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Which is the Dispersal Route of the Ancient Atlantic eels: Reanalysis of the Phylogeny of Freshwater Eels

Abstract

Recent molecular studies for the freshwater eels, the genus *Anguilla*, have revealed that the traditional systematics based on the morphological features is not appropriate. Nevertheless, the arguments regarding the dispersal route of the ancient Atlantic eels have not been brought into a conclusion either. In this study, we gathered all published Anguilliformes sequences and reanalyze the phylogenetic relationships of the genus *Anguilla*. The monophyletic clade with *A. mossambica* and Atlantic eels, main basis of the Tethys Seaway hypothesis, is not supported by any analyses. The final result confirms the realistic Central American gateway scenario, and also proposes the probability of the existence of a new *Anguilla* species in the genetic view.

Key words: Eel Phylogeny, Dipersal route

Molecular studies in freshwater eels have been conducted recently to provide more information other than the similar morphological characters between the eel species for analyzing their phylogenetic relationships[1-6]. These studies all agree with the four-groups scenario proposed by Ege[7] and suggest that the ancestors of Atlantic eels were derived from the western Pacific through the Tethys Sea to the paleo-Atlantic Ocean by the global tropic westward current[8,9] as indicated in Fig. 1. Lin[10] first recommended that the morphological features might be unstable or have occurred independently in different lineages during evolution and should not be appropriate for constructing the phylogenetic relationships as presented by Ege[7]. The following studies also support his result[11,12]. However, the arguments regarding the dispersal route of ancient Atlantic eels have not been brought yet into a conclusion.

The Tethys Seaway hypothesis depends on two bases, one is the lack of *Anguilla* species along the eastern margins of the Pacific and south Atlantic, the other is the monophyletic clade including Atlantic eels and the South African eel, *A. mossambica*, in the phylogenetic tree. Although previous studies suggest the Tethys Seaway hypothesis[2-4], the monophyletic clade did not appear in their phylogenetic results generated from 410 bp of the cytochrome *b* gene for 8 of 15 species. Subsequent studies[5,12] constructed the molecular phylogeny for all species of the genus *Anguilla* based on complete cytochrome *b* and partial 16S rRNA genes. The Atlantic eels and *A. mossambica* were thus clustered together in their parsimonious tree. Nevertheless, Lin et al.[11] analyzed complete cytochrome *b* and 12S rRNA genes for 11 species and argued against the existence of the monophyletic clade of Atlantic eels and *A. mossambica*.

Lin, Y. S., Y. P. Pho and C. S. Tzeng (2001) Which is the dispersal route of the ancient Atlantic eels: Reanalysis of the phylogeny of freshwater eels. J. Taiwan Fish. Res., **9**(1&2): 161-173.

Lin[10] first proposed the Central American gateway scenario instead of the Tethys Sea hypothesis and was supported by Lin et al.[11] They speculated that the ancestors of the Atlantic eels did not migrate by drifting through the Tethys Seaway at the leptocephali stage but instead trekked across the Central American Isthmus to the Sargasso Sea for spawning at the adult stage. This assumption provides a realistic explanation why *Anguilla* species did not settle along the eastern margins of the Pacific and south Atlantic. The divergence time of the genus *Anguilla* calculated by Lin et al.[11] also supports the Central American scenario but not the Tethys Seaway hypothesis.

In this study, we gather all the published molecular data of the genus *Anguilla* to reanalyze their phylogenetic relationships and discuss which dispersal route of the ancient Atlantic eels is probable. We also propose the probability of the existence of a new *Anguilla* species in the genetic view.

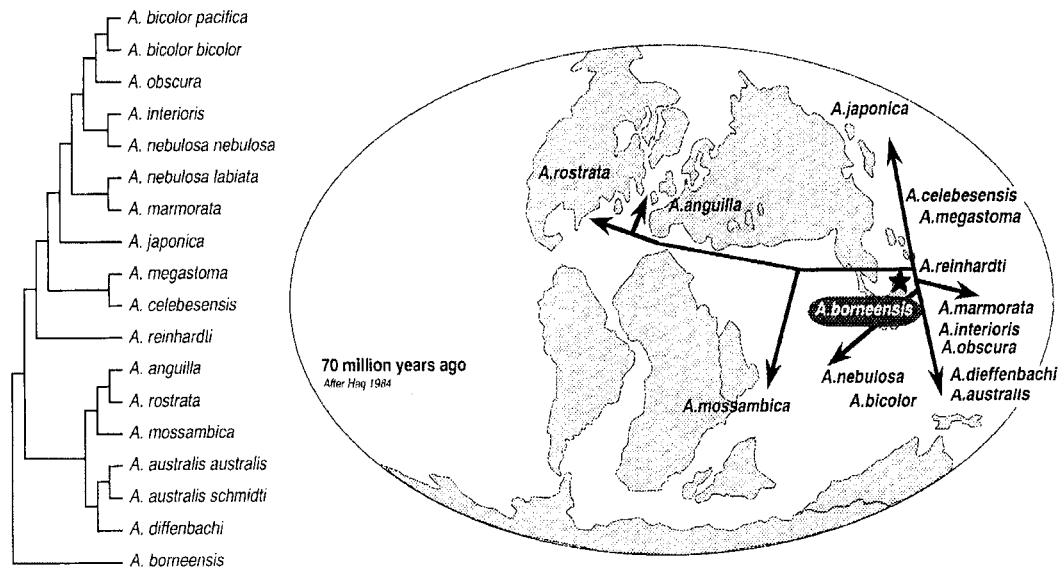

Fig. 1. Molecular phylogenetic relationships and historical dispersion of the genus *Anguilla* suggested by Aoyama et al. (12). *A. nebulosa* and *A. borneensis* are the synonyms of *A. bengalensis* and *A. malgumora*, respectively.

Materials and Methods

We collected 146 sequences of three mitochondrial genes including all the *Anguilla* species and 17 other species belong to Elopomorpha from the literature and GenBank for analyses (Table 1). *A. nebulosa* and *A. borneensis* in Aoyama et al.[12] are the synonyms of *A. bengalensis* and *A. malgumora*, respectively. The 16S rRNA sequence of *Stemonidium hypomelas* published by Aoyama et al.[12] and the cytochrome *b* and 12S rRNA sequences of *S. hypomelas* published by Inoue et al.[13] were considered as the same individual in this study. The 12S and 16S rRNA sequences were aligned respectively according to the secondary structures [14-16], whereas the cytochrome *b* sequences were aligned manually because all of them are of the same length (1140 bp). The base pair numbers deposited in Table 1 are according to the alignment results. One segment of 12S rRNA gene and seven segments of 16S rRNA gene could not be aligned exactly and were eliminated in the following analyses (nucleotide numbers: 191-194, 1144-1150, 1204-1207, 1523-1527, 1803-1805, 1865-1868, 2281-2284, and 2380-2395, in AB038556).

Table 1. Samples analyzed

Source	Species (Number of haplotypes)	Family	Gene [a]		
			Cytochrome b	12S rRNA	16S rRNA
Lin (10);	*Anguilla japonica* (2)	Anguillidae	1-1140	1-1023	-
Lin et al. (11);	*Anguilla marmorata* (2)	Anguillidae	1-1140	1-1023	-
AF006702-AF006719;	*Anguilla reinhardti* (2)	Anguillidae	1-1140	1-1023	-
AF074863-AF074866;	*Anguilla bicolor pacifica* (2)	Anguillidae	1-1140	1-1023	-
AF266482-AF266503;	*Anguilla bicolor bicolor* (1)	Anguillidae	1-1140	1-1023	-
AF267913	*Anguilla dieffenbachi* (1)	Anguillidae	1-1140	1-1023	-
	Anguilla australis (2)	Anguillidae	1-1140	1-1023	-
	Anguilla anguilla (2)	Anguillidae	1-1140	1-1023	-
	Anguilla rostrata (2)	Anguillidae	1-1140	1-1023	-
	Anguilla malgumora (2)	Anguillidae	1-1140	1-1023	-
	Anguilla mossambica (2)	Anguillidae	1-1140	1-1023	-
	Anguilla bengalensis labiata (1)	Anguillidae	1-1140	1-1023	-
	Ariosoma shiroanago major (1)	Congridae	1-1140	1-1023	-
Aoyama et al. (12);	*Anguilla celebesensis* (1)	Anguillidae	1-1140	97-1017	1-1525
AB021748-AB021783;	*Anguilla interioris* (1)	Anguillidae	1-1140	97-1017	1-1525
AB021885-AB021901	*Anguilla megastoma* (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla marmorata (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla nebulosa nebulosa (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla reinhardti (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla borneensis (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla japonica (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla rostrata (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla anguilla (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla mossambica (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla dieffenbachi (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla bicolor bicolor (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla bicolor pacifica (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla obscura (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla australis australis (1)	Anguillidae	1-1140	97-1017	1-1525
	Anguilla australis schmidti (1)	Anguillidae	1-1140	97-1017	1-1525
	Conger japonicus (1)	Congridae	-	-	1-1525
	Stemonidium hypomelas (1)	Serrivomeridae	-	-	1-1525
Inoue et al. (29); AB038556	*Anguilla japonica (1)*	Anguillidae	1-1140	1-1023	1-1586
Inoue et al. (13);	*Conger myriaster* (1)	Congridae	1-1140	1-1023	1-1586
AB038381; AB038410;	*Moringua edwardsi* (1)	Moringuidae	1-780	106-832	-
AB038414-AB038420;	*Kaupichthys hyproroides* (1)	Chlopsidae	1-780	106-832	-
AB049978-AB049991	*Gymnothorax kidako* (1)	Muraenidae	1-780	106-832	-
	Synaphobranchus kaupii (1)	Synaphobranchidae	1-780	106-832	-
	Ophisurus macrorhynchus (1)	Ophichthidae	1-780	106-832	-
	Nessorhamphus danae (1)	Derichthyidae	1-780	106-832	-
	Muraenesox bagio (1)	Muraenesocidae	1-780	106-832	-
	Nemichthys scolopaceus (1)	Nemichthyidae	1-780	106-832	-
	Nettastoma parviceps (1)	Nettastomatidae	1-780	106-832	-
	Stemonidium hypomelas (1)	Serrivomeridae	1-780	106-832	-
	Megalops cyprinoides (1)	Megalopidae	1-780	106-832	-
Bastro et al., (6)	*Anguilla japonica* (3)	Anguillidae			940-1586
AJ244B11-AJ244832	*Anguilla australis* (4)	Anguillidae			940-1586
	Anguilla rostrata (1)	Anguillidae			940-1586
-	*Anguilla anguilla* (5)	Anguillidae			940-1586
-	*Anguilla reimharti* (1)	Anguillidae			980-1586
-	*Anguilla mossambica* (1)	Anguillidae			1002-1586
-	*Anguilla marmorata* (4)	Anguillidae			940-1586
-	*Anguilla obscura* (1)	Anguillidae			1278-1586
-	*Conger oceanicus* (2)	Congridae			1006-1571
Forey et al., (1);	*Anguilla rostrata* (1)	Anguillidae			987-1556
X99183; x99185	*Echiophis puctitor* (1)	Ophichthidae			987-1556
X99187	*Ophichthus rex* (1)	Ophichthidae			987-1556

Note. [a] The base pair numbers are deposited according to the alignment results.

Table 2 illustrates that the cytochrome *b* gene sequence of *A. bicolor bicolor* published by Aoyama et al.[12] is incorrect. The first 457 bp of the sequence is similar to *A. mossambica* but *A. bicolor bicolor*. Cross-contamination of DNA samples might be the most likely explanation in this situation. The other similar event appeared in Bastrop et al.'s (2000) *A. mossambica* sample, which was contaminated with the sample of *A. reinhardti* as indicated by Aoyama et al.[12] and Lin et al.[11] (Table 3). Thus these two questionable sequences were eliminated from our data set analysis.

Table 2. The nucleotide difference matrix of cytochrome *b* gene among the sequences of *A. mossambica* and the two subspecies of *A. bicolor*.

	Number of nucleotide differences of cytochrome b gene															
	First 457 base pairs								*Last 683 base pairs*							
Sample	*[1]*	*[2]*	*[3]*	*[4]*	*[5]*	*[6]*	*[7]*	*[8]*	*[1]*	*[2]*	*[3]*	*[4]*	*[5]*	*[6]*	*[7]*	*[8]*
[1] *A. bicolor pacifica* 1 (Lin)	-	0	1	5	33	33	33	33	-	1	3	22	28	70	67	71
[2] *A. bicolor pacifica* 2 (Lin)		-	1	5	33	33	33	33		-	2	21	27	69	66	70
[3] *A. bicolor pacifica* (Aoyama)			-	4	33	33	33	33			-	19	25	68	65	69
[4] *A. bicolor bicolor* (Lin)				-	37	37	37	37				-	12	60	57	61
[5] *A. bicolor bicolor* (Aoyama)					-	0	1	0					-	59	56	60
[6] *A. mossambica* 1 (Lin)						-	1	0						-	3	5
[7] *A. mossambica* 2 (Lin)							-	1							-	6
[8] *A. mossambica* (Aoyama)								-								-

Table 3. The nucleotide difference matrix of 16S rRNA gene among the sequences of *A. mossambica* and *A. reinhardti*.

Sample	*Number of nucleotide differences of partial 16S rRNA gene (524 base pairs)*			
	[1]	*[2]*	*[3]*	*[4]*
[1] *A. mossambica* (Aoyama)	-	11	11	12
[2] *A. mossambica* (Bastrop)		-	0	1
[3] *A. reinhardti* (Aoyama)			-	1
[4] *A. reinhardti* (Bastrop)				-

We sorted out our data sets with the three mitochondrial genes combined or separated independently and managed them by two processes for not all the sequences in Table 1 are complete. The first one is keeping the complete sequences (or almost complete) with less species, and the other is including all the species for shorter segments. The number of transitional (ts) and transversional (tv) substitutions and Tamura-Nei distances[17] were calculated for all the data sets in the MEGA package Version 1.02[18]. The neighbor-joining method[19] was also implemented with 5,000 bootstrap replications[20] to construct the phylogenetic relationships. Besides the distance matrix methods, we also employed

Maximum Parsimony (MP) and Maximum Likelihood (ML) methods using PAUP* Version 4.0b8[21]. Alternative phylogenetic trees were compared with each other using the Kishino-Hasegawa[22], Templeton[23], and winning-sites tests, as implemented in PAUP*. Table 4 lists the data sets employed in the MP and ML analyses. The alternative tree topologies were prepared according to the phylogenetic relationships constructed by neighbor-joining method based on complete cytochrome *b* and 12S rRNA genes. We shuffled five certain clades in the constructed tree to form 105 possible alternative topologies. The ts/tv ratio, nucleotide frequencies, and proportion of invariable sites were estimated by PAUP* and the variable sites were assumed as Gamma distribution when ML was utilized. Other default parameter settings were applied.

Table 4. The data sets applied in the Maximum Parsimony and Maximum Likelihood analyses.

Applied gene	*Data set*												
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*XIII*
Cytochrome *b* gene	+	+				+	+	+	+			+	+
12S rRNA gene			+	+		+	+			+	+	+	+
16S rRNA gene					+			+	+	+	+	+	+
Applied species													
12 *Anguilla* sp. (Lin)	+	+	+	+		+	+						
17 *Anguilla* sp. (Aoyama)	+	+	+	+	+	+	+	+	+	+	+	+	+
Anguilla japonica (Inoue)	+	+	+	+	+	+	+	+	+	+	+	+	+
Conger myriaster (Inoue)	+	+	+	+	+	+	+	+	+	+	+	+	+
Ariosoma shiroanago major (Lin)	+	+	+	+		+	+						
Conger japonicus (Aoyama)					+								
Stemonidium hypomelas (Aoyama)					+				+		+		+
Stemonidium hypomelas (Inoue)		+		+			+		+		+		+

Results and Discussion

Fig. 2 illustrates the relationships between transitional and transversional substitutions among species in different genes. Comparing these three genes for the genus *Anguilla*, cytochrome *b* gene possesses the largest number of substitutions and is slightly saturated in transitions. The imperceptible saturation could be corrected by the distance estimation methods when constructing the distance based phylogenetic relationships. More substitutions provide more information to construct a reliable phylogeny. As this viewpoint, cytochrome *b* gene is better than the other two rRNA genes for constructing the phylogeny in the genus *Anguilla*. The highest multiple correlation coefficient (R^2) value, 0.7626, appears in 12S rRNA gene, and the second highest value, 0.5888, is in cytochrome *b* gene. A low R^2 value implies a nonlinear relationship, which would increase the bias when constructing the phylogenetic tree. The stem regions in the rRNA genes are conserved and stable in evolution, however, the loop regions evolve rapidly and could not be aligned well. The uncertain alignment with less informative sites would lead to ambiguous phylogenetic relationships especially by Maximum Parsimony method and should be avoided.

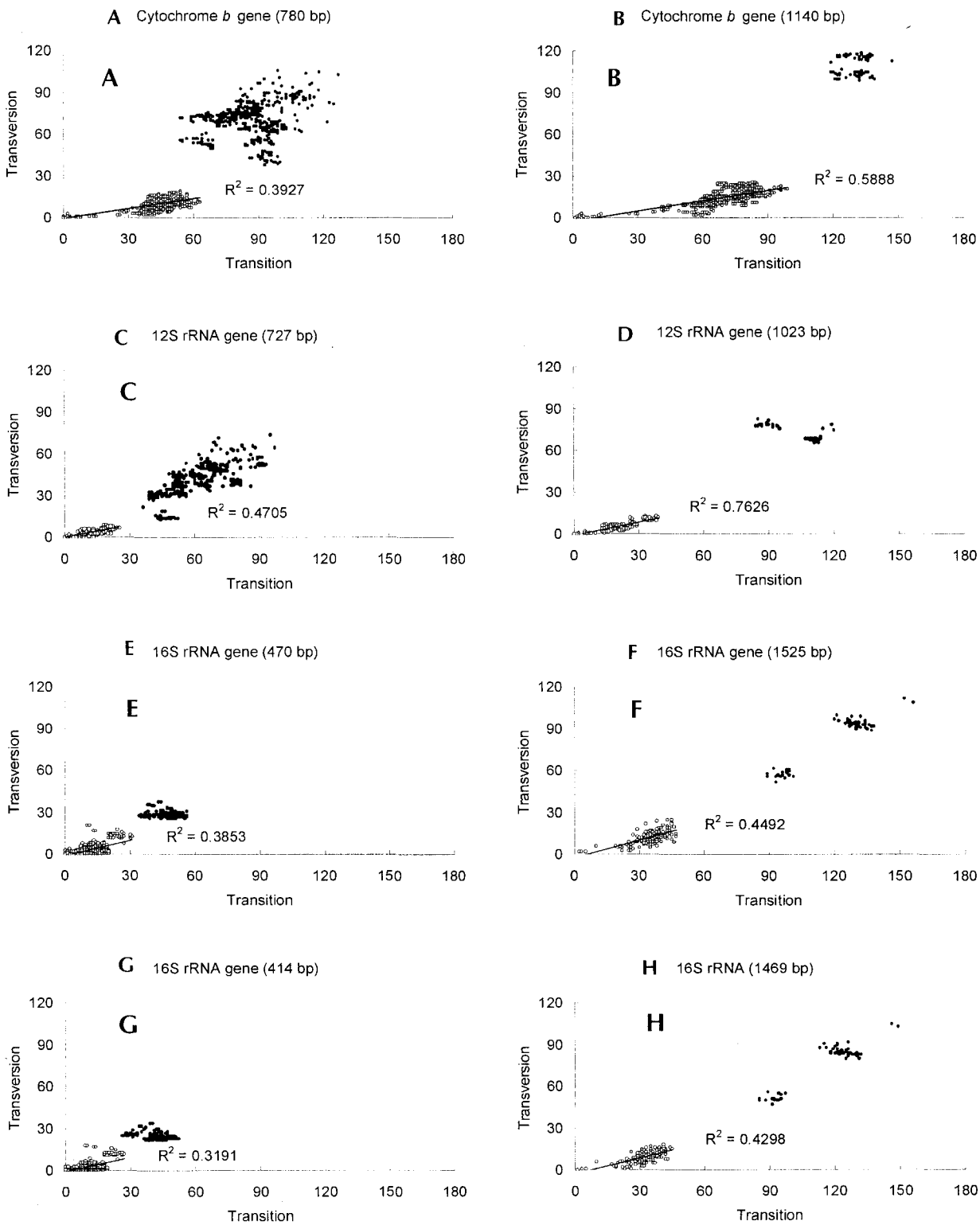

Fig. 2. The relationships between transitions and transversions. The open and solid circles represent the substitutions in the genus *Anguilla* and between Anguilliform families respectively. A, C, E, and G were generated from partial sequences with more species, and B, D, F, and H were from complete (almost complete) sequences with less species. The seven segments, which could not be aligned well in 16S rRNA gene, are kept in E and F, and eliminated in G and H. R^2 denotes the multiple correlation coefficient among the spots in the genus *Anguilla*.

Aoyama et al.[12] took stepwise phylogenetic analysis as follows. The 16S rRNA gene data set was first analyzed for all *Anguilla* species and the two outgroups mainly based on MP method. The second step constructed a most parsimonious tree from cytochrome *b* gene data to confirm the first step result. Finally a combined data set containing both the two genes using *A. malgumora* as the basal species resulted in a single most parsimonious tree as indicated in Fig. 1. As we previously indicated, 16S rRNA gene is not the best choice to be applied at the first step for less informative sites and the possibility of uncertain alignments. Moreover, the tree topology generated from 16S rRNA gene by Aoyama et al.[12] could not be repeated in this study. Different alignments in the two studies might be one possible reason, however, the parsimonious tree from cytochrome *b* gene, a constant 1,140 bp coding gene, by Aoyama et al.[12] could not be repeated either. *A. mossambica* is always clustered with *A. bicolor bicolor* or *A. australis*, but never forms a monophyletic clade with Atlantic eels.

To analyze the phylogenetic relationships in the genus *Anguilla*, cytochrome *b* and 12S rRNA genes might be more appropriate than 16S rRNA gene. Lin et al.[11] combined the former two genes and concluded that the monophyletic clade of *A. mossambica* and Atlantic eels does not exist. Fig. 3 represents the relationships of evolution rates among the three genes. The ratios between genes are kind of constant in the genus *Anguilla*. Nevertheless, when the distance is larger, the differences of rates between genes might be more significant. Combining more genes or elongating the sequences is suggested in the case.

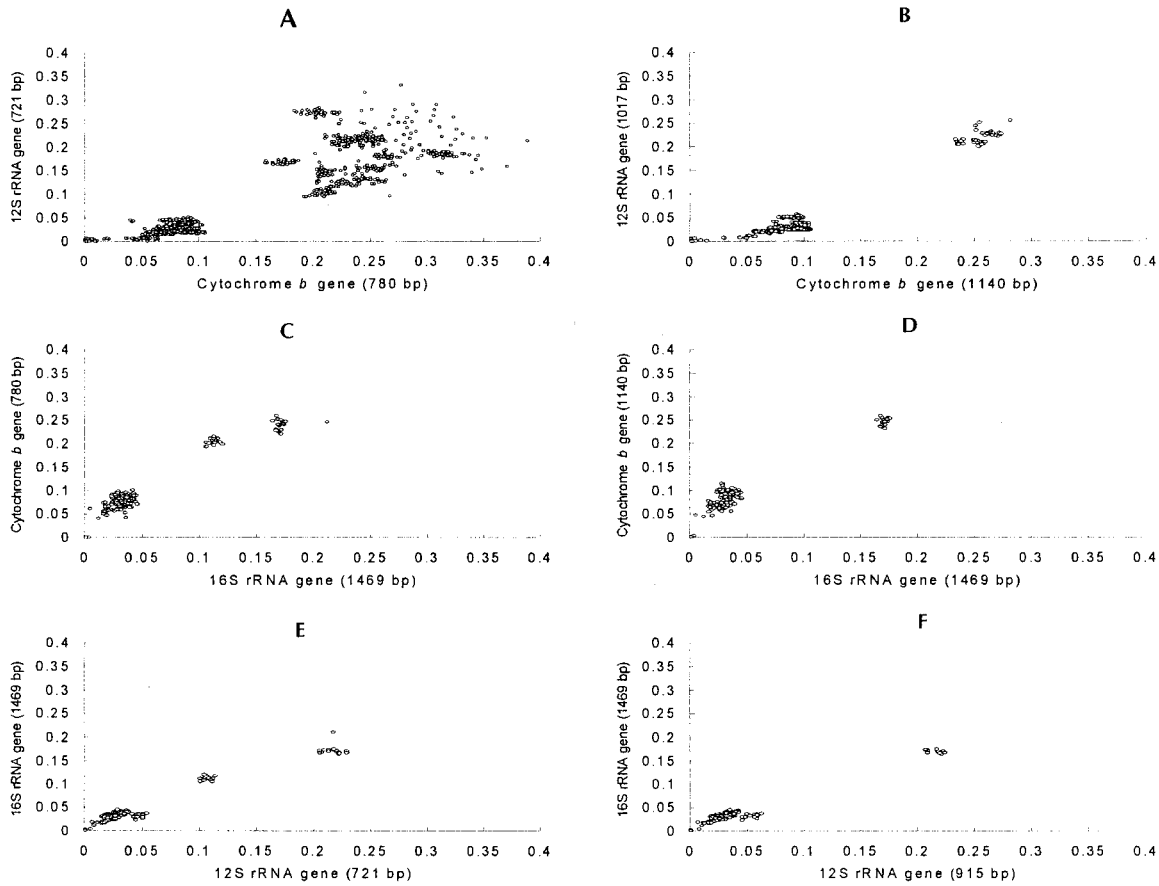

Fig. 3. The relationships of Tamura-Nei distances between genes. A, C, and E were generated from partial sequences with more species, and B, D, and H were from complete (almost complete) sequences with less species.

Subsequently we combined all available complete cytochrome *b* and 12S rRNA genes and constructed a neighbor-joining tree based on Tamura-Nei distances as illustrated in Fig. 4. Even including four new species, the topology still confirms the result proposed by Lin et al.[11]. Interestingly, the samples of *A. malgumora* from Lin et al.[11] and Aoyama et al.[12] are separated in the topology. The clustering implies that the samples from the two studies might be different species, which we will discuss later. Five stable clades were verified. They are *A. malgumora* by Aoyama et al.[12] (g), *A. mossambica* (m), *A. anguilla* and *A. rostrata* (A), *A. australis* and *A. dieffenbachi* (SP), and other species (X). Although this topology argues against the monophyletic clade of *A. mossambica* and Atlantic eels, the bootstrap values are not high enough to deny this assumption. Thus these five clades were shuffled to generate 105 tree topologies for MP and ML analyses using the Kishino -Hasegawa[22], Templeton[23], and winning-sites tests. Thirteen data sets were analyzed independently for different gene and different species composition (Table 4). The *P* values of the three tests, Kishino-Hasegawa, Templeton, and winning-sites tests, for Maximum Parsimony analyses were plotted in Fig. 5. The *P* values of Templeton test are similar to which of Kishino-Hasegawa test and the values of winning-sites test are slightly higher than the former two. Thus, Table 5 and Table 6 only represent the Kishino-Hasegawa values for Maximum Parsimony and Maximum Likelihood analyses, respectively.

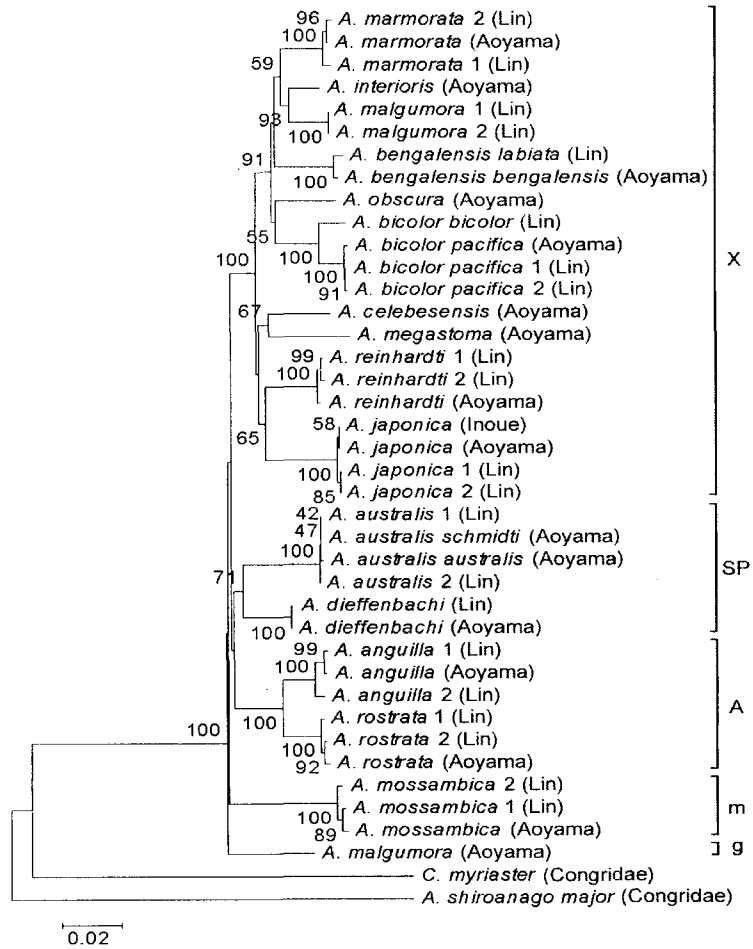

Fig. 4. The inferred neighbor-joining tree based on the Tamura-Nei distances from complete cytochrome *b* and 12S rRNA genes. The numbers at the nodes are bootstrap values from 5000 replicates. The scale bar represents the branch length. The notations characterize the clades employed in MP and ML tests.

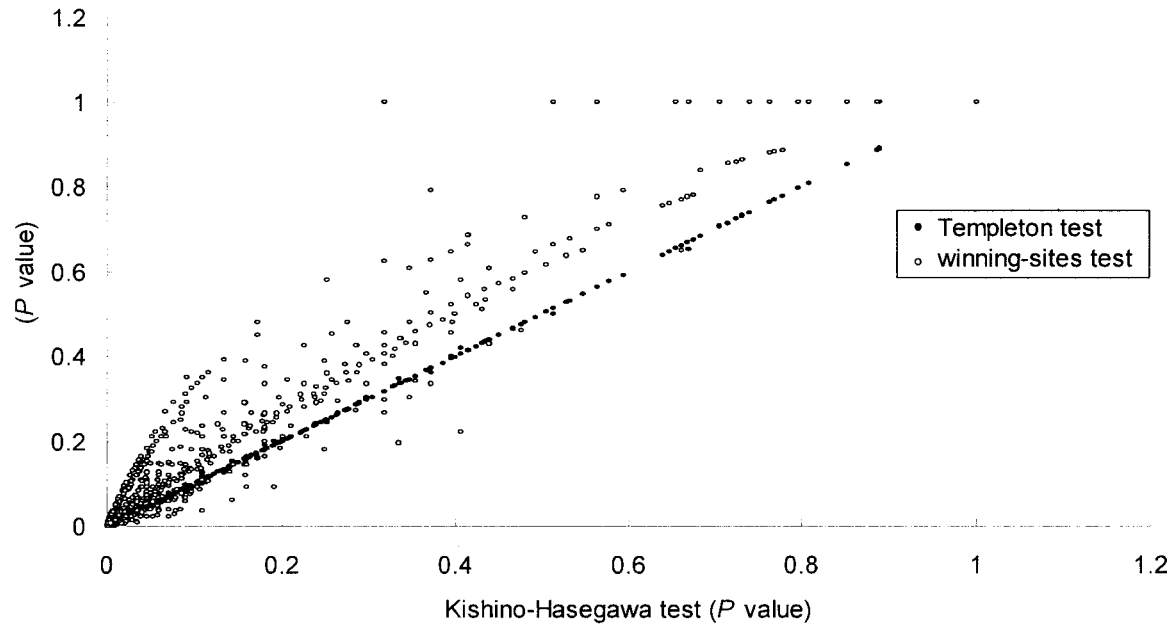

Fig. 5. The relationships of *P* values between Kishino-Hasegawa, Templeton, and winning-sites tests for MP analyses.

Table 5. The *P* values of Kishino-Hasegawa test for Maximum Parsimony analysis.

	Data set												
Tree topology	***I***	***II***	***III***	***IV***	***V***	***VI***	***VII***	***VIII***	***IX***	***X***	***XI***	***XII***	***XIII***
[001](X,(SP,(A,(m,g))))	0.1574	0.1442	0.7632	-	0.1574	0.4235	0.3305	0.2764	0.1944	0.2734	0.0588	0.3174	0.1025
[002](SP,(X,(A,(m,g))))	**0.0410**	**0.0233**	0.7632	0.5640	0.4144	0.1573	0.0705	**0.0286**	**0.0396**	0.2087	0.2231	0.0707	**0.0431**
[003](SP,(A,(X,(m,g))))	**0.0284**	0.0679	0.1574	0.1025	0.4144	**0.0284**	**0.0455**	**0.0477**	0.1048	0.0679	0.0555	**0.0455**	**0.0295**
[006](SP,((A,X),(m,g)))	**0.0284**	**0.0233**	0.1574	0.1025	0.4144	**0.0284**	**0.0163**	**0.0131**	**0.0280**	0.0679	0.0555	**0.0150**	**0.0086**
[007]((SP,X),(A,(m,g)))	**0.0481**	**0.0233**	0.7391	0.5640	0.0736	0.1573	**0.0482**	**0.0066**	**0.0040**	**0.0498**	**0.0342**	**0.0125**	**0.0032**
[045](g,(SP,(X,(A,m))))	0.3460	0.3460	0.3175	0.2484	0.2009	0.3712	0.4055	0.1167	0.0833	0.0782	**0.0326**	0.1905	0.0700
[051](g,(X,(A,(SP,m))))	0.1968	0.1968	0.1337	0.1574	0.3939	0.1444	0.1986	0.1167	0.0833	0.1599	0.0782	0.1797	0.0629
[052](g,(A,(X,(SP,m))))	0.4145	0.4145	0.2254	0.2515	0.3459	0.5317	0.4659	0.2394	0.2569	0.1496	0.0705	0.2451	0.1176
[053](g,(A,(SP,(X,m))))	-	-	0.1337	0.1574	0.2852	0.6832	0.5466	0.2972	0.3174	0.0956	**0.0396**	0.2386	0.1069
[059](g,((A,SP),(X,m)))	0.1798	0.1025	0.0707	0.0896	0.3175	0.2208	0.1700	0.0896	0.0593	0.0863	**0.0339**	0.1631	**0.0489**
[060](g,(m,(X,(A,SP))))	0.3175	-	0.2061	0.1574	0.4915	0.1573	0.4498	-	-	0.2394	**0.0498**	0.6745	0.2858
[062](g,(m,(A,(X,SP))))	0.4388	0.8085	0.7057	0.4056	0.3939	-	0.6832	0.4143	0.1798	0.4143	0.1025	0.7681	0.2624
[087](m,(X,(SP,(A,g))))	0.5318	0.5639	0.1025	0.5932	-	0.6618	0.5317	0.4309	0.8887	0.2851	0.5638	0.5775	0.5775
[088](m,(SP,(X,(A,g))))	0.4330	0.3361	0.1025	0.7964	0.3175	0.5466	0.3939	0.4387	0.7774	0.1656	0.5638	0.5775	0.5775
[091](m,((SP,X),(A,g)))	0.2208	0.3175	0.1798	0.7964	0.3175	0.4350	0.3459	0.3539	0.6683	0.3658	0.7390	0.6831	0.5317
[094](m,(X,(A,(SP,g))))	0.2010	0.3175	**0.0338**	0.3714	-	0.2279	0.1616	0.3271	0.7631	0.1968	0.4055	0.3692	0.3692
[101](m,(X,(g,(A,SP))))	0.1445	0.2395	**0.0081**	0.2515	-	0.1404	0.0606	0.1893	0.5272	0.0833	0.2060	0.1167	0.1167
[103](m,(g,(X,(A,SP))))	0.2088	0.7238	0.0833	0.5130	-	0.2569	0.2569	0.2889	-	0.3174	0.3174	0.4796	0.4796
[104](m,(g,(SP,(X,A))))	0.1938	0.7152	0.3175	0.6700	-	0.3174	0.4796	0.2889	-	0.6548	0.6548	0.7389	0.7389
[105](m,(g,(A,(X,SP))))	0.1938	0.7152	-	-	0.7056	0.5317	-	0.2499	0.8527	-	-	-	-

Note. The values less than 0.05 are denoted boldface.

Table 6. The *P* values of Kishino-Hasegawa test for Maximum Likelihood analysis.

Tree topology	*Data set*												
	I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*XIII*
[001](X,(SP,(A,(m,g))))	0.2656	0.2412	-	0.3298	0.5974	0.6175	0.6002	0.4867	0.7661	0.6992	0.5413	0.6143	0.5063
[002](SP,(X,(A,(m,g))))	0.2656	0.2556	-	-	-	0.6174	0.7018	0.8363	0.8902	-	-	-	-
[003](SP,(A,(X,(m,g))))	0.2663	0.3144	0.1041	0.1080	-	0.1359	0.1544	-	0.9276	0.2358	0.2127	0.3183	0.3239
[006](SP,((A,X),(m,g)))	0.2656	0.2558	0.1041	0.1080	-	0.1194	0.1074	0.8330	0.8903	0.2358	0.2127	0.3144	0.3019
[007]((SP,X),(A,(m,g)))	0.2655	0.2412	-	0.3291	0.4966	0.6170	0.6002	0.4867	0.7661	0.6990	0.5412	0.6145	0.5061
[045](g,(SP,(X,(A,m))))	0.5083	0.5222	0.0846	0.0754	0.7740	0.2237	0.2762	0.8603	-	0.2936	0.3167	0.4754	0.6181
[051](g,(X,(A,(SP,m))))	0.4231	0.4168	0.1069	0.0848	0.5878	0.1913	0.1947	0.3733	0.5700	0.1967	0.1763	0.1738	0.2044
[052](g,(A,(X,(SP,m))))	0.4287	0.4401	0.1967	0.1355	0.6043	0.3479	0.3231	0.4133	0.5737	0.4524	0.2904	0.3302	0.3253
[053](g,(A,(SP,(X,m))))	0.8039	0.8218	0.1825	0.1261	0.5931	0.4775	0.4319	0.4893	0.6306	0.4494	0.2727	0.3345	0.3369
[059](g,((A,SP),(X,m)))	-	-	0.0884	0.0721	0.2326	0.2790	0.2779	0.2883	0.4330	0.1156	0.0787	0.1323	0.1343
[060](g,(m,(X,(A,SP))))	0.7362	0.8576	0.1190	0.1060	0.1435	0.3078	0.3408	0.4741	0.6606	0.1912	0.0830	0.2632	0.2052
[062](g,(m,(A,(X,SP))))	0.5438	0.6546	0.3313	0.2118	0.1435	-	0.8881	0.3989	0.6153	0.3606	0.0747	0.3779	0.2678
[087](m,(X,(SP,(A,g))))	0.6272	0.4751	0.1844	0.4274	0.7314	0.4237	0.4934	0.5202	0.6846	0.4598	0.5884	0.3634	0.5648
[088](m,(SP,(X,(A,g))))	0.3840	0.3247	0.1991	0.4671	0.6255	0.3030	0.4251	0.4344	0.5506	0.4623	0.6028	0.3579	0.5218
[091](m,((SP,X),(A,g)))	0.3818	0.3247	0.2773	0.6718	0.6107	0.5003	0.6588	0.4338	0.5506	0.7677	0.7616	0.5204	0.6513
[094](m,(X,(A,(SP,g))))	0.5762	0.4042	0.0953	0.1801	0.5181	0.1980	0.1775	0.5987	0.4833	0.2313	0.2551	0.2963	0.3032
[101](m,(X,(g,(A,SP))))	0.6814	0.4744	0.0834	0.1690	0.3732	0.2149	0.2037	0.2934	0.4968	0.1163	0.1836	0.1343	0.2096
[103](m,(g,(X,(A,SP))))	0.6016	0.6029	0.1144	0.2221	0.1960	0.2435	0.3093	0.4714	0.6173	0.1925	0.2014	0.2674	0.2984
[104](m,(g,(SP,(X,A))))	0.4709	0.4801	0.1144	0.2221	0.1959	0.1868	0.2356	0.3966	0.5783	0.1924	0.2015	0.2571	0.2866
[105](m,(g,(A,(X,SP))))	0.4709	0.4801	0.2747	0.7842	0.1960	0.5434	-	0.3963	0.5783	0.3764	0.4261	0.3981	0.5054

In the 105 tree topologies, only the best tree in each analysis and the trees with all the *P* values not less than 0.05 were maintained in Table 5 and 6. Cytochrome *b* gene data sets support the topology (g,(A,SP,(X,m))). For the two rRNA genes, MP analyses prefer to pull *A. mossambica* out as the basal clade, but ML analyses favor an internal cluster with *A. mossambica* and *A. malgumora* together. Combining cytochrome *b* and 12S rRNA genes, the topology (g,m,(A,(X,SP))) has the highest probability. This topology is also supported by the MP analyses for the combination of the two rRNA genes, and for the combination of all the three genes, however, the ML analyses root the tree in different way, (SP,(X,(A,(m,g)))). The monophyletic clade of *A. mossambica* and Atlantic eels only appear in ML analysis for one data set, the combination with 16S rRNA and partial cytochrome *b* genes. However, other topologies also have considerably high *P* values in this data set. The MP analyses for the same data set prefer a monophyletic clade, (A,SP), and have low values for (A, m), 0.1167 and 0.0833.

Based on these results, we could conclude that the phylogenetic relationships of the genus *Anguilla* constructed from the three mitochondrial genes provide weak but considerable evidences arguing against the monophyletic clade of *A. mossambica* and Atlantic eels. The radiation of the genus *Anguilla* in a window of time caused the difficulty to distinguish the basal clades in the phylogenetic tree.

Noticeably, in Lin et al.'s[11] study the probability of Ishikawa et al.'s[5] tree, the same topology as Aoyama et al.'s[12], is less than 0.0001. The low probability is due to considering the two *A. malgumora* samples as the same species. Lin et al.[11] obtained 10 glass eel specimens of *A. malgumora* from the Philippines provided by Dr. W. N. Tzeng. The specimens were identified with Ege's[7] key (number of vertebrae and AD/TL) as described in Lin[10], and all of them were identified as the same species by sequencing. Aoyama et al.[12] collected one *A. malgumora* specimen from Borneo and measured the morphological features without the number of vertebrae. The phylogenetic relationships constructed in this study suggest that these two samples should be different species with similar morphological characters. To confirm this assumption, more specimens from these two samples and the type specimens of *A. malgumora* and *A. borneensis* should be included for further study.

Although we could not obtain significant evidence against the Tethys Seaway hypothesis from the phylogenetic analyses, the generation time of the genus *Anguilla* calculated by Lin et al.[11] provides a solid one. Given that the divergence time of Anguilliformes from the other orders of Euteleostei is around 143 million years ago, as indicated by fossil records[24,1], Lin et al.,[11]employed the transversional substitutions of cytochrome *b* and 12S rRNA genes to estimate the divergence time between Anguillidae and Congridae to be 119.7 million years, and the divergence time of the genus *Anguilla* to be 20.6 million years. The Poisson distances of cytochrome *b* genes also support this result. The divergence times were thus estimated as 120.0 and 18.8 million years, respectively. The ancient Tethys Sea was closed 30 million years ago, much earlier than the speciation event for the Atlantic eels (about 10 million years ago). In contrast, Northern and Southern America remained divided until the plate movement 5 million years ago. Barron and Peterson[25-27] also had doubt about the existence of the westward global circum-equatorial current, which was assumed in the Tethys Sea scenario.

Lin et al.[11] proposed a realistic hypothesis to explain how the ancestors of Atlantic eels dispersed through the Central American gateway into Atlantic Ocean and why the *Anguilla* species do not appear along the eastern margins of the Pacific and south Atlantic. South Pacific eels dispersed eastward from the Metropolis to their present habitats. However, in this region, the surface currents transport the leptocephali westward. In contrast, the adult eels descend and migrate eastwards for reproduction in the ocean, implying that their ancestors moved eastwards following the discovery of new spawning places. Jespersen[28] postulated that the spawning place of *A. obscura* lay around Tahiti. This implies that the distance between an appropriate spawning place in the South Pacific Ocean and the Central America could possibly be reduced to 7,000 km. In comparison, adult European eels migrate farther to Sargasso Sea for reproduction. Based on these facts, Lin et al.[11] postulated that the adults of ancient Atlantic eels migrated into the Atlantic Ocean through the Central American gateway in deep water. A strong eastward current 1,500 meters below the surface[27] in the tropical Pacific Ocean could make the migration more efficient. The settlers found new spawning places in the Sargasso Sea and were separated from the Pacific groups to become the ancestors of the current Atlantic eels. Consequently, the separation between the Atlantic and the Pacific groups must have been even earlier than the closure time of the Central American Isthmus, 5 million years ago. The westward ocean currents in the eastern Pacific Ocean would prevent the leptocephali being transported to America. The different ocean current system in south Atlantic or the lack of suitable spawning places also prevents *Anguilla* species dispersing into.

Fig. 6 summarizes the proposed evolutionary history of the genus *Anguilla*. The migration route of ancient Atlantic eels and the multiple radiation events as described by Lin et al.[11] are also illustrated. Combining all the available published molecular

data of the genus *Anguilla*, we could conclude that the possibility of the Central American gateway scenario is much higher than the Tethys Seaway hypothesis.

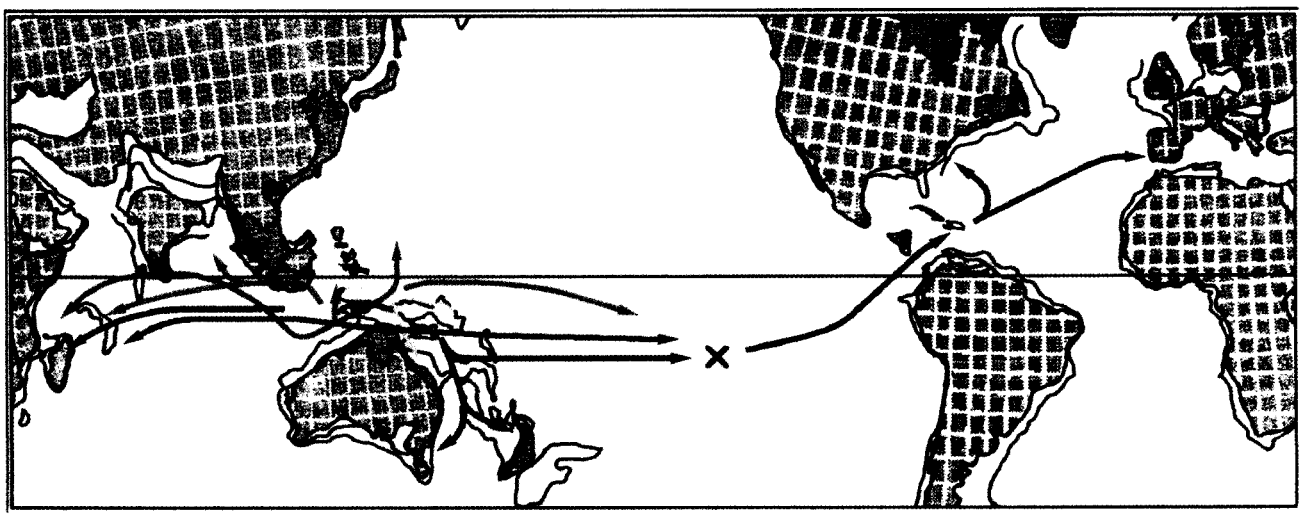

Fig. 6. The surface of the earth 20 million years ago modified from Barron et al. (30) and the possible migration routes of the *Anguilla* species modified from Lin et al. (11). The spawning place of *A. obscura* is near Tahiti, × (Jespersen 28), and the environment provides a possible step way for the migration of the Atlantic eels.

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