THE RAFFLES BULLETIN OF ZOOLOGY 2007 Supplement No. **14**: 51-59
Date of Publication: 31 Jan.2007

MOLECULAR PHYLOGENY OF PHILIPPINE FRESHWATER EELS *ANGUILLA* SPP. (ACTINOPTERYGI: ANGUILLIFORMES: ANGUILLIDAE) INFERRED FROM MITOCHONDRIAL DNA

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ABSTRACT. **–** There are no studies on phylogenetic analysis of the freshwater eels, *Anguilla* spp. in the Philippines at a molecular level and species identification of the eel is usually carried out through morphological features. In this study, mitochondrial DNA (mtDNA) was used to characterize the species and establish the phylogeny of the eels found in the Philippines. Genomic DNA was extracted from nine eels collected from the Cagayan River system and Chico River in Northern Luzon, Philippines. The 16S rRNA and cytochrome *b* genes of the mtDNA of the eels were amplified through polymerase chain reaction (PCR) and the resultant products were sequenced. Nucleotide sequences of the amplified regions of the 16S rRNA and cytochrome *b* consisted of 813 and 920 nucleotide base pairs, respectively. Based on the DNA analysis, two *Anguilla* species (including two *Anguilla* subspecies) were validated and identified from the samples namely, *A. marmorata, A. bicolor bicolor* and *A. bicolor pacifica*. *Anguilla marmorata* was the basal species of the lineage among the three species and *A. bicolr* is the most recently evolved species. Phylogenetic analysis indicates that the Philippine freshwater eels belong to the Indo-Pacific lneage and corroborates with the phylogenetic trees reconstructed from the mtDNA data from the GenBank for these eels. This study is the first to use molecular genetics to identify the freshwater eels in the Philippines.

KEY WORDS. – mtDNA, *Anguilla* spp., 16S rRNA, cytochrome *b*, PCR, phylogeny.

INTRODUCTION

Freshwater eels of the genus *Anguilla* are catadromous fishes that migrate between freshwater and marine environments. The eels have a multi-stage life cycle. They spawn in the open ocean, after which, their leaf-like larvae, leptocephalus, drift with the oceanic currents towards their continental growth habitat. The leptocephali metamorphose to glass eels in the continental shelf and further develop into elvers when they reach the river estuaries. The elvers eventually settle in the brackish coastal waters, estuaries and rivers to grow into yellow eels. During maturation, they become silver eels and return to their oceanic spawning grounds to reproduce and die (Tesch, 2003). Knowledge and background of the natural life history and speciation are the basis for sound fishery management or aquaculture industry development of eels. Elvers in the estuary have long been harvested for aquaculture and are now constantly depleted due to high demand. To guarantee a constant supply of elvers, researchers and eel farmers alike are now looking at the possibility of getting

Table 1. Primers used in the amplification of the 16S rRNA and cytochrome *b* gene seqences.

Cytochrome *b* gene (Han et al., 2002)	
H15341	5' – TGC TAA CGA CCT AGT GG – 3'
L151341	5' – CTA GTC AAC CTA CTA ATG GG – 3'
16S rRNA gene (Aoyama et al., 2001)	
L1374	5' – GAA GAA ATG GGC TAC ATT TTC TA – 3'
H2009	5' – CCT AAG CAA CCA GCT ATA AC – 3'
L1854	5' – AAA CCT CGT ACC TTT TGC AT – 3'
H2582	5' – ATT GCG CTA CCT TTG CAC GGT – 3'

stocks from the Southeast Asian region (Tseng et al., 2001; Tzeng, 2004).

There are about 18 species of anguillid eels in the world. Most of them are tropical species residing in the Indo-Pacific region (Tesch, 2003). The Philippine archipelago lies within the Indo-Pacific region, near the major spawning grounds of both the tropical eels and the temperate eel (*Anguilla japonica*) in the western waters of the Marianas Islands. With the influence of the North Equatorial Current and its archipelagic nature, many freshwater eels thrive and succeed in building separate populations in the Philippines, especially in the large rivers and estuarine areas of Northern Luzon. It has been known that the Indo-Pacific region was the origin of the speciation of the freshwater eels of the genus *Anguilla* (Aoyama & Tsukamoto, 1997; Lin et al., 2001). The ancestors of both temperate and tropical eels originated from the Indo-Pacific region, particularly in the archipelagic area of Indonesia, Malaysia and the Philippines.

In the past, mainly morphological characters were used for inferring fish phylogenetic relationships (to understand their speciation and evolution). In the case of freshwater eels, it is difficult to differentiate the species because of the similarity in external morphology. Therefore, the reconstructed phylogenetic trees based on morphology were controversial due to the complex evolutionary changes in either morphological or physiological characters (Bastrop et al., 2000). Recent advances in molecular biology have changed this situation. Firstly, the blueprint of all organisms is written in the DNA and it is now possible to study the evolutionary relationships of organisms by comparing their DNA sequences. Secondly, species identification based on molecular genetics is more reliable than morphological characteristics which are often affected strongly by the environment.

In the Philippines, freshwater eels have different natural populations scattered throughout the whole country. It is an important food fish for many of the indigenous people of the Northern Philippines and some regard this group of fish as mystical because of its long life and largeness in size. Although this is the case, there has been no documentation of the basic biology of eels such as evolutionary history and phylogenetic studies in the Philippines, where these fishes are quite abundant and geographically near to their spawning ground. This study aims to identify the eel species collected in the Philippines and to validate their identity and determine their phylogenetic relationship by analyzing the 16S rRNA and cytochrome *b* genes of the mitochondrial DNA (mtDNA).

MATERIALS AND METHODS

Sample collection. **–** A total of 24 freshwater eels were collected from the Cagayan River system and Chico River (Fig. 1). Morphological characteristics of the eel, such as the presence of mottles on skin and length of fins, were used to establish a basis for preliminary identification of the species. The morphometric measurements gathered were as follows: total length (TL), head length (H), pre-dorsal fin length (PD), pre-anal fin length (PA), distance between verticals from origin of dorsal fin to anus (AD) and percent (%) values of AD in proportion to TL. A piece of muscle tissue (25 - 50 g) from each of the eels was minced and placed in a 1.5 ml microfuge tubes (Axygen Inc. USA) containing absolute

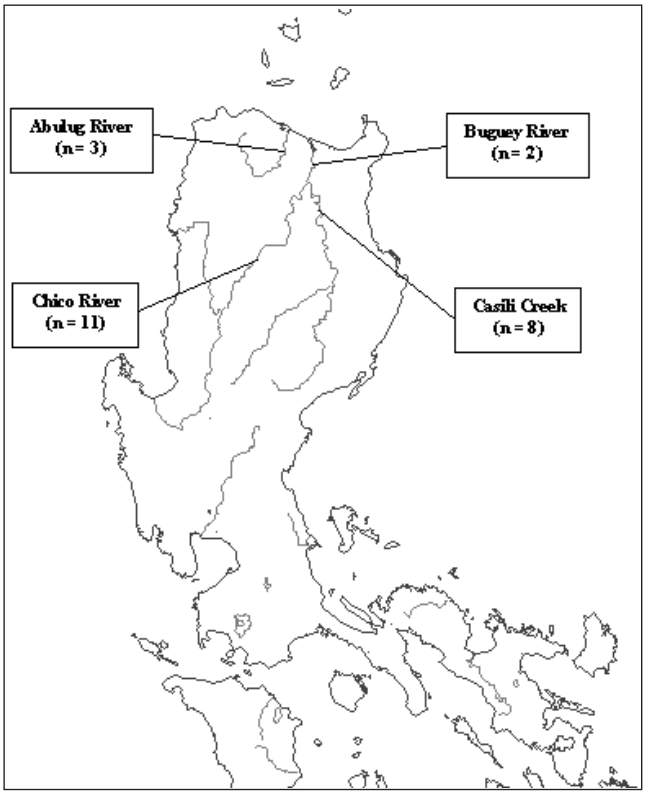

Fig 1. Map of Luzon Island, Philippines showing the sampling sites and sample size (n).

Table 2. Characteristics of the eel specimens collected from Buguey River, Casili Creek and Abulug River that are part of the Cagayan River system.

Specimen Nos.	Species Name	Characteristics	Sampling Sites
1 - 11	*Anguilla marmorata*	Mottled and long finned	Chico River, Kalinga Province
12 - 13	*Anguilla bicolor pacifica*	Plain and short finned	Buguey River, Cagayan Province
14 - 21	*Anguilla bicolor bicolor*	Plain and short finned	Casili Creek, Cagayan Province
22 - 24	*Anguilla bicolor pacifica*	Plain and short finned	Abulug River, Cagayan Province

Table 3. Morphometric measurements of the eel samples.

Specimen Nos.	Mean ± Standard Deviation (SD)					
	TL (cm)	H (cm)	PA (cm)	PD (cm)	AD (cm)	AD/TL (%)
1 - 11	32.33 ± 4.68	4.51 ± 0.65	13.92 ± 1.99	8.74 ± 1.27	5.17 ± 0.75	15.93 ± 0.31
12 - 13	39.50 ± 2.12	5.53 ± 0.32	16.40 ± 0.57	15.80 ± 0.85	0.60 ± 0.28	1.54 ± 0.80
14 - 21	41.00 ± 8.25	5.76 ± 1.15	16.92 ± 3.40	16.40 ± 3.30	0.51 ± 0.11	1.25 ± 0.18
22 - 24	45.17 ± 11.64	6.33 ± 1.63	18.57 ± 4.75	18.07 ± 4.67	0.50 ± 0.10	1.12 ± 0.16

TL = total length; H = head length; PA = pre-anal fin length; PD = pre-dorsal fin length; AD = distance between the verticals from the origin of the dorsal fin to the anus; AD/TL = percentage of AD in proportion to TL.

ethanol. The tissues were brought to the Institute of Fisheries Science, College of Life Science, National Taiwan University, Taipei, Taiwan for mitochondrial DNA (mtDNA) analysis.

DNA extraction, PCR amplification and sequencing. – Extraction of DNA from the muscle tissue samples was carried out using the Genemark tissue and cell genomic purification kit (Genemark Technology Co. Ltd.). Mitochondrial 16S rRNA and cytochrome *b* genes were amplified by polymerase chain reaction (PCR) and using primers from Aoyama et al. (2001) and Han et al. (2002) as shown in Table 1. The PCR amplification procedures were as follows: pre-denaturation at 94°C for 2 minutes, 30 - 35 cycles of denaturation (94°C for 1 minute), annealing (53 - 57°C for 1 minute) and extension (72°C for 1 minute); and final extension at 72°C for 10 minutes. The PCR products were sent for sequencing at Seeing Bioscience Ltd., Taipei, Taiwan. The sequences were submitted to the nucleotide database of GenBank (www.ncbi.nlm.nih.gov) with the following accession numbers: DQ093409, DQ093410, DQ093411, DQ093412, DQ093413, DQ093414, DQ093415, DQ093416 and DQ093417.

Phylogenetic Analysis. – Nucleotide sequences were aligned initially using the computer package, DAMBE (Xia, 2000; Xia & Xie, 2001). The phylogenetic tree was constructed based on the neighbor-joining tree (NJ) method with Kimura 2-Parameter distances using the Molecular Evolutionary Genetics Analysis version 2.1 (MEGA2) computer software package (Kumar et al., 2001).

RESULTS

Morphological characteristics of the eels. – Specimens number 1 to 11 collected from the Chico River in Kalinga Province had mottles on the skin and long fins. On the other hand, specimens number 12 to 24 collected from the Cagayan River system in Cagayan Province did not possess mottled skin but they were plain-skinned and short-finned. The species of the eels which were identified based on morphology are listed on Table 2.

A summary of the morphometric measurements of the samples is presented in Table 3. The TL of *Anguilla marmorata* ranges from 26.5 cm to 38.8 cm, H from 3.7 cm to 5.4 cm, PA from 11.4 cm to 16.7 cm and PD from 7.2 cm to 10.5 cm. For *A. bicolor bicolor,* TL ranges from 32.0 cm to 54.0 cm, H from 4.5 cm to 7.6 cm, PA from 13.2 cm to 22.3 cm and PD from 12.8 cm to 21.6 cm. For *A. bicolor pacifica,* TL ranges from 37.0 cm to 58.5 cm, H ranges from 5.2 cm to 8.2 cm, PA from 15.2 cm to 24.0 cm and PD, from 14.8 cm to 23.4 cm.

AD/TL (%) was an effective criterion to classify the species into short- and long-finned eels. The eels with AD/TL (%) less than 5% were classified as short-finned eels and those with values greater than 5% were described as long-finned eels. *Anguilla marmorata* were long-finned since their AD/TL values ranged from 15.84% to 16.29% (Table 3). Whereas, *A. bicolor bicolor* were classified as short-finned species because their AD/TL values ranged from 1.11% to 1.30%. *Anguilla bicolor pacifica* were also classified as a short-finned species with AD/TL values ranging from 0.97% to 2.10%.

***Substitution rate of 16S rRNA and cytochrome* b. -** The nucleotide substitution rate of the mtDNA (R) was examined with the formula, R = transition / transversion. The nucleotide substitution rate of the 16S rRNA partial gene sequences of the four Philippine eels selected from the same population are presented in Table 4. The alignment of 16S rRNA region consists of 813 bp with some gaps and unresolved sites. The highest rate of nucleotide substitutions (10.272) was found between specimen 13 and *A. dieffenbachii* (NC_006538) involving 32 bp. On the other hand, the lowest substitution rate (0.500) involving three bp was found between specimens

Table 4. Nucleotide substitution rate of 16S rRNA partial sequences of four selected specimens of this study with other *Anguilla* species and an outgroup (*Misgurnus anguillicaudatus*).

Species Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
[1]	0.00																					
[2]	1.13	0.00																				
[3]	1.10	1.00	0.00																			
[4]	1.11	2.75	2.96	0.00																		
[5]	1.13	2.51	3.09	4.53	0.00																	
[6]	1.18	1.86	2.26	3.23	0.75	0.00																
[7]	1.13	1.97	2.35	2.28	2.16	1.77	0.00															
[8]	1.13	4.13	4.95	2.48	3.23	2.51	3.23	0.00														
[9]	1.21	3.69	4.66	4.13	4.61	3.07	1.70	5.17	0.00													
[10]	1.21	4.00	3.63	3.09	2.20	2.04	2.28	3.54	6.61	0.00												
[11]	1.11	1.55	1.58	1.14	2.07	1.93	1.64	1.68	1.64	1.68	0.00											
[12]	1.13	5.66	7.90	3.89	2.64	1.89	2.04	11.32	5.79	3.47	1.84	0.00										
[13]	1.21	3.84	3.50	2.96	2.37	1.91	2.16	3.38	6.09	?	1.74	3.27	0.00									
[14]	1.24	2.40	2.70	2.56	3.23	2.58	2.63	3.23	3.42	3.23	1.82	5.17	3.09	0.00								
[15]	1.01	1.43	1.61	1.53	1.53	1.31	2.44	2.06	1.10	1.44	1.35	1.29	1.36	1.71	0.00							
[16]	1.21	3.09	3.84	3.54	2.85	2.03	1.84	3.77	17.38	5.44	1.43	4.08	5.10	3.69	1.49	0.00						
[17]	1.20	1.98	2.33	1.80	2.37	2.47	1.82	1.86	3.36	2.83	1.30	2.63	2.70	1.88	1.61	2.51	0.00					
[18]	1.16	1.80	1.86	1.00	2.43	2.43	1.44	2.04	2.40	1.96	1.02	2.04	1.86	1.77	1.01	2.37	1.51	0.00				
[19]	1.18	1.96	2.37	3.39	1.00	?	1.86	2.63	3.24	2.17	2.01	2.03	2.04	2.69	1.39	2.18	2.58	2.56	0.00			
[20]	1.18	2.06	2.47	3.54	1.26	?	1.96	2.75	3.42	2.30	2.08	2.18	2.17	2.80	1.46	2.33	2.69	2.70	?	0.00		
[21]	1.15	1.66	1.97	2.63	0.67	0.50	1.58	2.15	2.43	1.74	1.77	1.58	1.63	2.24	1.21	1.70	2.15	2.05	?	0.50	0.00	
[22]	1.21	5.40	7.20	3.90	2.64	1.89	2.17	10.27	6.15	3.48	1.77	?	3.27	4.74	1.53	3.82	2.40	2.04	1.89	2.04	1.47	0.00

[1] = *Misgurnus anguillicaudatus;* [2] = *A. australis schmidtii*; [3] = *A. australis australis*; [4] = *A. anguilla*; [5] = *A. bicolor bicolor*; [6] = *A. bicolor pacifica*; [7] *A. celebesensis*; [8] = *A. dieffenbachii*; [9] = *A. interioris*; [10] = *A. bengalensis labiata*; [11] = *A. malgumora*; [12] = *A. marmorata*; [13] = *A. nebulosa nebulosa*; [14] = *A. mossambica*; [15] = *A. megastoma;* [16] = *A. obscura*; [17] = *A. reinhardtii*; [18] = *A. rostrata*; [19] = specimen number 13 (*A. bicolor pacifica*); [20] = specimen number 15 (*A. bicolor pacifica*); [21] = specimen number 14 (*A. bicolor pacifica*); [22] = specimen number 1 (*A. marmorata*).

Table 5. Nucleotide substitution rate of cytochrome *b* gene partial sequence of five selected specimens of this study with other *Anguilla* species and an outgroup (*Misgurnus anguillicaudatus*).

Species Code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
[1]	0.00																							
[2]	?	0.00																						
[3]	5.26	5.34	0.00																					
[4]	3.29	3.34	4.55	0.00																				
[5]	2.74	2.79	3.87	20.45	0.00																			
[6]	4.51	4.59	6.11	4.98	4.51	0.00																		
[7]	5.18	5.29	5.36	3.59	3.05	3.49	0.00																	
[8]	3.83	3.89	5.85	5.52	4.41	7.18	3.45	0.00																
[9]	4.53	4.60	5.85	5.97	5.22	10.15	3.78	11.84	0.00															
[10]	6.48	6.37	7.23	3.62	3.24	5.48	5.53	4.13	4.83	0.00														
[11]	4.67	4.75	6.21	5.43	4.79	11.95	4.95	8.37	17.65	4.76	0.00													
[12]	3.57	3.64	4.34	3.76	3.57	9.39	3.73	4.51	5.55	4.61	5.74	0.00												
[13]	4.84	4.84	6.20	2.97	2.80	4.97	4.59	3.86	4.48	5.94	4.54	4.75	0.00											
[14]	4.53	4.60	5.85	5.97	5.22	10.15	3.78	11.84	?	4.83	17.65	5.55	4.48	0.00										
[15]	3.40	3.45	3.45	7.32	5.76	6.09	2.70	8.26	6.76	2.95	5.51	4.24	3.61	6.76	0.00									
[16]	6.16	6.27	5.14	3.65	3.32	11.72	6.51	5.33	6.26	5.55	5.92	7.66	4.67	6.26	3.68	0.00								
[17]	5.84	5.94	7.93	3.87	3.50	5.07	5.95	4.61	5.07	5.94	5.34	4.13	5.30	5.07	3.23	5.34	0.00							
[18]	4.19	4.26	5.54	5.53	4.88	8.53	5.21	6.61	11.49	4.31	3.02	4.95	4.00	11.49	4.71	4.85	4.82	0.00						
[19]	4.60	4.67	6.12	5.43	4.79	11.72	4.86	8.15	17.27	4.68	?	5.64	4.47	17.27	5.51	5.78	5.26	3.53	0.00					
[20]	3.24	3.29	4.48	1.00	6.12	5.97	3.45	6.76	7.18	3.62	6.51	4.38	2.97	7.18	9.39	3.98	3.87	6.64	6.51	0.00				
[21]	2.74	2.79	3.94	21.49	?	4.51	3.05	4.51	4.91	3.13	4.50	3.57	2.90	4.91	5.88	3.19	3.56	4.60	4.50	6.47	0.00			
[22]	4.19	4.12	3.50	3.15	2.72	5.33	4.33	3.85	3.83	3.83	4.10	4.21	3.34	3.83	2.79	6.48	3.56	3.73	4.02	3.45	2.63	0.00		
[23]	2.79	2.84	3.87	20.45	?	4.42	2.98	4.41	5.02	3.18	4.60	3.50	2.85	5.02	5.76	3.25	3.50	4.69	4.60	6.12	?	2.68	0.00	
[24]	1.26	1.26	1.19	1.15	1.16	1.18	1.19	1.26	1.30	1.25	1.22	1.22	1.26	1.30	1.16	1.28	1.33	1.21	1.22	1.20	1.16	1.22	1.15	0.00

[1] = *Anguilla. australis schmidtii*; [2] = *A. australis australis*; [3] = *A. anguilla*; [4] = *A. bicolor bicolor*; [5] = *A .bicolor pacifica*; [6] = *A. celebesensis*; [7] = *A. dieffenbachia;* [8] = *A. interioris*; [9] = *A. bengalensis labiata*; [10] = *A. malgumora*; [11] = *A. marmorata*; [12] = *A. megastoma*; [13] = *A. mossambica*; [14] = *A. nebulosa nebulosa*; [15] = *A. obscura;* [16] = *A. reinhardtii;* [17] = *A .rostrata*; [18] = specimen number 4 (*A. marmorata*); [19] = specimen number 2 (*A. marmorata*); [20] = specimen number 17 (*A. bicolor bicolor*); [21] = specimen number 20 (*A. biclor pacifica*); [22] = *A. japonica Taiwan*; [23] = specimen number 21 (*A.bicolor bicolor*); [24] = *Misgurnus Anguillicaudatus*.

14 and 15 as well as between specimen 14 and *A. bicolor pacifica* (NC_006535). No values were obtained between the following: specimens 13 and 15, specimens 13 and 14, specimen 13 and *A. bicolor pacifica* (NC_006535) and specimen 15 and *A. bicolor pacifica* (NC_006535).

The nucleotide substitution rates of the partial sequence of cytochrome *b* gene of all eel species are shown in Table 5. All of the cytochrome *b* regions of the Philippine eels (*Anguilla marmorata*, *A. bicolor bicolor* and *A. bicolor pacifica*) used in this study were 920 bp. The highest value (21.295) of the rate of transitions over transversions was between specimen 20 and *A. bicolor bicolor* (NC_006534). On the other hand, the lowest value of nucleotide substitutions (1.002) was found between specimen 17 and *A. bicolor bicolor* (NC_006534). There was no substitution between specimen 2 and *A. marmorata*, specimens 20 and 21, specimen 20 and *A. bicolor pacifica* (NC_006535) and specimen 21 and *A. bicolor pacifica* (NC_006535).

***Phylogenetic relationships.* –** The molecular phylogenetic identification of the freshwater eel samples from the Philippines was carried out. From the phylogenetic tree inferred from the 16S rRNA region of the identified species, three specimens (numbers 13, 14 and 15), belonged to the same branch, indicating that these eels were from the same lineage of *A. bicolor pacifica* (Fig. 2) and specimen 1 (*A. marmorata*) was joined at the same branch with *A. marmorata* (NC_006540).

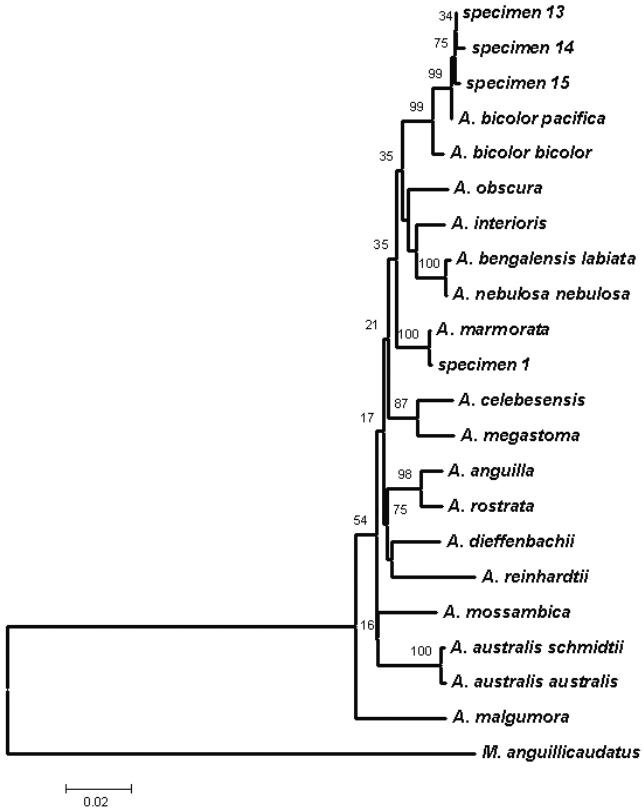

Fig 2. Phylogenetic tree inferred from 16S rRNA gene using neighbor-joining method. Specimen numbers 13, 14 and 15 of *Anguilla bicolor pacifica* were collected from the Cagayan River system and specimen number 1 of *A. marmorata* was collected from Chico River.

To test the reliability of the inferred tree topology, determination of bootstrap values was performed in which values above 70% were considered highly reliable. Results obtained show that specimens 13, 14 and 15 have a 75% bootstrap value. In line with this, *A. bicolor pacifica* (NC_006535) and the branch consisting of the three eel samples had a bootstrap value of 99%. It can be inferred fairly that these eels were the same species identified as *A. bicolor pacifica*. Specimen 1 (*A. marmorata*) and *A. marmorata* (NC_006540) are supported by the highest bootstrap value of 100%.

A phylogenetic tree was also constructed for cytochrome *b* regions of the eels using the same tree-building method, distance matrix method and bootstrapping (Fig. 3). The phylogenetic tree shows that specimen 21, identified as *A. bicolor pacifica*, was clustered with *A. bicolor pacifica* (NC_006535) with a 100% bootstrap value. Specimen number 17 (*A. bicolor bicolor*) was strongly branched with *A. bicolor bicolor* (NC_006534) with a bootstrap value of 100%. Specimens 2 and 4 (*A. marmorata*) were clustered with *A. marmorata* (NC_006540) species, supported by high bootstrap values of 77% and 100%, respectively.

To develop a more comprehensive reconstruction of the phylogenetic tree, the sequenced data of 16S rRNA and cytochrome *b* genes were combined and analyzed accordingly

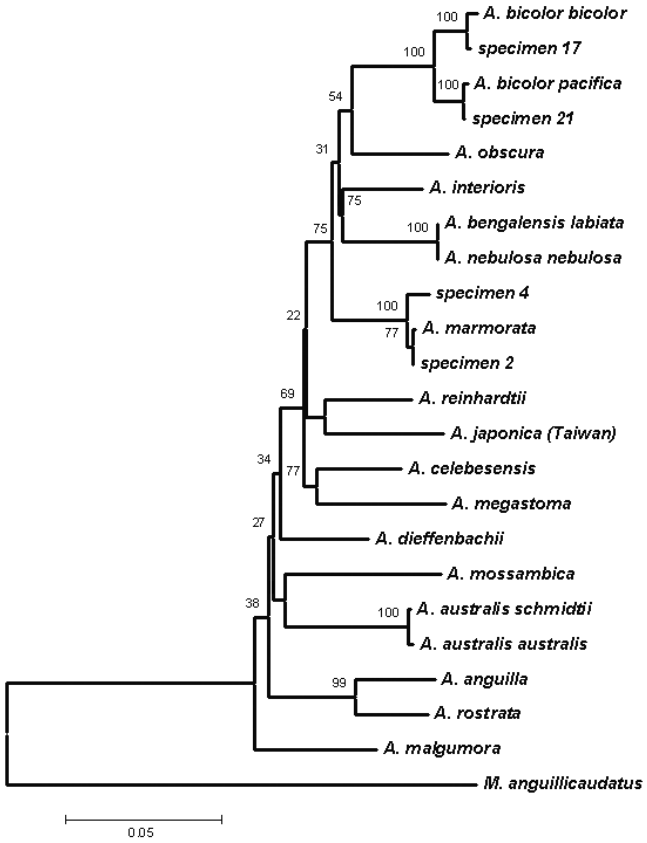

Fig 3. Phylogenetic tree inferred from the cytochrome *b* coding region of the mtDNA using neighbor-joining method. Specimen number 17 (*Anguilla bicolor bicolor*), specimen number 21 of *A. bicolor pacifica* was collected from the Cagayan River System and specimen numbers 2 and 4 of *A. marmorata* were collected from Chico River.

(Fig. 4). Results show that specimens 13 and 20 were strongly clustered with *A. bicolor pacifica* (NC_006535), supported by a high 100% bootstrap value. Similarly, specimen 1 was clustered with *A. marmorata* (NC_006540) and also supported by a 100% bootstrap value. The phylogenetic inference was more robust if an outgroup was included in the analysis. In this study, *Misgurnus anguillicaudatus* of the family Cobitidae (order Cypriniformes) was used as the outgroup for the phylogenetic analysis.

DISCUSSION

Species identification. **–** The conventional way of identifying species of fishes is by morphological characteristics. In the case of freshwater eels, identification based on morphological descriptions seems to be misleading because these fishes resemble each other. Although Watanabe (2001) indicated that the variegated skin color pattern and the distance between the verticals through the anus and origin of the dorsal fin in percent of total length are the best suited taxonomic key characters for the discrimination of group species, these characteristics are not species-specific.

In this study, two species of Philippine eels, *Anguilla marmorata* and *A. bicolor*, were accurately identified based on morphological and morphometric features. Interestingly, all eels identified as *A. marmorata* were from the same sampling site, the Chico River and all *A. bicolor* species were from the Cagayan River system. The identification of *A. marmorata* based on morphological features, such as distinct

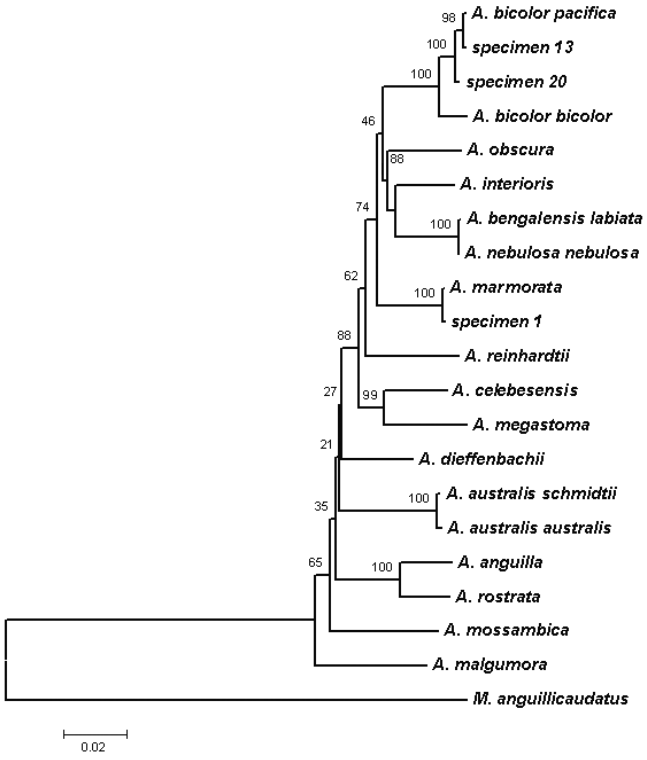

Fig 4. Phylogenetic tree inferred from the combined cytochrome *b* and 16S rRNA gene sequence data. Specimen numbers 13 and 20 of *Anguilla bicolor pacifica* were collected from the Cagayan River System and specimen number 1 of *A. marmorata* was collected from Chico River.

variegations on the skin and a long dorsal fin was reliable. On the other hand, discriminating between the subspecies of *A. bicolor* was more difficult since the morphological characteristics were indistinguishable and the morphometric measurement differences were almost negligible. This is corroborated by Watanabe (2001) who mentioned that the morphological approach for discriminating subspecies was difficult and often led to misidentification of species.

Species identification based on the colour pattern and fin length was not reliable for discriminating similar species of freshwater eels. Environmental factors can influence the morphological characters of the eels, thereby affecting the reliability of the identification (Huang et al., 2001; Han et al., 2002). Morphological characteristics were not sufficient for systematic analysis because of overlapping and similar features among species. Thus, freshwater eel identification needs to be supported by molecular characterization. To validate the identification of Philippine eels based on morphological features, the present study carried out the molecular characterization of the eels using partial sequences of the 16S rRNA and cytochrome *b* genes of the mitochondrial DNA. DNA sequencing provides a tool for the detailed and relatively accurate identification of eel species, particularly when there are difficulties in differentiating the species based on morphological characteristics (Huang et al., 2001; Lin et al., 2002).

The present study is the first molecular characterization of the freshwater eels in the Philippines based on the cytochrome *b* and 16S rRNA gene sequences. There were two species (including two sub-species) identified among the samples: *A. marmorata, A. bicolor bicolor* and *A. bicolor pacifica* respectively. Interestingly, no *A. japonica* (Japanese eel) was found in the collected samples, although the Philippines lies along the migratory pathway of this species (Cheng & Tzeng, 1996). In the study of Cheng & Tzeng (1996), on the timing of metamorphosis and estuarine arrival of *A. japonica*, the Japanese eel leptocephali are still incapable of undergoing metamorphosis as revealed by otolith microstructure. When these leptocephali approach the Philippine territories, it is still too early for them to undergo metamorphosis and thus, they continue to drift by the upward Kuroshio Current, toward the East and Northeast Asian countries.

Genetic variation. **–** Pairwise comparisons, sequence alignment and DNA analysis of the 16S rRNA gene and cytochrome *b* gene partial sequences show low values of nucleotide differences. Billington & Herbert (1991) and Sang et al. (1994) argued that selective pressures on the fishery stocks may result in genetic divergence. Hence, the low values of the nucleotide differences indicate that the pressures (e.g., fishing pressures, exploitation and natural barriers) influencing the population of Philippine eels are correspondingly low which may be the reason why these genes are conserved among the two species.

The subspecies of *A. bicolor* demonstrated large differences in cytochrome *b* sequences, that corresponded to interspecies levels, but showed only slight differences in the 16S rRNA

gene sequences. Similar findings were reported by Aoyama et al. (2001). The existence of a larger number of species-specific nucleotide positions makes cytochrome *b* a more suitable marker for genetic identification.

Phylogenetic relationships. **–** This study presents the first reported phylogenetic analysis for freshwater eels in the Philippines, based on the mitochondrial DNA (mtDNA) sequence data. mtDNA analysis is a very useful tool for molecular phylogenetics because of its special features. The wide range of evolutionary rates between different regions of the mtDNA makes it a suitable tool to accomplish genetic studies among taxa of several fish groups at multiple taxonomic levels (Karaiskou et al., 2003; Inoue et al., 2003). The genes used in this study were cytochrome *b* and 16S rRNA. The cytochrome *b* is a widely used molecular marker because of its relatively slow rate of evolution; it has been used to evaluate phylogenetic relationships among freshwater eels and other fishes (Han et al., 2002; Aoyama et al., 2001; Bastrop et al., 2000; Lin et al., 2001; Karaiskou et al., 2003).

The 16S rRNA phylogenetic tree showed that the samples from the Cagayan River system formed a clade with *A. bicolor pacifica* while samples from the Chico River formed a clade with *A. marmorata*, with robust bootstrap support. The same results were also obtained with the cytochrome *b* gene, only that some samples from the Cagayan River formed a clade with *A. bicolor bicolor*.

Phylogeography. **–** Phylogenetic relationships of animals reflect historical biogeographic factors (Avise, 1987). In this study, all of the samples formed a clade under the Indo-Pacific lineage. Under this lineage, *A. marmorata* appeared to be the most basal species (most ancestral species). For both gene phylogenies, *A. marmorata* was placed as most ancestral branch, suggesting that this lineage originated in the Indo-Malayan region. *A. marmorata* is the most widely-distributed freshwater eel species (Miller et al., 2001) and it forms different distinct populations around the Southeast Asian region. As indicated by Aoyama et al. (2001), freshwater eels might have originated in the Indo-Pacific area. These findings were also supported by Tsukamoto & Aoyama (1998) and Tsukamoto et al. (2002) stating that two-thirds of the recognized 18 *Anguilla* species and subspecies inhabit the tropics in the Western Pacific and this may be geographical origin of the genus *Anguilla*. From other phylogenetic studies of freshwater eels (Bastrop et al., 2000; Aoyama et al., 2001; Lin et al., 2001; Minegishi et al., 2005), no *A. marmorata* samples were collected from the Philippines. Therefore, this is the first study to molecularly characterize *A. marmorata* from the Philippines.

CONCLUSIONS

The present study aims to provide a molecular phylogeny and identification of the anguillid or freshwater eels (*Anguilla* spp.) found in the Philippines. Evolutionary relationships and genetic characterization are the basic information of natural populations of fish. In the present study, this information of Philippine freshwater eels was elucidated. Freshwater eels found in the Northern Luzon Island belonged to the Indo-Pacific group and among these, the *Anguilla bicolor* species were the least speciated. Since this study is a preliminary and pioneering molecular characterization of freshwater eels in the Philippines, it is suggested that more comprehensive studies of freshwater eels in the Philippines, especially on the characterization and population genetic structure, be carried out.

ACKNOWLEDGMENTS

We are thankful to Dr. Tzeng's research team at the Fisheries Biology Laboratory of the Institute of Fisheries Science, College of Life Sciences, National Taiwan University for their help in this study. We are also grateful to Engr. Perfecto Garcia and his family for their assistance during the sampling of eels in the Cagayan Province area. The travel of Mr. Brian Wade Jamandre was supported by the Santeh Feeds Corporation Scholarship grant, Office of the Student Affairs of the Central Luzon State University and Freshwater Aquaculture Center - College of Fisheries Production fund.

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