Coastal Marine Science 32(1): 19–29, 2008

The use of morphological and molecular genetic variations to evaluate subspecies issues in the genus *Anguilla*

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Received 30 November 2007; Accepted 7 April 2008

Abstract — To examine the validity of the subspecies of *Anguilla nebulosa*, *A. bicolor* and *A. australis*, variations in morphological and molecular genetic characters were compared between geographically separate samples of these three species with reference to populations of *A. marmorata* and the 11 other species of *Anguilla*. There were statistically significant differences in the total number of vertebrae between the subspecies of each of the three species. The genetic distances (0.003–0.007) of the mitochondrial 16S ribosomal RNA gene between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis* were smaller than those of any combinations of all anguillid species (0.012–0.057). The genetic distance between the *A. australis* subspecies was smallest (0.003) among the three species, and those between the two subspecies of both *A. bicolor* (0.007) and *A. nebulosa* (0.006) were larger than those among populations of *A. marmorata* (0–0.003). Morphological and molecular genetic variations between each of the three subspecies pairs and among the populations of *A. marmorata* were much less than those at the species level. This study is a first step towards evaluating the subspecies issues of the genus *Anguilla*, but more studies on the population structure, spawning areas and migration routes of all species are needed to fully understand the taxonomic status within the genus *Anguilla*.

Key words: *Anguilla*, subspecies, *A. nebulosa*, *A. bicolor*, *A. australis*, vertebrae, mtDNA, 16SrRNA, control region, PCR-RFLP

Introduction

The freshwater eels, genus *Anguilla* Schrank, 1798, are one of the most unique eel groups in the Anguilliformes, since it is the only genus in which all species show catadoromus migrations between the sea and freshwater or estuaries in their life history. Their leaf-like transparent larvae, termed leptocephali, passively drift on currents from their spawning areas offshore to their growth habitats in freshwater or estuaries. This type of larval transport can result in large-scale dispersal, which could be affected by shifts in oceanic currents and result in transport of larvae outside of their typical species range. This situation makes some of Ege's (1939) classification of the genus *Anguilla* unclear, because he used geographic distribution as an essential taxonomic character (See Watanabe et al. 2004).

The genus *Anguilla* is currently recognized as comprising 15 species, three of which are further divided into two subspecies (Ege 1939, Castle and Williamson 1974). Of these, *A. nebulosa* McClelland, 1844 was divided into *A. nebulosa nebulosa* McClelland, 1844 and *A. nebulosa labiata* (Peter 1852), *A. bicolor* McClelland, 1844, into *A. bicolor bicolor* McClelland, 1844 and *A. bicolor pacifica* Schmidt, 1928, and *A. australis* Richardson, 1841, into *A. australis australis* Richardson, 1841 and *A. australis schmidtii* Phillipps, 1925 (Ege 1939). This traditional classification was based on several morphological differences, such as the body proportions, dentition and number of vertebrae, found between the two allopatric groups within species (Fig. 1).

Ege (1939) suggested that *A. marmorata* Quoy and Gaimard, 1824, which has the widest geographic distribution among the 15 species of the genus *Anguilla*, included three primary "races". A recent molecular genetic study (Ishikawa et al. 2004) and morphological (Watanabe et al. in press) studies recognized several different populations in this species. On the other hand, three temperate species, *A. anguilla* (Linnaeus, 1758), *A. rostrata* (Lesueur, 1817), and *A. japonica* Temminck and Schlegel, 1846, are thought to comprise panmictic populations despite their rather broad geographic distributions (e.g. Tesch 1977). Indeed, recent molecular genetic studies did not detect any genetic differences among distantly separated geographic samples in these temperate anguillid eels in the northern hemisphere: the European eel, *A. anguilla* (Lintas et al. 1998, Dannewitz et al. 2005), the American eel, *A. rostrata* (Avise et al. 1986, Wirth and Bernatchez 2003), and the Japanese eel, *A. japonica* (Sang et al. 1994, Ishikawa et al. 2001). However, there were also two studies which suggested the possibility of genetic differentiation within *A. anguilla* (Wirth and Bernatchez

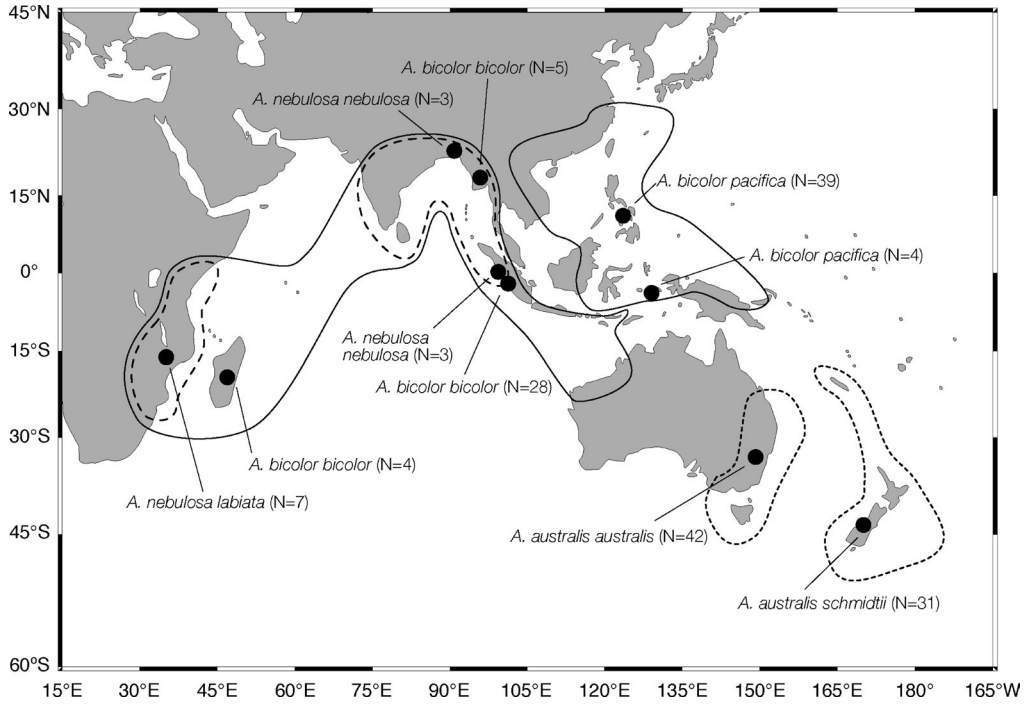

Fig. 1. Distribution of the two subspeces of *A. nebulosa* (broken lines), *A. bicolor* (straight lines) and *A. australis* (doted lines) based on Ege (1939), and the collection localities (solid circle) of specimens used in this study.

2001) and *A. japonica* (Tseng et al. 2006), so this problem is still unsolved.

These considerations show that there are some anguillid species that comprise panmictic populations, while there are some others with multiple populations. In addition, there are species that have subspecies in the genus *Anguilla*. However, the definition of taxonomic categories has not been well validated in anguillid species and the relative status between subspecies and populations is unclear (the term "race" used by Ege (1939) is essentially the same as a "population"). The purpose of this study was to quantitatively compare the variation and differences in morphological and molecular genetic characters between recognized taxonomic (species or subspecies) and ecological (population) units to understand the biological significance of the subspecies of the genus *Anguilla*.

Materials and Methods

Vertebral counts

The total number of vertebrae (TV) was examined as a morphological character because TV is one of the important characters of taxonomy in the genus *Anguilla* (Ege 1939, Tabeta et al. 1976, Watanabe et al. 2005, 2006, in press). A comparison of TV between each of the three subspecies pairs was done to reconfirm morphological differences between the two subspecies of *A. nebulosa*, *A. bicolor* and *A. australis*. Part of the data used for this comparison were 166 specimens (total length: 151–1248 mm) that were collected from 10 localities from 1 June 1995 to 2 March 2002 (Fig. 1, Table 1): *A. nebulosa nebulosa* (N=6), Sumatra, Indonesia and Bangladesh; *A. nebulosa labiata* (N=7), Malawi, Africa; *A. bicolor bicolor* (N=37), Madagascar, Sumatra and Myanmar; *A. bicolor pacifica* (N=43), Philippines and Ambon Island, Indonesia; *A. australis australis* (N=42), Australia; *A. australis schmidtii* (N=31), New Zealand. One specimen of *A. nebulosa labiata* could not be used for morphological analysis because of heavy damage. TV was counted using radiographs (soft-X, Softex Co., Ltd.). This data was called the original TV data set. The TV data of each subspecies were tested for differences in their distributions using the Kolmogorov-Smirnov two-sample test program of StatView (version 5.0) for Macintosh (SAS Institute Inc., Chicago, Illinois), with $P<0.05$ as the criterion for significance.

In addition, we used TV data of a total 2079 specimens of *A. nebulosa nebulosa* (N=90), *A. nebulosa labiata* (N=140), *A. bicolor bicolor* (N=500), *A. bicolor pacifica* (N=324) *A. australis australis* (N=461) and *A. australis schmidtii* (N=564) described in Ege (1939) (Table 2-B) and these data were also analyzed in the present study. These data were called Ege's TV data set.

It is well known that meristic characters such as the TV, fin rays, and gill rakers as well as external morphological characters vary geographically among populations of fishes (Uiblein 1995, O'Reilly and Horn 2004, Kim et al. 2006). However, it should be noted that there is often a relationship between meristic characters, especially TV, and latitude,

Table 1. Collection localities and number of specimens of *Anguilla nebulosa nebulosa*, *A. nebulosa nebulosa*, *A. bicolor bicolor*, *A. bicolor pacifica*, *A. australis australis*, *A. australis schmidtii* and *A. marmorata* examined in the study. * 11 specimens of *A. australis australis* were from the National Museum of Natural History, Washington DC, United States (fish numbers: 344896, 344897). TV: The total number of vertebrae, 16S: Genetic distance for 16SrRNA, RFLP: PCR-RFLP analysis for the control region.

Species or subspecies name	Number of specimens	Collection locality	Collection date	Total length (mm)	Experiments
A. nebulosa nebulosa	3	Bangladesh	1995/6/1	309–567	TV, RFLP
	3	Sumatra Island, Indonesia	1997/2/28–3/4	178–505	TV, RFLP
Subtotal	6				
A. nebulosa labiata	1	Malawi	1998/10/3	358	TV, 16S, RFLP
	5	Malawi	1998/11/6–11	255–1248	TV, RFLP
	1	Malawi	1999/9/23	<500	RFLP
Subtotal	7				
A. bicolor bicolor	1	Sumatra Island, Indonesia	1993/8/23	470	TV
	18	Sumatra Island, Indonesia	1996/5/28	319–576	TV
	9	Sumatra Island, Indonesia	1997/2/24–3/12	302–568	TV, RFLP
	5	Myanmar	1999/3/23	504–673	TV, RFLP
	3	Madagascar	1994/9/9	384–490	TV, RFLP
	1	Madagascar	1996/12/20	491	TV, RFLP
Subtotal	37				
A. bicolor pacifica	34	Philippines	1996/1/11	214–385	TV
	9	Amdon Island, Indonesia	1997/12/20	151–611	TV, RFLP
Subtotal	43				
A. australis australis	13	Australia	1996/8/14	293–362	TV
	18	Australia	1998/1/8–14	484–654	TV, RFLP
	11	Australia*		191–474	TV
Subtotal	42				
A. australis schmidtii	8	New Zealand	1996/9/5–11	338–474	TV
	4	New Caledonia	1997/11/7–8	249–349	TV
	4	New Zealand	1999/8/2	375–530	TV, RFLP
	15	New Zealand	1999/8/2	311–677	TV
Subtotal	31				
A. marmorata	3	Madagascar	1994/9/9	363–623	16S
	3	Japan	1993/8/8–9	476–1034	16S
	1	Sulawesi Island, Indonesia	1993/9/2	311	16S
	4	Tahiti	1996/8/6	704–1177	16S
Subtotal	11				

which is known as Jordan's rule (Jordan 1892). This tendency for TV to increase with latitude is often regarded as an association between TV and water temperature (Fowler 1970, Hubbs 1992). In the case of eels of the genus *Anguilla*, the spawning areas that are known for the temperate species *A. anguilla*, *A. rostrata*, and *A. japonica* (Schmidt 1925, McCleave et al. 1987, Tsukamoto 1992, 2006) and the tropical species *A. bicolor*, *A. borneensis*, *A. celebesensis*, and *A. marmorata* (Jespersen 1942, Miller et al. 2002, Aoyama et al. 2003) are all in tropical to subtropical regions with similar water temperatures. In addition, both *A. anguilla* and *A. rostrata* spawn in the same area (McCleave et al. 1987), but their TV are different with very little overlap (*A. anguilla*: range 110–119, mean 114.7; *A. rostrata*: range 103–111, mean 107.2; Ege 1939). Therefore, because of the similar temperatures at the spawning areas of most anguillids, it seems likely that variations of TV in eels are derived from genetic rather than environmental factors. Furthermore, the apparent population structure of *A. marmorata* (Watanabe et al. in press) and the subspecies of *A. australis* (Watanabe et al. 2006) based on molphological differences in TV roughly correspond with the population structure of *A. marmorata* (Ishikawa et al. 2004) and *A. australis* (Shen and Tzeng 2007, but see Dijkstra and Jellyman 1999) inferred from molecular genetic analysis. This evidence suggests that the genetic differences in mean TV are correlated with the amount

of evolutionary divergence among populations of eels. We focused on the degree of the differences in mean TV between subspecies of *A. nebulosa*, *A. bicolor* and *A. australis*, between *A. anguilla* and *A. rostrata*, and among populations of *A. marmorata* in both TV data sets, since the degree of the difference in mean TV likely reflects the evolutionary time scale of the divergences between taxa or groups in the genus *Anguilla*.

Genetic distance for 16SrRNA

We focused on the mitochondrial 16S ribosomal RNA gene (16SrRNA) and the control region as molecular genetic characters. The 16SrRNA is a relatively conservative gene (Meyer 1993) and is frequently used for phylogenetic analysis and identification studies of the species of *Anguilla* (Aoyama et al. 2000, 2001, Watanabe et al. 2005) and the control region is used for studies of the population structure of species of *Anguilla* (Ishikawa et al. 2001, 2004). The genetic distances (Kimura 1980) of 16SrRNA were examined by PHYLIP 3.5 to compare genetic distances among 143 populations of *A. marmorata* (N=11: Madagascar (N=3), Ogasawara (N=3), Sulawesi (N=1) and Tahiti (N=4)), between the two subspecies of *A. nebulosa*, *A. bicolor* and *A. australis* (N=6, 1 specimen per subspecies), and among the 15 species of the genus *Anguilla* (N=15, 1 specimen per species). For the genetic distance comparison at the species level, a single specimen of *A. nebulosa nebulosa*, *A. bicolor bicolor*, and *A. australis australis* was used for *A. nebulosa*, *A. bicolor* and *A. australis*. The data of these specimens for 13 anguillid species except for *A. nebulosa labiata* and *A. marmorata* from Aoyama et al. (2001) were obtained from the DDBJ/EMBL/GenBank (Accession Nos. AB021748-AB021783). *Anguilla nebulosa labiata* (N=1) collected from Malawi and *A. marmorata* (N=10) from 4 different localities (Madagascar, Ogasawara, Sulawesi and Tahiti) were used for the 16SrRNA sequence determinations. Since 5 different populations of *A. marmorata* were reported (Ishikawa et al. 2004), which were Madagascar, Sumatra, North Pacific, Fiji and Tahiti populations, we used 10 specimens of *A. marmorata* to examine genetic differences among 3 of the populations that were Madagascar (N=3), North Pacific (Ogasawara: N=3, Sulawesi: N=1), and Tahiti (N=3).

DNA extraction and the sequencing of 16SrRNA for 11 specimens of *Anguilla nebulosa labiata* and *A. marmorata* were conducted following Aoyama and Tsukamoto (1997). Briefly, total genomic DNA was isolated and purified using phenol-chloroform-isoamyl alcohol (25 : 24 : 1, volume/volume) twice with diethyl ether, then concentrated by ethanol precipitation. A fragment of the 16SrRNA gene was amplified using the polymerase chain reaction (PCR) with oligonucleotide primers that were nested in the 16SrRNA: L1374, H2009, L1854, H2582 (the four primers: Aoyama et al. 2001), L1854 and H3058 (the two primers: Miya and Nishida 1996). Amplification parameters were 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 60 s. Double-stranded DNA products from PCR were purified by filtration through a Microcon-100 (Amicon Inc.) filter and sequenced following the manufacture's protocol (Applied Biosystems Japan Ltd.). Sequences were obtained from the light and heavy strands of each fragment amplified for verification. Sequences of *A. nebulosa labiata* (1557 bp) and *A. marmorata* (1191–1194 bp) have been submitted to DDBJ/EMBL/GenBank, under Accession Nos. AB303369-AB303380.

PCR-RFLP analysis for the control region in subspecies

Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis was done to examine geographic and genetic differences in each subspecies of *A. nebulosa*, *A. bicolor*, and *A. australis*. Among all specimens collected in this study, a total of 60 specimens were used for PCR-RFLP analysis: *A. nebulosa nebulosa* (N=4), Sumatra Island, Indonesia and Bangladesh; *A. nebulosa labiata* (N=7), Malawi; *A. bicolor bicolor* (N=18), Madagascar, Sumatra and Myanmar; *A. bicolor pacifica* (N=9), Ambon Island, Indonesia; *A. australis australis* (N=18), Australia; *A. australis schmidtii* (N=4), New Zealand.

Pieces of liver tissue were excised from each eel and minced in 95% ethanol or in a buffer consisting of 8 M urea, 10 mM tris-HCl ph 8.5, 125 mM NaCl, 50 mM EDTA and 1% volume/weight of sodium dodecyl sulfate (Aoyama and Tsukamoto 1997). The remainder of each eel specimen was preserved in 5 to 20% formalin for morphological analysis. Total genomic DNA extraction was carried out as mentioned above. A fragment of the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes and a part of the control region was amplified by PCR with a pair oligonucleotide primers, L15774 (Shields and Kocher 1991) and H16498 (Kocher et al. 1989). The PCR was carried out with the GeneAmp PCR system 2400 (Perkin-Elmer, Inc.), with a 25 μl reaction volume containing 13.8 μl of sterile distilled water, 2.5 μl 10xPCR buffer (Perkin-Elmer, Inc.), 2.5 μl dNTP (deoxynucleotide triphosphate) at 2 mM, 5 μl of each primer at 5 μM, 0.2 μl of *Taq* DNA polymerase (Perkin-Elmer, Inc.) and 1 μl of total DNA. Amplification parameters were 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 60 s. The PCR products were cleaved by the seven restriction enzymes, *Alu* I, *Eco* T22I, *Hha* I, *Hinc* II, *Mbo* I, *Msp* I and *Mva* I (Takara Shuzo Co., Ltd.). PCR-RFLP analysis and data analysis followed Watanabe et al. (2005). Restriction procedures were carried out in a 20 μl final volume containing 5 μl PCR product, 10 units of restriction enzyme, and 2 μl of restriction enzyme buffer supplied by the manufacturers and incubated at 37°C overnight. RFLP was detected and compared with the positions of size markers (ϕX174-*Hinc* II digest, Takara

Shuzo Co., Ltd.) using electrophoresis on 1% agarose gels with ethidium bromide staining. A 0–1 data matrix was constructed based on the absence (0) or presence (1) of the following 12 restricted fragment lengths (Watanabe et al. 2005): 1 (>1057 bp), 2 (=1057), 3 (>770, <1057), 4 (=770), 5 (>612, <770), 6 (=612), 7 (>495, <612), 8 (=495), 9 (>392, <495), 10 (≥291, ≤392), 11 (>210, <291), 12 (≤210). Hierarchical cluster analysis was carried out on this binary data set using SPSS (version 6.1) for Macintosh (SAS Institute Inc., Chicago, Illinois) with the squared Euclidian distance measure, and UPGMA clustering (unweighted pair-group method using arithmetic averages).

Results

Total number of vertebrae

The TV range and mean of the original TV data set for *A. nebulosa nebulosa* were 106 to 111 (mean±standard deviation: 108.0±1.7), *A. nebulosa labiata*, 110 to 114 (111.8±1.7), *A. bicolor bicolor*, 106 to 111 (109.3±1.3), *A. bicolor pacifica*, 104 to 109 (107.0±1.2), *A. australis australis*, 109 to 115 (112.2±1.3) and *A. australis schmidtii*, 108 to 114 (111.4±1.5) (Table 2).

The TV range and mean of Ege's TV data set for *A. nebulosa nebulosa* were 106 to 112 (109.1±1.2), *A. nebulosa labiata*, 107 to 115 (111.3±1.3), *A. bicolor bicolor*, 106 to

Table 2. The total number of vertebrae (TV) of *Anguilla nebulosa nebulosa, A. nebulosa labiata, A. bicolor bicolor, A. bicolor pacifica, A. australis australis* and *A. australis schmidtii* from this (A) and Ege's (1939) (B) studies.

TV	*A. nebulosa nebulosa*	*A. nebulosa labiata*	*A. bicolor bicolor*	*A. bicolor pacifica*	*A. australis australis*	*A. australis schmidtii*
A)						
115					1	
114		1			6	1
113		2			11	8
112					14	4
111	1	1	8		5	10
110		2	9		4	6
109			11	5	1	
108	3		6	9		2
107	1		2	15		
106	1		1	10		
105				3		
104				1		
Total	6	6	37	43	42	31
Mean	108.0	111.8	109.3	107.0	112.2	111.4
SD	1.7	1.7	1.3	1.2	1.3	1.5
B)						
116					2	
115		2			22	4
114		4	1		77	21
113		17	3		155	99
112	4	33	35		129	198
111	7	49	87	2	63	164
110	21	26	157	2	11	65
109	32	7	124	37	2	12
108	18	1	75	85		1
107	7	1	13	107		
106	1		5	66		
105				17		
104				6		
103				2		
Total	90	140	500	324	461	564
Mean	109.1	111.3	109.7	107.1	112.6	111.7
SD	1.2	1.3	1.3	1.2	1.2	1.1

114 (109.7±1.3), *A. bicolor pacifica*, 103 to 111 (107.1±1.2), *A. australis australis*, 109 to 116 (112.6±1.2) and *A. australis schmidtii*, 108 to 115 (111.7±1.1) (Table 2).

The TV ranges of Ege's TV data set were wider than those of the original TV data set. However, the mean of TV values of these two data sets were almost the same with the largest differences being in *A. nebulosa nebulosa* (this study: 108.0, Ege's study: 109.1). The main reason for the difference between the two data sets was that the original TV data set (N=6) included fewer specimens than Ege's TV data set (N=90).

There were significant differences in the TV distributions (Kolmogorov-Smirnov two-sample test, $P<0.05$) between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis*, in the original TV data set, as well as in Ege's TV data set.

Genetic distances for 16SrRNA

Genetic distances in the 16SrRNA sequences among the 15 species ranged from 0.012 (*A. anguilla* vs. *A. rostrata*) to 0.057 (*A. borneensis* vs. *A. nebulosa*) (Fig. 2-A, Table 3). The genetic distance (0.012) between *A. anguilla* and *A. rostrata* was the lowest value among the 15 species pairs. Subspecies pairs showed genetic distances of 0.003 (*A. australis australis* vs. *A. australis schmidtii*), 0.006 (*A. nebulosa nebulosa* vs. *A. nebulosa labiata*) and 0.007 (*A. bicolor bicolor* vs. *A. bicolor pacifica*) (Fig. 2-B). These values for subspecies pairs were all lower than the lowest value of genetic distance between species (*A. anguilla* vs. *A. rostrata*). The genetic distances among the 10 specimens of *A. marmorata* including three different populations were from 0 to 0.003

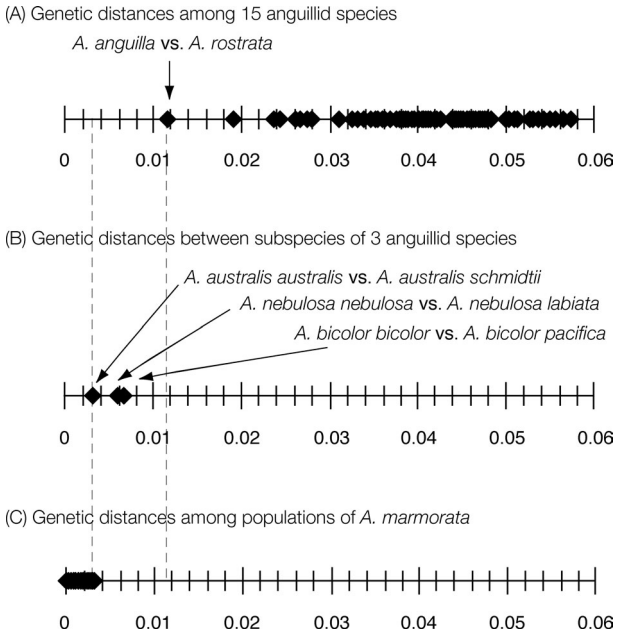

Fig. 2. Genetic distance among 15 species of *Anguilla* (A), among populations of *A. marmorata* (B) and between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis* (C).

Table 3. Genetic distances between each species of the genus *Anguilla*. CEL: *A. celebesensis*, INT: *A. interioris*, MEG: *A. megastoma*, NEB: *A. nebulosa*, MAR: *A. marmorata*, REI: *A. reinhardtii*, BOR: *A. borneensis*, JAP: *A. japonica*, ROS: *A. rostrata*, ANG: *A. anguilla*, DIE: *A. dieffenbachii*, MOS: *A. mossambica*, BIC: *A. bicolor*, OBS: *A. obscura*, AUS: *A. australis*.

Species	CEL	INT	MEG	NEB	MAR	REI	BOR	JAP	ROS	ANG	DIE	MOS	BIC	OBS
INT	0.040													
MEG	0.028	0.038												
NEB	0.045	0.019	0.043											
MAR	0.042	0.025	0.038	0.026										
REI	0.036	0.039	0.035	0.040	0.039									
BOR	0.051	0.056	0.051	0.057	0.055	0.053								
JAP	0.040	0.039	0.036	0.041	0.035	0.037	0.051							
ROS	0.039	0.043	0.036	0.046	0.038	0.034	0.046	0.035						
ANG	0.037	0.045	0.034	0.040	0.035	0.031	0.041	0.032	0.012					
DIE	0.045	0.048	0.044	0.050	0.044	0.038	0.056	0.043	0.037	0.033				
MOS	0.046	0.042	0.041	0.047	0.043	0.035	0.050	0.040	0.040	0.033	0.039			
BIC	0.044	0.026	0.042	0.028	0.026	0.041	0.054	0.039	0.043	0.039	0.047	0.048		
OBS	0.041	0.024	0.043	0.027	0.025	0.041	0.053	0.040	0.043	0.040	0.045	0.047	0.026	
AUS	0.047	0.046	0.045	0.048	0.042	0.039	0.054	0.042	0.038	0.034	0.042	0.035	0.046	0.047

(Fig. 2-C), which were also lower than the genetic distances among any pairs of the 15 species (0.012–0.057) and the two subspecies pairs of *A. nebulosa* (0.006) and *A. bicolor* (0.007), but were the same as the genetic distance for the *A. australis* subspecies pair (0.003).

PCR-RFLP analysis of the control region

Dendrograms were obtained from the cluster analysis using a 0–1 data matrix for specimens of each subspecies pair (Fig. 3). *Anguilla nebulosa nebulosa* and *A. nebulosa labiata* were clearly divided into respective groups. However, one specimen of *A. nebulosa labiata* from Malawi had a different pattern compared to the restriction fragment lengths of the other *A. nebulosa* specimens. *Anguilla bicolor bicolor*

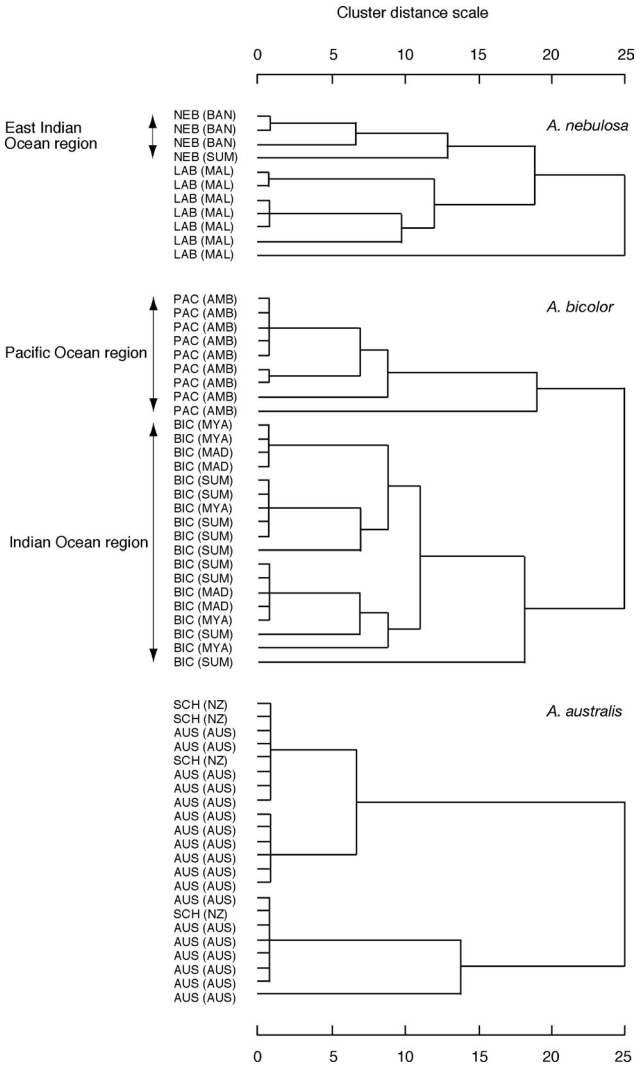

Fig. 3. Dendrograms obtained from the cluster analysis of PCR-RFLP analysis of the control region of *A. nebulosa*, *A. bicolor* and *A. australis*. NEB: *A. nebulosa nebulosa*, LAB: *A. nebulosa labiata*, BIC: *A. bicolor bicolor*, PAC: *A. bicolor pacifica*, AUS: *A. australis australis*, SCH: *A. australis schmidtii*. Sample location in parentheses, BAN: Bangladesh, SUM; Sumatra, MAL: Malawi, AMB: Ambon Island, 547 MYA: Myanmar, MAD: Madagascar, NZ: New Zealand, AUS: Australia.

and *A. bicolor pacifica* were clearly divided into two groups that corresponded to the Pacific Ocean region (Ambon) and the Indian Ocean region (Madagascar, Sumatra and Myanmar). Specimens from both sides of the Indian Ocean (west side: Madagascar, east side: Sumatra and Myanmar) were well mixed in a clade. *Anguilla australis australis* and *A. australis schmidtii* were not divided and were mixed.

Discussion

Morphological characters

The results showed that there was a clear difference in TV between the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis*. Ege (1939) also pointed out other morphological differences in proportional and meristic characters between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis*, but did not statistically test those. Watanabe et al. (in press) also found that there was a difference in TV among populations of *A. marmorata*. The size of the difference in mean TV (7.5) between *A. anguilla* and *A. rostrata* was larger than those between each of the subspecies pairs of *A. nebulosa* (2.2), *A. bicolor* (2.5) and *A. australis* (0.9), and among populations of *A. marmorata* (0.1–0.8) (Table 4). This suggested that the evolutionary divergence times between *A. anguilla* and *A. rostrata* were larger than those between the two subspecies of *A. nebulosa*, *A. bicolor* and *A. australis*, and among populations of *A. marmorata*. Furthermore, the sizes of the differences in mean TV between each of the subspecies pairs of *A. nebulosa* and *A. bicolor* were the same and were larger than those between the two subspecies of *A. australis* and among populations of *A. marmorata*. This suggests that the evolutionary divergence times between each of the subspecies pairs of *A. nebulosa* and *A. bicolor* are larger than those between the two subspecies of *A. australis* and among populations of *A. marmorata*. This estimation agreed with the molecular phylogenetic tree constructed using sequence data of the total mtDNA genome of all 15 species and 3 subspecies of *Anguilla* (Minegishi et al. 2005) because *A. nebulosa* and *A. bicolor* were also separated into respective subspecies earlier than the two subspecies of *A. australis*.

Molecular genetic characters

This study is the first to compare the genetic distances between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis*, among the 15 species in the genus *Anguilla*, and among populations of *A. marmorata*. The genetic distance in 16SrRNA between *A. anguilla* and *A. rostrata* was the lowest among species, but was higher than those between each of the subspecies pairs of *A. australis*, *A. nebulosa* and *A. bicolor* and among populations of *A. marmorata*. Furthermore, genetic distances in 16SrRNA between each of the subspecies pairs of *A. nebulosa* and *A. bicolor* were the

Table 4. Means and differences in the total number of vertebrae (TV) between *Anguilla anguilla* and *A. rostrata*, between the two subspecies of 3 species, and among populations of *A. marmorata*.

Species, subspecies or populations	Mean TV	Difference	References
A. anguilla	114.7	7.5	Ege, 1939
A. rostrata	107.2		Ege, 1939
A. bicolor bicolor	109.7	2.5	Ege, 1939
A. bicolor pacifica	107.2		Ege, 1939
A. nebulosa nebulosa	109.1	2.2	Ege, 1939
A. nebulosa labiata	111.3		Ege, 1939
A. australis australis	112.6	0.9	Ege, 1939
A. australis schmidtii	111.7		Ege, 1939
A. marmorata		0.1–1.8	
Madagascar	105.6		Watanabe, unpublished data
Sumatra	105.3		Watanabe, unpublished data
North Pacific	104.6		Watanabe, unpublished data
Fiji	105.7		Watanabe, unpublished data
Tahiti	106.4		Watanabe, unpublished data

same and were higher than those between the two subspecies of *A. australis* and among populations of *A. marmorata*. These results suggest that the degree of morphological and molecular genetic differences or evolutionary divergence time between each of the subspecies pairs of *A. nebulosa* and *A. bicolor* are lower or newer than those between *A. anguilla* and *A. rostrata*. It seems reasonable to suppose that the two subspecies of *A. nebulosa* and *A. bicolor* are already divided into two reproductively isolated groups, which possessed genetic differences more than twice those of the populations of *A. marmorata*. Furthermore, it seems reasonable to suppose that the two subspecies of *A. australis* have about the same genetic differences as among populations of *A. marmorata*. The same estimations could be found by the results of the morphological character of TV.

PCR-RFLP analysis using the control region also clearly showed genetic differences between each of the subspecies pairs of *A. nebulosa* and *A. bicolor*. However, there was no clear separation between *A. australis australis* and *A. australis schmidtii* in the PCR-RFLP analysis. This result was same as in the study of Dijkstra and Jellyman (1999) who sequenced the control region (611 base pairs) of mtDNA of *A. australis australis* and *A. australis schmidtii* to test the hypothesis that there was no genetic difference between the two subspecies. They found no statistically significant difference and concluded that these two subspecies appeared to share a common gene pool. In addition, the genetic differences of 16SrRNA between *A. australis australis* and *A. australis schmidtii* were as small as those among populations of *A. marmorata*. However, Watanabe et al. (2005) reported that *A. australis* had greater genetic differences between the two subspecies than within the other 14 species. Watanabe et al. (2006) also found a significant difference between the two subspecies of *A. australis* in 16 external morphological characters and 3 meristic characters including TV. Furthermore, a recent molecular genetic study using microsatellite DNA indicated that populations of *A. australis* in eastern Australia and in New Zealand may be reproductively isolated from one another (Shen and Tzeng 2007). To test the molecular differences and population structure of these two subspecies more precisely, we need to also analyze other types of nuclear DNA, which may more directly affect the morphological differences, using more rapidly evolving genetic markers such as Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphisms (SNPs).

In the present study, there was no significant difference in the relatively conservative mtDNA 16SrRNA gene sequences in *A. marmorata*. This result was the same as the study by Ishikawa et al. (2004). However, there were clear genetic differences in the control region in different populations of *A. marmorata* (Ishikawa et al., 2004). This suggested that recently isolated populations of *A. marmorata* have not yet accumulated species-specific genetic markers in their mtDNA 16SrRNA sequences, but have accumulated population-specific genetic markers in the control region. Furthermore, Ishikawa et al. (2004) reported that sequence differences in the control region of the mtDNA of 162 individuals of *A. marmorata* varied from 0% to 10.8%. The highest value was comparable to that between the two Atlantic species, *A. anguilla* and *A. rostrata* (10.0%), and much larger than that between the two subspecies of *A. bicolor* (6.2%). These observations suggest that the degree of differences in the control region may vary widely depending on the size of the species range and the locations of their spawning areas.

Comparison between morphological and molecular genetic characters

Comparisons of the morphological and molecular ge-

netic differences between *A. anguilla* and *A. rostrata*, between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis*, and among populations of *A. marmorata* in the present study indicated the same three things. (1) The morphological and molecular genetic differences between *A. anguilla* and *A. rostrata* were bigger than those between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis* and among populations of *A. marmorata*. (2) The morphological and molecular genetic differences between each of the subspecies pairs of *A. nebulosa* and *A. bicolor* were bigger than those between the two subspecies of *A. australis* and among populations of *A. marmorata*. (3) The morphological and molecular genetic differences between subspecies of *A. australis* were the same as those among populations of *A. marmorata*, except for in the control region.

Validity of subspecies

The analyses of the present study found that there were morphological and molecular genetic differences between each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis*. Considering the morphological and molecular genetic differences and reproductively isolated ecological units (populations), there are three possible taxonomic solutions for the subspecies problems of the genus *Anguilla*: (1) Because recent molecular genetic (Ishikawa et al. 2004, Minegishi et al. in press) and morphological (Watanabe et al. in press) studies recognized several populations in *A. marmorata* by finding small morphological and molecular genetic divergences in comparison to those of *A. anguilla* and *A. rostrata*, the two subspecies of *A. nebulosa*, *A. bicolor* and *A. australis* should be regarded merely as populations of each species. (2) If the validity of the two subspecies of *A. nebulosa*, *A. bicolor* and *A. australis* are to be accepted, the populations of *A. marmorata* with similar differences should also be regarded as subspecies. (3) If focusing on the reproductive isolation of populations of the genus *Anguilla*, which without considering the small differences in morphological and molecular genetic characters, each population should be regarded as a species, and the subspecies of *Anguilla* should also be species.

The morphological and molecular differences that have been found and the allopatric distributions of each of the subspecies pairs of *A. nebulosa*, *A. bicolor* and *A. australis* agrees with subspecies concept defined by Mayr and Ashlock (1991). However, to use and expand the use of the subspecies concept to *A. marmorata* as in case (2), it will require the use of 2 taxonomic (species and subspecies) and 1 ecological (population) units. The case of (1) or (3) would be simpler concepts than the case of (2) because these cases use just species and population designations without any subspecies. Furthermore, some biologists have suggested that the subspecies as a category rank should be abolished (Wilson and Brown 1953, Burt 1954, Hagmeier 1958).

We need further studies on the population structure of all species in the genus *Anguilla* using many more specimens because there have been no studies of the population structure of tropical eels except for *A. marmorata*. Furthermore, studies on the spawning and recruitment areas of species other than *A. anguilla*, *A. rostrata* and *A. japonica* are needed as a first step, because understanding the exact patterns of their migration loops, which consist of the migrations between different habitats for spawning and for growth (Tsukamoto and Aoyama 1998, Tsukamoto et al. 2002), will be important for the determining population structures and the taxonomy of the genus *Anguilla*. Taxonomy of this genus can be discussed more completely after more detailed results are obtained in studies on the population structure, spawning areas and migration routes of all species, which will be key factors in determining the taxonomy of the genus *Anguilla*.

Acknowledgements

This study could not have been possible without the generous assistance in sampling expeditions, for which we sincerely thank the following colleagues: S. Ishikawa, T. Sato, M. Ohashi, H. Chagi, A. Kawada, C. Hayward, K. Ogawa, T. Otake, T. Inagaki, T. Arai, T. Yoshinaga, S. Sasai, and T. Isono. The paper greatly benefited from critical comments and suggestions on an early draft by M. J. Miller. We thank M. Oya for her assistance in many aspects of the project. S. W. was partly supported by the Ito Foundation for Promotion of Ichthyology, a Sasakawa Scientific Research Grant from the Japan Science Society, and Research Fellowships for Young Scientists (No. 18780143) from the Japan Society for the Promotion of Science. This study was supported partly by Grants-in-aid (No. 07306022, 08041139, 11691177 and 12NP0201) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; "Research for the Future" Program No. JSPS-RFTF 97L00901 from the Japan Society for the Promotion of Science; Touwa Shokuhin Shinkoukai; and the Eel Research Foundation of Nobori-kai.

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