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MOLECULAR PHYLOGENY AND GENETIC DIVERSITY OF FRESHWATER *ANGUILLA* EELS IN INDONESIAN WATERS BASED ON MITOCHONDRIAL SEQUENCES

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PHYLOGENETIC RELATIONSHIPS
GENETIC DIVERSITY
TROPICAL EELS
INDONESIAN WATERS
ANGUILLA SPP.

ABSTRACT. – Indonesian waters, have been proposed by biologists to be the origin of freshwater eel anguillid. The objective of this study was to investigate molecular phylogenetic relationships and genetic diversity of the seven Indonesian eel taxa based on the cyt b gene. Each eel species was identified by comparison with published sequences, namely *Anguilla celebesensis, A. interioris, A. borneensis, A. marmorata, A. bicolor bicolor, A. b. pacifica* and *A. nebulosa nebulosa*. A total of 129 different haplotypes were identified from this study, most of them for the first time, showing haplotype and nucleotide diversities of 0.98 and 4.57 %, respectively. *A. bicolor* was clearly found to be composed of two subspecies, *A. b. bicolor* and *A. b. pacifica*. The first subspecies was split into two major clades supported by a high bootstrap value, with each clade characterized by two diagnostic nucleotides. *A. marmorata* was also split into two clades, supported by a high bootstrap value. This first study of phylogenetic relationships and genetic diversity from all *Anguilla* taxa inhabiting Indonesian waters, based on 1115 specimens, is necessary for a local management and conservation of this valuable resource in terms of both biodiversity and economic development.

BACKGROUND

Freshwater eels are catadromous species characterized by a growth stage in estuaries, rivers and lakes and spawning far offshore after adult migrations of sometimes thousands of kilometers from their growth habitat (Tesch 1977). After hatching, the larvae (leptocephali) are passively transported by oceanic currents, to their growth habitat, where they metamorphose into glass eels before migrating via the continental shelf to estuaries (Tesch 1977, Mochioka 2003).

Tsukamoto & Aoyama (1997) proposed a dispersal theory to explain eel speciation. A migration loop consists of active adult migration for spawning and larvae passive transportation to the feeding and growing habitat of their parents. Accidental drift of larvae by a global circumequatorial current is likely responsible for a westward expansion of their growth habitat. After growing in the new habitat, if some adults have found a new spawning site, a new migration loop is established and new subspecies, then species, will subsequently appear because of spawning site isolation.

The worldwide geographic distribution of eels appears to be related to the subtropical circulation of the ocean, with most species being located on the western side of the Atlantic, Pacific and Indian Oceans (Tesch 1977). Warm westward flowing equatorial currents reach and flow along the east coast of each continent, whereas the west coasts are swept by cold currents originating from high southern latitudes.

The origin of freshwater anguillid eels, *Anguilla* genus, is a question that has always intrigued scientists. The western Pacific region, including Indonesian waters, have been proposed by biologists to be the origin of freshwater eels (Tsukamoto & Aoyama 1998, Aoyama & Tsukamoto 1997, Aoyama *et al*. 2001). This is supported by the fact that two thirds of the 19 identified *Anguilla* species and subspecies inhabit the tropics while only five species are found in temperate regions (but they reproduce in tropical regions). Seven of the 19 species and subspecies occur in the western Pacific around Indonesia (Tsukamoto & Aoyama 1998). However, information on tropical eels that occupy waters around Indonesia is still limited.

The phylogeny of *Anguilla* eels is based on the pioneer studies of Ege (1939) using morphological characters. He divided the *Anguilla* genus into four groups. First, the variegated species with broad undivided maxillary and mandibular bands of teeth (*A. celebesensis, A. interioris* and *A. megastoma*); second, variegated species with a toothless longitudinal groove in the maxillary and mandibular bands of teeth (*A. nebulosa, A. marmorata* and *A. reinhardti*); third, species without variegated markings and with a long dorsal fin (*A. anguilla, A. rostrata, A. mossambica, A. borneensis, A. japonica* and *A. dieffenbachii*) and fourth, species without variegated markings and with

a short dorsal fin (*A. bicolor, A. obscura* and *A. australis)*. Ege suggested that the first group is the most ancestral.

Classical methods using morphological characters rapidly appeared to be insufficient in identifying eel groups and species as compared to the molecular approach (Aoyama 2003, Watanabe *et al*. 2005). Molecular phylogenetic research has generated numerous more or less concordant publications, and lastly, the complete mtDNA sequence of all eel taxa produced a comprehensive phylogeny and successfully resolved controversial topologies, while confirming *A. mossambica* as being the basal species (Minegishi *et al*. 2005).

While Indonesian waters are in the eel area of origin, information about their distribution, evolution and phylogenetic relationships is still limited. The objective of this study was to investigate molecular phylogenetic relationships and genetic diversity of the seven Indonesian eel taxa sampled at 27 Indonesian localities, based on the cyt b gene.

MATERIALS AND METHODS

Specimen collection and species identification: A total of 1115 specimens were collected around Indonesia during field trips from 2008 to 2011, covering the entire geographic distribution range of the *Anguilla* genus in Indonesian waters (Fig. 1). Specimens were collected in estuaries along the Indian and Pacific Ocean coasts and around Arafuru and Celebes Seas. All specimens were at the yellow eel subadult stage, except specimens from Cimandiri River (code: Pel) which were glass eels.

A clip of tissue from the anal fin was taken on the 1115 specimens and stored in absolute alcohol for DNA analysis.

In a first step, species assignation was done based on three morphological characters: (i) the horizontal distance between the anterior origins of dorsal and anal fins (= short-fin *vs* long-fin), (ii) the breadth of the maxillary bands according to the number of teeth counted in the mid part of maxilla and (iii) the presence or absence of marbled body coloration of silver eel (for more explanation, see Fahmi *et al*. 2013).

DNA extraction: Total genomic DNA was extracted using a gSYNC Mini Kit (Tissue) from Geneaid. The protocol, according to the manufacturer's recommendations, can be found in the publication of Fahmi *et al*. (2013).

Semi-multiplex species identification: In order to select, for analysis, an equilibrated number of individuals per species, each of the 1115 specimens was first identified using a semi-multiplex PCR protocol according to Fahmi *et al*. (2013), using nine species-specific primers included in one PCR reaction. The diagnostic DNA bands (Fahmi *et al*. 2013) were observed under UV light and photographed with a digital Canon camera. This determination, applied to the 1115 specimens, has already been published (Fahmi *et al*. 2012).

Among the 1115 specimens that were identified, we selected 213 specimens (Table I) for sequencing of the whole cyt b gene. The choice was driven by the best representation of each species and each region. Except for limited captures as *A. borneensis* (3 specimens), at least 5 individuals were sequenced for each station and each species.

PCR amplification and sequencing: The cyt b fragment was amplified by polymerase chain reaction (PCR). The PCR primers were designed especially for this study based on sequences of all species of the *Anguilla* genus published in GenBank (access numbers AP007233-AP007249). The published whole cyt b sequences were aligned with the Clustal W computer program (Mega 5.0 software) in order to determine the common sequence zones among all species. A primer pair, i.e. F-EEL-

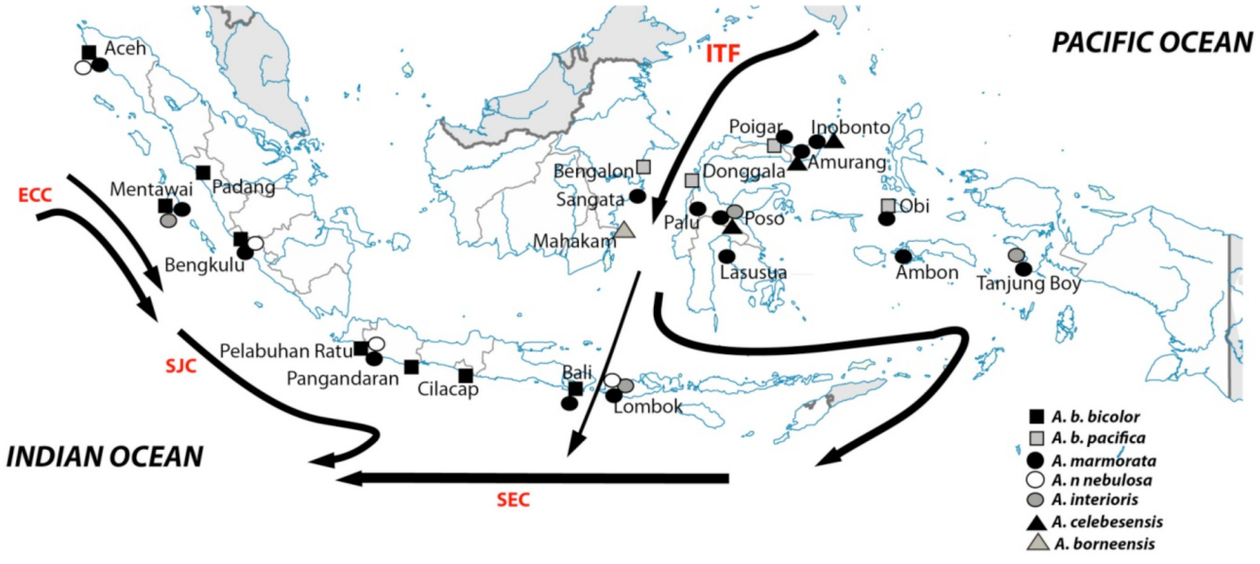

Fig. 1. – Sampling location for this study. The current flow pattern in Indonesia waters, probably influencing the spread of eel larvae, is given through simplified arrows, adapted from Wretzky (2005). ITF (Indonesian Throughflow), SEC (South Equatorial Current), ECC (Equatorial Countercurrent), SJC (South Java Current).

Table I. – Sample characteristics. N° = station numbers used in the phylogenetic tree (Fig. 2); Code = codes of the station names used in the map (Fig. 1). Geographic coordinates are GPS (WGS84) decimal degrees.

Species	N°	Code	Location	Nbr	Date	GPS Coordinate
Anguilla bicolor bicolor	**1**	Ace	Estuary of Tadu, Aceh	8	Jan 2010	4.052734N, 96.239095E
(Nbr=65)	**2**	Pad	Estuary of Bungus River, Padang	16	Jun 2011	0.904510N, 100.345265E
	3	Men	Sea Waters of Mentawai	1	Mar 2012	2.432980S, 99.931922E
	4	Ben	Estuary of Ketaun River, Bengkulu, Sumatera	2	Aug 2008	3.380419S, 101.813796E
	5	Pel	Estuary of Cimadiri, Pelabuhan Ratu, Java	22	Dec 2009	6.985042S, 106.538769E
	6	Cil	Estuary of Cilacap, Java	5	Jun 2009	7.752917N, 109.013627E
	7	Pang	Estuary of Pangandaran, Java	5	Aug 2009	7.698894S, 108.652016E
	8	Bal	Estuary of Mengereng River, Bali	6	Jun 2009	8.471516S, 115.631141E
Anguilla bicolor pacifica	**9**	Poig	Estuary of Poigar, North Celebes	3	Nov 2011	1.010857N, 124.297675E
(Nbr=16)	**10**	Dong	Estuary of Donggala River, Celebes	6	Oct 2008	0.667046S, 119.745792E
	11	Obi	Obi Island, Maluku	3	Nov 2010	1.470287S, 127.637849E
	12	Beng	Estuary of Bengalon River, Borneo	4	Sep 2009	0.731979N, 117.735842E
Anguilla marmorata	**1**	Ace	Sea Waters of Smeulue	2	Mar 2012	2.735306N, 96.221788E
(Nbr=94)	**3**	Men	Sea Waters of Mentawai	7	Mar 2012	1.473035S, 98.870895E
	4	Ben	Estuary of Ketaun River, Bengkulu, Sumatera	6	Aug 2008	3.380419S, 101.813796E
	5	Pel	Estuary of Cimadiri, Pelabuhan Ratu, Java	7	Dec 2009	6.985042S, 106.538769E
	8	Bal	Estuary of Mengereng River, Bali	9	Jun 2009	8.471516S, 115.631141E
	13	Lom	Estuary of Labuan Haji River, Lombok	6	Jun 2009	8.753792N, 116.784744E
	14	Sang	Estuary of Sangata River, Borneo	6	Oct 2010	0.438157N, 117.611260E
	15	Las	Estuary of Lasusua River, Celebes	10	Oct 2012	3.512352N, 120.877051E
	16	Pos	Estuary of Poso River, Celebes	10	Oct 2010	1.388840N, 120.749909E
	17	Pal	Estuary of Tantena River,Clebes	6	Mar 2012	0.884551S, 119,858616E
	18	Amb	Estuary of Tanah merah, Seram, Ambon	5	Aug 2010	2.980005N, 130.010454E
	11	Obi	Obi Island, Maluku	3	Nov 2010	1.470287S, 127.637849E
	19	Pap	Ware mare River, Papua	10	Nov 2010	7.418732S, 138.910563E
	20	Amu	Estuary of Amurang, North Celebes	2	Nov 2010	1.181486N, 124.563303E
	9	Poig	Estuary of Poigar, North Celebes	2	Nov 2011	0.998700N, 124.243768E
	21	Ino	Estuary of Inobonto, North Celebes	3	Jan 2012	0.922279N, 124.088919E
Anguilla celebesensis	**4**	Ben	Estuary of Ketaun River, Bengkulu, Sumatera	1	Aug 2008	3.380419S, 101.813796E
(Nbr=12)	**16**	Pos	Estuary of Poso River, Celebes	7	Oct 2010	1.388840S, 120.749909E
	20	Amu	Estuary of Amurang, North Celebes	2	Nov 2010	1.181486N, 124.563303E
	21	Ino	Estuary of Inobonto, North Celebes	2	Jan 2012	0.922279N, 124.088919E
Anguilla interioris	**3**	Men	Sea Waters of Mentawai	7	Mar 2012	2.432980S, 99.931922E
(Nbr=15)	**13**	Lom	Estuary of Labuan Haji River, Lombok	1	Jun 2009	8.753792S, 116.784744E
	16	Pos	Estuary of Poso River, Celebes	2	Oct 2010	1.388840S, 120.749909E
	19	Pap	Ware mare River, Papua	5	Nov 2010	7.418732S, 138.910563E
Anguilla borneensis *(Nbr=3)*	**22**	Mah	Estuary of Mahakam River, Borneo	3	Oct 2011	0.413258S, 117.565888E
Anguilla nebulosa nebulosa	**1**	Ace	Estuary of Tadu, Aceh	1	Jan 2010	4.052734N, 96.239095E
(Nbr=8)	**4**	Ben	Estuary of Ketaun River, Bengkulu, Sumatera	1	Aug 2008	3.380419S, 101.813796E
	5	Pel	Estuary of Cimadiri, Pelabuhan Ratu, Java	5	Dec 2009	6.985042S, 106.538769E
	13	Lom	Estuary of Labuan Haji River, Lombok	1	Jun 2009	8.753792S, 116.784744E
Total				**213**		

Cytb: 5'-CCA CCG TTG TAA TTC AAC-3' and R-EEL-Cytb: 5'-AAG CTA CTA GGC TTA TC-3', was designed and amplified a fragment of approximately 1184 bp. PCR was carried out according to the protocol given in Fahmi *et al*. (2013). The PCR products were checked on 1.5 % agarose gel electroporesis and stained with 1 % Cyber Safe for 35 min. The migrated DNA was visualized using a blue light under a digital camera.

The sequencing reactions were performed by the private company Genoscreen (Lille, France) using an ABI Prism sequencer (ABI, Applied Biosystem Inc). The sequences were then managed using MEGA ver.5.1 software.

Genetic diversity: The genetic diversity of each species was estimated using the unbiased haplotype diversity parameter (*h*) (Nei 1987) and the nucleotide diversity (π) (Nei & Jin 1989). These were calculated with DNA Sequence Polymorphism software (DnaSP ver 5.1). Using the Hasegawa-Kishino-Yano (i.e. *HKY*) method (Shimodaira & Hasegawa 1999), the best model of nucleotide substitution analyzed under maximum likelihood was *HKY+G+I*; where G is the gamma evolutionary rate among sites and I is the fraction of evolutionarily invariable sites.

The null hypothesis of neutral marker evolution was tested using the Tajima D test (Tajima 1989) with 10,000 permutations. A negative Tajima's D parameter value indicates population expansion. Population expansion analysis were implemented with DnaSP ver 5.1 software.

Phylogenetic analysis: Following Inoue *et al*. (2004), the sequence of the sawtooth eel (*Serrivomer sector,* Serrivomeridae; AP007250) was used as outgroup. Nucleotide sequences of cyt b genes from all specimens analyzed here (213 individuals), all species of the *Anguilla* genus available in GenBank used as taxonomic reference (AP007233-AP007249: 19 species and subspecies) and three species used as outgroups (different genera) were aligned and edited using the Clustal W software program and later checked by eye. Sequence regions in which the site homology was questionable in the alignment were omitted from the analysis.

The molecular phylogenetic tree was constructed with the Molecular Evolution Genetic Analysis (MEGA ver. 5.1) software package (Kumar *et al*. 2008). Neighbor-joining (NJ) methods were chosen and the reliability of each branch was assessed by bootstraps with 1000 replications. Analyses of evolutionary divergence between and within species were conducted using the Kimura 2- parameter model in MEGA ver. 5.1 software.

RESULTS

Cyt b sequence diversity within seven Anguilla *species and subspecies in Indonesia*

After alignment, the 1184 bp sequences were reduced to 1040 bp in order to reach a common length in the 213 sequences analyzed. There were several questionable aligned sites. These regions were omitted from the following analysis so that 950 bp were exploited with a total of 198 polymorphic sites (20.84 %).

Table II presents the nucleotide substitutions in cyt b sequences. Based on the *HKY+G+I* model (Table II), no significant difference in base composition was found, while the frequencies of each nucleotide were not balanced: adenine (A) 30.3 %, tymine (T) 27.1 %, cytosine (C) 28.5 % and guanine (G) had a low frequency of 14 %. The transitions and transversion rate ratios were 0.006 for purines and 26.802 for pyrimidines, while the overall transition/transvertion bias was $R = 6.4$.

The 213 individuals analyzed showed 129 different haplotypes (see supplementary table), 124 of which had never been observed before. *A. marmorata* (n = 94; 16

Table II. – Matrix nucleotide substitution based on *HKY+G+I* model.

From\To	A	T	C	G
A	–	0.0166	0.0175	0.1227
T	0.0186	–	0.2504	0.0086
C	0.0186	0.2381	–	0.0086
G	0.2661	0.0166	0.0175	–

Table III. – Genetic diversity and neutral test of species/sub species of tropical eel genus *Anguilla* from Indonesia waters.

Species/subspecies	n	p	Nhp	h	π (%)	Tajima's D test
A. marmorata	92	16	44	0.937 ± 0.013	0.861 ± 0.002	-1.9511*
A. interioris	13	4	11	0.974 ± 0.051	0.541 ± 0.000	-0.8798
A. n. nebulosa	7	4	6	0.953 ± 0.111	0.302 ± 0.000	-1.6226*
A. b. pacifica	18	4	11	0.935 ± 0.048	1.012 ± 0.006	-2.2838*
A. b. bicolor	66	8	45	0.931 ± 0.026	1.060 ± 0.002	-1.8541*
A. celebesensis	14	4	9	0.923 ± 0.058	0.544 ± 0.000	-0.4112
A. borneensis	3	1	3	1.000 ± 0.000	0.128 ± 0.000	n.d.
Total	213		129	6.653 ± 0.307	4.448 ± 0.010	–
Average	–		–	0.950 ± 0.044	0.635 ± 0.001	–

Note: number of sequence (n), number of populations (p), number of haplotypes (Nhp), haplotype diversity (h) and nucleotide diversity (π), * statistical significants (p = 0.005).

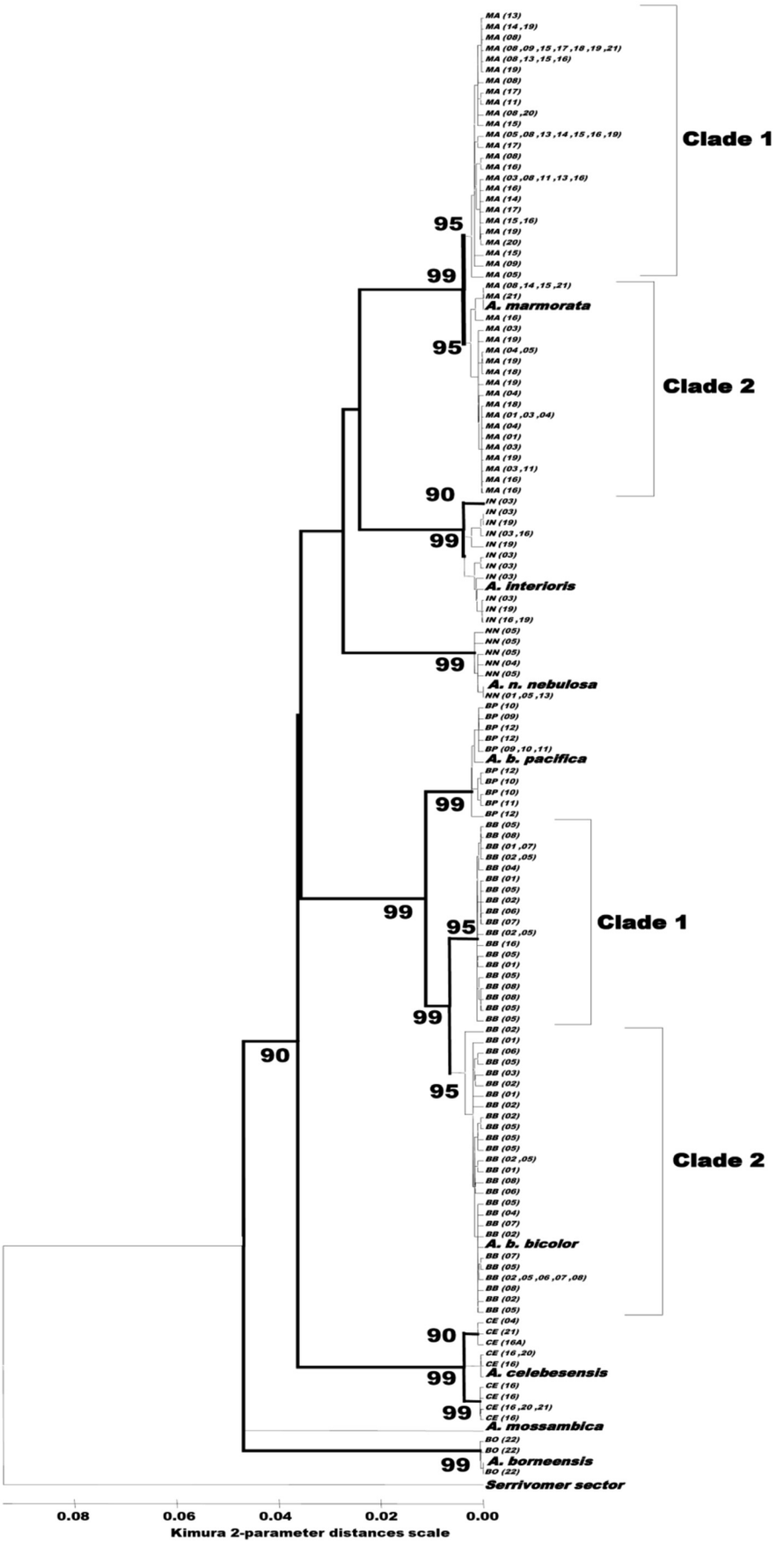

Fig. 2. – Neighbor Joining (NJ) phylogenetic tree of all sequences produced in this study based on 950 bp of mitochondrial DNA cyt b gene fragment under Kimura 2-parameter model (up 75 %). BB = *Anguilla bicolor bicolor*; BO = *A. borneensis*; BP = *A. b. pacifica*; CE = *A. celebesensis*; IN = *A. interioris*; MA = *A. marmorata*; NN = *A. nebulosa nebulosa*. The numbers between parentheses are the station number (Table I, second column). Complete species names indicate the reference sequences provided by Genbank. Only well sustaining bootstrap values are indicated (> 70%).

localities) presented 43 different haplotypes more or less shared among samples. In *A. interioris* (n = 15, 4 localities), 11 haplotypes were found; 5 in *A. n. nebulosa* (n = 8, 4 localities); 10 in *A. b. pacifica* (n = 16, 4 localities); 45 in *A. b. bicolor* (n = 65, 8 localities), 8 in *A. celebesensis* (n = 12, 4 localities) and 2 in *A. borneensis* (n = 3, 1 locality).

The genetic diversity for each species is shown in Table III. The range of haplotype diversity (*h*) within species ranged from 0.92 to 1.00 and almost all species had a high level of haplotype diversity (average 0.95). The range of sequence divergence or nucleotide diversity (π) within each species was between 0.13 and 1.06 % (lower π levels were found in *A. borneensis*). The overall nucleotide diversity (π) was 4.45 %.

Negative Tajima's *D* test values were obtained for all tested species, thus rejecting the null hypothesis of neutral evolution of the cyt b marker. This indicated that most populations of eel species in Indonesian waters have been in expansion except *A. interioris* and *A. celebesensis* (negative value of the test, but not significant). The test for *A. borneensis* has not been performed because of a too small size sample.

Phylogenetic relationships based on the cyt b sequence

The phylogenetic tree of each produced haplotype was constructed using the NJ method and based on the Kimura 2-parameter, using *Serrivomer sector* as outgroup. The phylogenetic tree was tested via 1000 bootstrap replications (Fig. 2).

Two diverged **A. bicolor** *subspecies in Indonesia*

The NJ phylogenetic tree confirmed significant genetic differentiation between *A. b. bicolor* and *A. b. pacifica*, supported by a strong bootstrap value (Fig. 2).

A. b. pacifica consisted of populations from the eastern part of Indonesia waters (Bengalon, Donggala, Poigar and Obi; see locations in Fig. 1). The nucleotide divergence within this taxon ranged from 0 to 3.1 % (Table IV). This sub-species had one dominant haplotype (HapEel-paci15, see supplementary table) shared by all populations except Bengalon.

A. b. bicolor was found in western Indonesian waters (Aceh, Padang, Bengkulu, Pelabuhan Ratu, Cilacap, Pangandaran, Mentawai and Bali; see locations in Fig. 1). Nucleotide differentiation within this subspecies ranged from 0.2 to 2.6 % (Table IV). *A. b. bicolor* also had a dominant haplotype (HapEel-bico59) that was shared by most samples. Allopatric divergence provoked mutations on cyt b sequences of *A. b. bicolor* in 16 nucleotides out of 1040. Nonsynonymous mutations (n = 3) were also found in cyt b sequences between both subspecies.

A wide distribution of **A. marmorata**

A. marmorata was found at nearly all the sampling locations except Padang, Cilacap, Pangandaran and Bengalon, probably due to their low sample sizes (4 to 16 eels analyzed per location). The alignment of 950 bp of cyt b from 92 *A. marmorata* individuals collected at 16 locations gave 99 polymorphic sites (10.42 %). The nucleotide diversity in each sample ranged from 0.08 to 6.9 %. The nucleotide diversity average was 0.973 ± 0.004 %. The *A. marmorata* branch of the NJ phylogenetic tree (Fig. 2) was supported by 99 % of the bootstrap replications. This branch split into two clades, each supported by 95 % of the bootstrap replications. Interclade divergences included a nonsynonymous mutation where isoleucine (I) in clade 1 changed into methionine (M) due to the codon 120 mutation (Table V).

Table IV. – Intraspecific genetic differentiation measured within *A. bicolor* species.

No	Sub species	Sample site	$n_{specimen\ analyzed}$	Nhp	π
1	*A. b. bicolor*	Aceh	6	6	0.871
2	*A. b. bicolor*	Padang	16	10	0.572
3	*A. b. bicolor*	Bengkulu	2	2	1.344
4	*A. b. bicolor*	Pelabuhan Ratu	27	22	2.632
5	*A. b. bicolor*	Pangandaran	5	4	0.557
6	*A. b. bicolor*	Cilacap	5	5	0.729
7	*A. b. bicolor*	Bali	6	5	0.723
8	***A. b. pacifica***	**Bengalon**	**4**	**4**	**0.464**
9	***A. b. pacifica***	**Donggala**	**8**	**6**	**0.247**
10	***A. b. pacifica***	**Obi**	**2**	**1**	**0.000**
11	***A. b. pacifica***	**Poigar**	**4**	**4**	**3.439**
	Total	16	85	69	11.578
	Average			6.27	1.053

* Nucleotide divergence (π) of *A. b. bicolor* (n = 67) = 1.061 % and *A. b. pacifica* (n = 18) = 1.037 %

A. marmorata showed three common haplotypes (HapEel-mar76, HapEel-mar82, HapEel-mar106) that were shared among all samples. This species also had private haplotypes that were found only in specific geographical regions, such as HapEel-mar106 which was only found in western Indonesian waters and was absent in eastern waters, or HapEel-mar76 which was found only in eastern Indonesian waters and was absent in western waters (see supplementary table).

DISCUSSION

This is the first study of phylogenetic relationships and genetic diversity based on mitochondrial DNA cyt b genes from all *Anguilla* taxa inhabiting Indonesian waters. These data are necessary for a local management and conservation of this valuable resource in terms of both biodiversity and economic development. According to Avise *et al*. (1986), providing an adequate description of the genetic structure of populations, reconstructing a genetic architecture and deducing evolutionary factors are key objectives in population genetics.

Among Indonesian eels, some species have a narrow distribution and a short migration loop, while others are distributed in an Indo-Pacific range from Africa to the Galapagos Archipelago (Gagnaire *et al*. 2009, Minegishi *et al*. 2012). Seven freshwater eel species inhabit Indonesian waters: *A. bicolor* (two subspecies: *A. b. bicolor* and *A. b. pacifica*), *A. marmorata*, *A. celebesensis*, *A. borneensis*, *A. interioris*, *A. nebulosa nebulosa* and *A. obscura* (Ege 1939, Castle & Williamson 1974, Fahmi *et al*. 2012, 2013, Fahmi 2013). The first two species are of interest for research and development because they have the largest distribution and highest abundance (Fahmi 2013), which means they have considerable economic potential.

Genetic diversity

The seven species of Indonesian tropical eels showed higher haplotype diversity (h) at the cyt b locus (range 0.93 to 1.00) than that of the temperate *Anguilla anguilla* species in Europe: ranging from 0.62 to 0.82 (Daemen *et al*. 2001) and estimated at 0.78 (Avise *et al*. 1986). However, this diversity is quite similar to that of the temperate Asian species *A. japonica*: 0.97 and 0.96 (Sang *et al*. 1994 and Ishikawa *et al*. 2001, respectively).

The haplotype variation we observed at the cyt b locus was 63.4 % when considering 129 haplotypes, 124 of which are new haplotypes that have never been observed before, from 213 eel specimens. This haplotype diversity is lower than in other species such as Japanese eel (98 %, n = 55, Ishikawa *et al*. 2001). The molecular marker used in this study (cyt b) showed a high level of polymorphic sites (20.48 %) as compared with the D-loop locus in other *Anguilla* species, i.e. *A. japonica*: 11 % (Sang *et al*. 1994) and 17 % (Ishikawa *et al*. 2001) and *A. anguilla*: 0.4 % (Daemen *et al*. 2001). Sequence divergence in this study (0.1-1.06 %) was lower than in *A. japonica* (1.1-1.6 %, Ishikawa *et al*. 2001) and higher than in *A. anguilla* (0.2-0.5 %, Daemen *et al*. 2001).

Table V. – Intraspecific genetic differentiation measured within *A. marmorata* species.

No	Code	Sample site	$n_{specimen\ analyzed}$	Nhp	π
1	Ace	Aceh	2	2	6.910 ± 0.03455
2	Men	Mentawai	8	5	0.295 ± 0.00073
3	Ben	Bengkulu	7	4	0.084 ± 0.00028
4	Pel	Pelabuhan Ratu	7	4	2.221 ± 0.01246
5	Bal	Bali	12	10	1.401 ± 0.00902
6	Lom	Lombok	6	5	0.313 ± 0.00103
7	Las	Lasusua	9	6	0.288 ± 0.00060
8	Pal	Palu	4	4	0.400 ± 0.00086
9	Pos	Poso	9	8	0.538 ± 0.00107
10	Sang	Sangata	5	5	0.365 ± 0.00079
11	Poig	Poigar	3	2	0.192 ± 0.00090
12	Amu	Amurang	2	2	0.384 ± 0.00192
13	Ino	Inobonto	3	3	0.448 ± 0.00083
14	Obi	Obi	3	3	0.576 ± 0.00202
15	Amb	Ambon	3	3	0.576 ± 0.00193
16	Pap	Papua	9	8	0.581 ± 0.00073
	Total	16	92	74	15.572 ± 0.0697
	Average			4.63	0.973 ± 0.0044

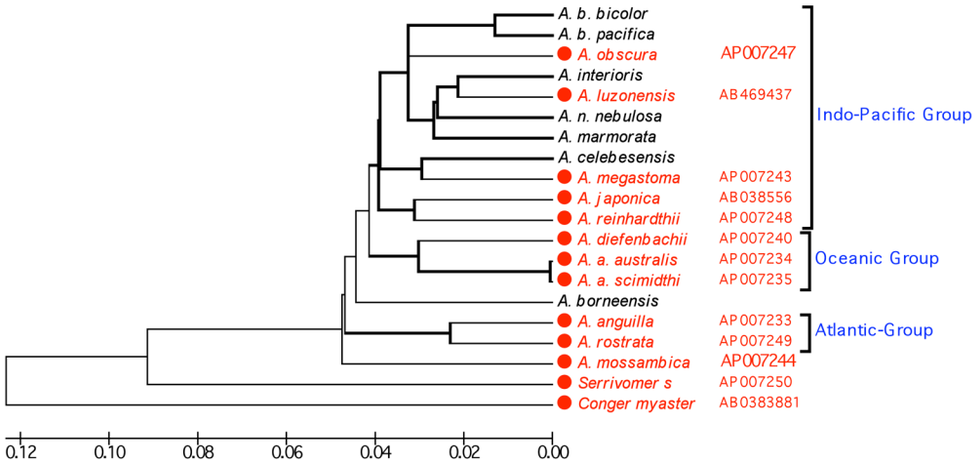

Fig. 3. – Dendrogram of the genetic distance of Indonesian tropical eels based on the cyt b sequence. Grey font: cyt b sequence from this study, and black font: based on sequences from GenBank.

Phylogenetics relationship

The first molecular phylogenetic investigation on the *Anguilla* genus was conducted by Tagliavini *et al*. (1996) based on a 475 bp segment of the cytochrome b (cyt b) gene from five species (*A. anguilla, A. rostrata, A. mossambica, A. japonica* and *A. marmorata*). Then Aoyama & Tsukamoto (1997) and Tsukamoto & Aoyama (1998) constructed phylogenies based on a 410 bp segment of the cyt b gene from eight species (*A. celebesensis, A. marmorata, A. mossambica, A. japonica, A. reinthardti, A. rostrata, A. anguilla* and *A. australis*). Thereafter, two molecular phylogenetic studies were published with more data: Lin *et al*. (2001) examined mitochondrial 12S rRNA and cyt b genes from 12 species and Aoyama *et al*. (2001) sequenced mitochondrial 16S rRNA and cyt b genes for all 18 taxa of the genus that are currently known. Both studies presented almost the same topology, showing species clusters corresponding to their geographic range.

The tree topology given in Fig. 2 is almost the same as that constructed by Minegishi *et al*. (2005) based on the whole mt-DNA sequence (15000 bp). Based only on cyt b sequences, but using published complementary species, the topology shown in Figure 3 also revealed three clades according to the geographic distribution: the Atlantic group (*A. anguilla* and *A. rostrata*), the Oceanic group (*A. dieffenbachii, A. australis australis* and *A. a. schmidtii*) and the Indo-Pacific group (*A. celebesensis, A. megastoma, A. nebulosa nebulosa, A. bicolor bicolor* and *A. b. pacifica*). We can conclude that the cyt b marker that was used in this study is very reliable for determining *Anguilla* phylogenetic relationships.

New knowledge on two remarkable species

As expected, *A. bicolor* was split into two subspecies with very high bootstrap values. Interestingly, the *A. b. bicolor* cluster (bootstrap value 99 %, Fig. 2) was divided into two clades, also supported by a high bootstrap (99 and 95 %, respectively), with each clade having two diagnostic nucleotides. This is similar to the observation of Minegishi *et al*. (2012), highlighting two mitochondrial sublineages in the Indian Ocean that do not coincide with the geographical distribution. It was hypothesized that after a first allopatric isolation, a possible secondary contact occurred in this region, thus distributing the two sublineages equally (Minegishi *et al*. 2012).

Indonesian *A. marmorata* populations were also split into two sublineages (Fig. 2). This splitting includes a nonsynonymous mutation whereby isoleucine was changed into methionine. One of the best molecular studies of this species was recently published (Gagnaire *et al*. 2011). Applying the AFLP method (based on the analysis of more than 850 loci) on the whole species range (from Madagascar to French Polynesia, i.e. 18,000 km), Indonesian marble eels appeared to be at the crossroads of three sublineages: North Pacific (NPO), South Pacific (SPO) and South West Indian (SWIO) sublineages. Less demonstrative because it was limited to the Indian Ocean, a previous publication (Gagnaire *et al*. 2009) showed the existence of two stocks at the two ends of the Indian Ocean using 16S rRNA gene of the mtDNA and SNP and microsatellites of the nDNA. These observations are now, for the first time, confirmed by mtDNA cyt b sequence analysis. Among the 16 localities where *A. marmorata* was found, 10 hosted the two clades in appearant sympatry, 4 showed only clade 1, and 2 only clade 2. The 4 private sites of clade 1 are mostly located north of Celebes (with the exception of the Lombok station in the southernmost part of the Flores Sea). On the contrary, the 2 private localities where only clade 2 is found are situated north of Sumatra, along the Indian Ocean coasts. These geographic positions suggest that clade 1 corresponds to NPO and clade 2 to SWIO.

Finally, beyond the confirmation and improvement in the distribution and phylogeny of Indonesian eels, the present study summarizes current knowledge obtained through molecular methods. This should now enable integrated management of the resource and the conservation of species that are vulnerable because they inhabit limited areas. Currently, freshwater eels have been harvested intensively in most regions of Indonesia. In order to limit this destruction, the Indonesian government banned glass eel exportation and established fisheries regulation. Vulnerable species were impossible to distinguish from very common species like *A. marmorata* or *A. bicolor* when captured in estuaries sympatry at the glass eel stage. The present analysis introduces the possibility to establish different rules according to the species and their abundance.

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Supplementary data
Table S1 : Distribution and characteristics of the sequences used in Fig. 2 (including new and reference sequences)

Haplotype	Species	Genbank Accession	Population	Reference
AP007238.1	*A. borneensis*	**AP007238.1**	MAHAKAM	**Minegishi *et al*. (2005)**
HapEel-born01	*A. borneensis*	HG965452	MAHAKAM	this study
HapEel-born02	*A. borneensis*	HG965453	MAHAKAM	this study
HapEel-cel03	*A. celebesensis*	HG965454	BENGKULU	this study
HapEel-cel04	*A. celebesensis*	HG965455	INOBONTO	this study
HapEel-cel05	*A. celebesensis*	HG965456	POSO	this study
AP007239.1	*A. celebesensis*	**AP007239.1**	POSO, AMURANG	**Minegishi *et al.* (2005)**
HapEel-cel06	*A. celebesensis*	HG965457	POSO	this study
HapEel-cel07	*A. celebesensis*	HG965458	POSO	this study
HapEel-cel08	*A. celebesensis*	HG965459	POSO	this study
HapEel-cel09	*A. celebesensis*	HG965460	POSO, AMURANG	this study
HapEel-cel10	*A. celebesensis*	HG965461	POSO	this study
AP007237.1	***A. b. pacifica***	**AP007237.1**		**Minegishi *et al*.(2005)**
HapEel-paci11	*A. b. pacifica*	HG965462	BENGALON	this study
HapEel-paci12	*A. b. pacifica*	HG965463	BENGALON	this study
HapEel-paci13	*A. b. pacifica*	HG965464	BENGALON	this study
HapEel-paci14	*A. b. pacifica*	HG965465	BENGALON	this study
HapEel-paci15	*A. b. pacifica*	HG965466	DONGGALA, POIGAR, OBI	this study
HapEel-paci16	*A. b. pacifica*	HG965467	DONGGALA	this study
HapEel-paci17	*A. b. pacifica*	HG965468	DONGGALA	this study
HapEel-paci18	*A. b. pacifica*	HG965469	DONGGALA	this study
HapEel-paci19	*A. b. pacifica*	HG965470	OBI	this study
HapEel-paci20	*A. b. pacifica*	HG965471	POIGAR	this study
AP007236.1	***A. b. bicolor***	**AP007236.1**		**Minegishi *et al.* (2005)**
HapEel-bico21	*A. b. bicolor*	HG965472	PELABUHANRATU	this study
HapEel-bico22	*A. b. bicolor*	HG965473	BALI	this study
HapEel-bico23	*A. b. bicolor*	HG965474	PADANG, PELABUHANRATU	this study
HapEel-bico24	*A. b. bicolor*	HG965475	ACEH, PANGANDARAN	this study
HapEel-bico25	*A. b. bicolor*	HG965476	BENGKULU	this study
HapEel-bico26	*A. b. bicolor*	HG965477	ACEH	this study
HapEel-bico27	*A. b. bicolor*	HG965478	PELABUHANRATU	this study
HapEel-bico28	*A. b. bicolor*	HG965479	PADANG	this study
HapEel-bico29	*A. b. bicolor*	HG965480	CILACAP	this study
HapEel-bico30	*A. b. bicolor*	HG965481	PANGANDARAN	this study
HapEel-bico31	*A. b. bicolor*	HG965482	PADANG, PELABUHANRATU	this study
HapEel-bico32	*A. b. bicolor*	HG965483	POSO	this study
HapEel-bico33	*A. b. bicolor*	HG965484	PELABUHANRATU	this study
HapEel-bico34	*A. b. bicolor*	HG965485	ACEH	this study
HapEel-bico35	*A. b. bicolor*	HG965486	PELABUHAN RATU	this study
HapEel-bico36	*A. b. bicolor*	HG965487	BALI	this study
HapEel-bico37	*A. b. bicolor*	HG965488	BALI	this study
HapEel-bico38	*A. b. bicolor*	HG965489	PELABUHAN RATU	this study
HapEel-bico39	*A. b. bicolor*	HG965490	PELABUHAN RATU	this study
HapEel-bico40	*A. b. bicolor*	HG965491	PADANG	this study
HapEel-bico41	*A. b. bicolor*	HG965492	ACEH	this study

Haplotype	Species	Genbank Accession	Population	Reference
HapEel-bico42	*A. b. bicolor*	HG965493	CILACAP	this study
HapEel-bico43	*A. b. bicolor*	HG965494	PELABUHAN RATU	this study
HapEel-bico44	*A. b. bicolor*	HG965495	MENTAWAI	this study
HapEel-bico45	*A. b. bicolor*	HG965496	PADANG	this study
HapEel-bico46	*A. b. bicolor*	HG965497	ACEH	this study
HapEel-bico47	*A. b. bicolor*	HG965498	PADANG	this study
HapEel-bico48	*A. b. bicolor*	HG965499	PADANG	this study
HapEel-bico49	*A. b. bicolor*	HG965500	PELABUHAN RATU	this study
HapEel-bico50	*A. b. bicolor*	HG965501	PELABUHAN RATU	this study
HapEel-bico51	*A. b. bicolor*	HG965502	PELABUHAN RATU, PADANG	this study
HapEel-bico52	*A. b. bicolor*	HG965503	PELABUHANRATU	this study
HapEel-bico53	*A. b. bicolor*	HG965504	ACEH	this study
HapEel-bico54	*A. b. bicolor*	HG965505	BALI	this study
HapEel-bico55	*A. b. bicolor*	HG965506	CILACAP	this study
HapEel-bico56	*A. b. bicolor*	HG965507	PELABUHAN RATU	this study
HapEel-bico57	*A. b. bicolor*	HG965508	PADANG	this study
HapEel-bico58	*A. b. bicolor*	HG965509	BENGKULU	this study
HapEel-bico59	*A. b. bicolor*	HG965510	CILACAP, PADANG, PANGANDARAN, BALI, PELABUHAN RATU	this study
HapEel-bico60	*A. b. bicolor*	HG965511	PANGANDARAN	this study
HapEel-bico61	*A. b. bicolor*	HG965512	PANGANDARAN	this study
HapEel-bico62	*A. b. bicolor*	HG965513	PELABUHAN RATU	this study
HapEel-bico63	*A. b. bicolor*	HG965514	BALI	this study
HapEel-bico64	*A. b. bicolor*	HG965515	PADANG	this study
HapEel-bico65	*A. b. bicolor*	HG965516	PELABUHAN RATU	this study
HapEel-neb66	*A. n. nebulosa*	HG965517	PELABUHAN RATU	this study
HapEel-neb67	*A. n. nebulosa*	HG965518	PELABUHAN RATU	this study
HapEel-neb68	*A. n. nebulosa*	HG965519	BENGKULU	this study
AP007246.1	*A. n. nebulosa*	**AP007246.1**	ACEH, LOMBOK, PELABUHAN RATU	**Minegishi *et al*. (2005)**
HapEel-neb69	*A. n. nebulosa*	HG965520	PELABUHAN RATU	this study
HapEel-neb70	*A. n. nebulosa*	HG965521	PELABUHAN RATU	this study
HapEel-mar71	*A. marmorata*	HG965522	LOMBOK	this study
HapEel-mar72	*A. marmorata*	HG965523	PAPUA, SANGATA	this study
HapEel-mar73	*A. marmorata*	HG965524	BALI	this study
HapEel-mar74	*A. marmorata*	HG965525	PAPUA	this study
HapEel-mar75	*A. marmorata*	HG965526	POSO, BALI, LOMBOK, LASUSUA	this study
HapEel-mar76	*A. marmorata*	HG965527	AMBON, LASUSUA, PALU, PAPUA, BALI, POIGAR, INOBONTO	this study
HapEel-mar77	*A. marmorata*	HG965528	BALI	this study
HapEel-mar78	*A. marmorata*	HG965529	PALU	this study
HapEel-mar79	*A. marmorata*	HG965530	OBI	this study
HapEel-mar80	*A. marmorata*	HG965531	BALI, AMURANG	this study
HapEel-mar81	*A. marmorata*	HG965532	LASUSUA	this study
HapEel-mar82	*A. marmorata*	HG965533	BALI, LASUSUA, LOMBOK, PAPUA, SANGATA, PELABUHAN RATU, POSO	this study
HapEel-mar83	*A. marmorata*	HG965534	PALU	this study
HapEel-mar84	*A. marmorata*	HG965535	BALI	this study
HapEel-mar85	*A. marmorata*	HG965536	POSO	this study

Haplotype	Species	Genbank Accession	Population	Reference
HapEel-mar86	*A. marmorata*	HG965537	LOMBOK, BALI, MENTAWAI, POSO, OBI	this study
HapEel-mar87	*A. marmorata*	HG965538	POSO	this study
HapEel-mar88	*A. marmorata*	HG965539	SANGATA	this study
HapEel-mar89	*A. marmorata*	HG965540	PALU	this study
HapEel-mar90	*A. marmorata*	HG965541	LASUSUA, POSO	this study
HapEel-mar91	*A. marmorata*	HG965542	PAPUA	this study
HapEel-mar92	*A. marmorata*	HG965543	AMURANG	this study
HapEel-mar93	*A. marmorata*	HG965544	LASUSUA	this study
HapEel-mar94	*A. marmorata*	HG965545	POIGAR	this study
HapEel-mar95	*A. marmorata*	HG965546	PELABUHAN RATU	this study
AP007242.1	*A. marmorata*	**AP007242.1**	BALI, LASUSUA, SANGATA, INOBONTO	**Minegishi *et al*. (2005)**
HapEel-mar96	*A. marmorata*	HG965547	INOBONTO	this study
HapEel-mar97	*A. marmorata*	HG965548	POSO	this study
HapEel-mar98	*A. marmorata*	HG965549	MENTAWAI	this study
HapEel-mar99	*A. marmorata*	HG965550	PAPUA	this study
HapEel-mar100	*A. marmorata*	HG965551	PAPUA	this study
HapEel-mar101	*A. marmorata*	HG965552	AMBON	this study
HapEel-mar102	*A. marmorata*	HG965553	BENGKULU, PELABUHAN RATU	this study
HapEel-mar103	*A. marmorata*	HG965554	PAPUA	this study
HapEel-mar104	*A. marmorata*	HG965555	BENGKULU	this study
HapEel-mar105	*A. marmorata*	HG965556	AMBON	this study
HapEel-mar106	*A. marmorata*	HG965557	BENGKULU, MENTAWAI, PELABUHAN RATU, ACEH	this study
HapEel-mar107	*A. marmorata*	HG965558	BENGKULU	this study
HapEel-mar108	*A. marmorata*	HG965559	ACEH	this study
HapEel-mar109	*A. marmorata*	HG965560	MENTAWAI	this study
HapEel-mar110	*A. marmorata*	HG965561	PAPUA	this study
HapEel-mar111	*A. marmorata*	HG965562	MENTAWAI, OBI	this study
HapEel-mar112	*A. marmorata*	HG965563	POSO	this study
HapEel-mar113	*A. marmorata*	HG965564	POSO	this study
AP007241.1	***A. interioris***	**AP007241.1**		**Minegishi *et al.* (2005)**
HapEel-int114	*A. interioris*	HG965565	MENTAWAI	this study
HapEel-int115	*A. interioris*	HG965566	PAPUA	this study
HapEel-int116	*A. interioris*	HG965567	PAPUA, POSO	this study
HapEel-int117	*A. interioris*	HG965568	MENTAWAI	this study
HapEel-int118	*A. interioris*	HG965569	MENTAWAI	this study
HapEel-int119	*A. interioris*	HG965570	MENTAWAI	this study
HapEel-int120	*A. interioris*	HG965571	PAPUA	this study
HapEel-int121	*A. interioris*	HG965572	MENTAWAI, POSO	this study
HapEel-int122	*A. interioris*	HG965573	MENTAWAI	this study
HapEel-int123	*A. interioris*	HG965574	PAPUA	this study
HapEel-int124	*A. interioris*	HG965575	MENTAWAI	this study