of all living *Anguilla* species and which began radiating about 20 Mya.

In summary, unlike temperate eels, it is possible that some tropical eels are distributed locally with limited population sizes. With this in mind, more species of eels may yet be discovered. The phylogeny of all currently recognized *Anguilla* species resolved in this study reasonably illustrates the evolutionary history and morphological transformation of freshwater eels. Combining our results and previous geographical studies (Barron and Peterson 1989 1991), the Central American Isthmus hypothesis (Lin et al. 2001) is supported by various types of evidence. The genus *Anguilla* might be ancient (Patterson 1993, Parenti 2008); however, the divergence of extant species is likely to have been relatively recent (~20 Mya). Our results also suggest that multiple radiation events have occurred in the Indo-Pacific region and continue moving forward.

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