Molecular Phylogeny of Osteoglossoids: A New Model for Gondwanian Origin and Plate Tectonic Transportation of the Asian Arowana

Yoshinori Kumazawa and Mutsumi Nishida†*

*Department of Earth and Planetary Sciences, Nagoya University, Nagoya, Japan; and †Ocean Research Institute, University of Tokyo, Tokyo, Japan

One of the traditional enigmas in freshwater zoogeography has been the evolutionary origin of *Scleropages formosus* inhabiting Southeast Asia (the Asian arowana), which is a species threatened with extinction among the highly freshwater-adapted fishes from the order Osteoglossiformes. Dispersalists have hypothesized that it originated from the recent (the Miocene or later) transmarine dispersal of morphologically quite similar Australasian arowanas across Wallace's Line, but this hypothesis has been questioned due to their remarkable adaptation to freshwater. We determined the complete nucleotide sequences of two mitochondrial protein genes from 12 osteoglossiform species, including all members of the suborder Osteoglossoidei, with which robust molecular phylogeny was constructed and divergence times were estimated. In agreement with previous morphology-based phylogenetic studies, our molecular phylogeny suggested that the osteoglossiforms diverged from a basal position of the teleostean lineage, that heterotidines (the Nile arowana and the pirarucu) form a sister group of osteoglossines (arowanas in South America, Australasia, and Southeast Asia), and that the Asian arowana is more closely related to Australasian arowanas than to South American ones. However, molecular distances between the Asian and Australasian arowanas were much larger than expected from the fact that they are classified within the same genus. By using the molecular clock of bony fishes, tested for its good performance for rather deep divergences and calibrated using some reasonable assumptions, the divergence between the Asian and Australasian arowanas was estimated to date back to the early Cretaceous. Based on the molecular and geological evidence, we propose a new model whereby the Asian arowana vicariantly diverged from the Australasian arowanas in the eastern margin of Gondwanaland and migrated into Eurasia on the Indian subcontinent or smaller continental blocks. This study also implicates the relatively long absence of osteoglossiform fossil records from the Mesozoic.

Introduction

Freshwater fishes form an important aspect of biogeographical studies, because they do not disperse easily through saltwater areas, and thus their evolution may be tightly linked to the geological histories of landmasses on which the evolution took place (Bănărescu 1990, pp. 11–55; Lundberg 1993). In such studies, it is very useful to have molecular clocks that can reliably date the corresponding evolutionary events. Previous studies (Thomas and Beckenbach 1989; Martin, Naylor, and Palumbi 1992; Adachi, Cao, and Hasegawa 1993; Martin and Palumbi 1993) have consistently suggested that molecular clocks run much more slowly in fishes than in mammals, presumably due to the lower metabolic rates and/or increased selectional constraints on the protein sequence evolution in poikilothermic fishes. However, a clock suitable for dating old divergences among bony fishes well over 100 MYA has not been fully developed, partly due to the supposed methodological difficulty in estimating large molecular distances by correcting multiple substitutions and the relative paucity of reliable fossil-based estimates of divergence times for bony fishes.

We have recently shown that gamma-corrected distances based on amino acid sequences of two mitochondrial protein genes, i.e., NADH dehydrogenase subunit 2 (ND2) and cytochrome *b* (cytb) genes, provide a good estimate of pairwise distances between rather distantly related animals (Kumazawa, Yamaguchi, and Nishida 1999; Kumazawa and Nishida 2000). These distances were then used to calibrate molecular clocks of bony fishes under some reasonable assumptions and to suggest that the familial radiation of perciform fishes considerably predated the Cretaceous/Tertiary boundary, after which their fossil records concertedly appear (Kumazawa, Yamaguchi, and Nishida 1999).

The present study focuses on fishes of the order Osteoglossiformes, one of the primary freshwater fish groups that are strictly intolerant of saltwater (Bănărescu 1990, pp. 48–55, 62–66). The osteoglossiforms are considered basal teleosts that preserve primitive anatomical features (e.g., the toothed tongue bones), but their individual members show peculiar specializations in morphology (e.g., elongate anal and dorsal fins), physiology (e.g., the air-breathing function of the swim bladder), and behavior (e.g., mouth brooding) (Nelson 1994, pp. 90–97; Greenwood and Wilson 1998). These specializations or adaptations in morphology have contributed to obscure phylogenetic relationships of the osteoglossiforms (Bonde 1996; Li and Wilson 1996 and references therein). In a standard classification by Nelson (1994, pp. 90–97), they were divided into two suborders, i.e., Osteoglossoidei and Notopteroidei (see table 1). The former comprises arowanas (the family Osteoglossidae) and the butterflyfish (the only species in the Pantodontidae), whereas the latter includes mooneyes (Hiodontidae), Old World knifefishes (Notopteridae), elephantfishes (Mormyridae), and the aba (the only species in the Gymnarchidae). Extant osteoglossiforms are adapted to various (mostly tropical or subtropical) fresh-

Key words: Osteoglossiformes, teleost fish, mitochondrial DNA, historical biogeography, fossil-based divergence time, molecular clock.

Address for correspondence and reprints: Yoshinori Kumazawa, Department of Earth and Planetary Sciences, Graduate School of Science, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8602, Japan. E-mail: h44858a@nucc.cc.nagoya-u.ac.jp.

Mol. Biol. Evol. 17(12):1869–1878. 2000

Table 1
Classification and Geographical Distribution of Extant Species in the Order Osteoglossiformes Based on Nelson (1994, pp. 90–97)

Classification	Geographical Distribution
Suborder Osteoglossoidei	
Family Osteoglossidae	
Subfamily Osteoglossinae	
Scleropages formosus (Asian arowana)	Southeast Asia
Scleropages jardinii (northern barramundi)	Northern Australia and New Guinea
Scleropages leichardti (spotted barramundi)	Eastern Australia
Osteoglossum bicirrhosum (silver arowana)	South America
Osteoglossum ferreirai (black arowana)	South America
Subfamily Heterotidinae	
Heterotis niloticus (Nile arowana)	West Africa and Nile
Arapaima gigas (pirarucu)	South America
Family Pantodontidae	
Pantodon buchholzi (butterflyfish)	West Africa
Suborder Notopteroidei	
Superfamily Hiodontoidea	
Family Hiodontidae	
Hiodon tergisus (mooneye)	North America
Hiodon alosoides (goldeye)	North America
Superfamily Notopteroidea	
Family Notopteridae	
Four genera and eight species (Old World knifefishes)	West Africa to Southeast Asia
Superfamily Mormyroidea	
Family Mormyridae	
Eighteen genera and 198 species (elephantfishes)	Tropical Africa and Nile
Family Gymnarchidae	
Gymnarchus niloticus (aba)	Tropical Africa and Nile

NOTE.—All species of the suborder Osteoglossoidei, two species of the family Notopteridae (*Chitala ornata* and *Papyrocranus* sp.), and two species of the family Mormyridae (*Campylomormyrus elephas* and *Marcusenius* sp.) were sequenced in this study.

water habitats in continents of Gondwanian origin with some exceptions, i.e., hiodontids in North America, two notopterid genera from South to Southeast Asia, and the Asian arowana in Southeast Asia (Nelson 1994, pp. 90–97).

The Asian arowana (*Scleropages formosus*) has acquired a special status in Japan and some East Asian countries as a very popular but extremely expensive aquarium fish, which has led to its inclusion among species threatened with extinction (Goh and Chua 1999, pp. 17–24). Several types of *S. formosus* with different color patterns inhabit separate regions of Southeast Asia (Borneo, Sumatra, and Indochina) that were probably connected through freshwater habitats during the Pleistocene glacial ages (Goh and Chua 1999, pp. 17–24). An interesting question about this species from a biogeographical standpoint arises from the fact that two other species of the same genus inhabit Australasia (Australia and New Guinea), which is generally considered to belong to a zoogeographical region different from that of Southeast Asia (Bănărescu 1995, pp. 1349–1354). Was it possible for the arowanas of the primary freshwater fish category to disperse across Wallace's Line? This has been one of the greatest enigmas in freshwater zoogeography (Bănărescu 1990, p. 159; 1995, pp. 1349–1354, 1397).

Molecular phylogenetic approaches may provide new insights into this question. To our knowledge, however, molecular phylogeny of the osteoglossiforms has not been fully investigated, except for mormyrid electric fishes (Alves-Gomes 1999; Lavoué et al. 2000). In this study, we thus analyzed the osteoglossoid phylogeny and divergence times using the two mitochondrial protein sequences expected to function as useful molecular markers for these issues.

Materials and Methods

Samples and Sequence Determination

We determined the complete nucleotide sequences of the ND2 and cytb genes for 12 osteoglossiform species consisting of all extant species of the suborder Osteoglossoidei and two genera from each of the families Notopteridae and Mormyridae (see table 1 for classification and geographic distribution of these species). Although hiodontids and the aba were not sampled in this study, these 12 species cover most major groups of the order Osteoglossiformes. Fish specimens were obtained from either other investigators or local shops. The Asian arowana is a species threatened with extinction and thus protected by CITES (Goh and Chua 1999, pp. 9–16). For this species, we used two individuals, here designated Asian arowana 1 and Asian arowana 2, that had died after being legally imported from Indonesia to Japan. They were cultivated individuals of a type of the so-called Bornean ''Red arowana''.

Amplification and sequence determination of the ND2 and cytb genes were carried out as previously described (Kumazawa, Yamaguchi, and Nishida 1999). Primers designed to amplify and sequence the same genes of perciforms (Kumazawa, Yamaguchi, and Nishida 1999) were useful in the present study. Amplified

Table 2
Bootstrap Support for Each Nodal Relationship of the Osteoglossiform Phylogeny by Different Methods

Method	Nodal Relationship Shown in Figure 1											
	a	b	c	d	e	f	g[a]	h	i	j	k	l
Nucleotide sequence												
Maximum parsimony (MP) ...	82	100	92	100	71	93	45	100	77	66	77	62
Neighbor joining (NJ).....	69	72	42	100	86	94	(32)	100	64	26	71	46
Maximum likelihood (ML)....	91	94	52	100	88	100	48	100	98	77	65	93
Amino acid sequence												
MP	67	76	63	100	69	80	(27)	100	80	69	79	52
NJ	90	75	42	100	95	95	46	100	95	57	87	53
ML	76	91	71	100	92	100	48	100	95	96	86	92

Note.—MP trees from nucleotide sequences were obtained by excluding transition substitutions at third codon positions, which are prone to be saturated (Kocher et al. 1995), and weighting transversion substitutions at first and second codon positions three times. Several other proportions for the weighting were also tested to confirm the robustness of the MP tree topology obtained (data not shown). MP trees from amino acid sequences were obtained by weighting all types of substitutions equally. Only first and second codon positions were used to obtain NJ trees from nucleotide sequences. The Tamura-Nei (Tamura and Nei 1993) Gamma distance option of njboot was selected because of an unbalanced base frequency (data not shown) and a strong transition/transversion bias (see below). NJ trees from amino acid sequences were obtained with the Amino Poisson-Gamma distance option of njboot. Note that bootstrap values in this case correspond to those shown in figure 1. ML trees were constructed with MOLPHY, version 2.3, with the local rearrangement search starting from a topology given by the heuristic (star decomposition) search. Only the first and second codon positions of the nucleotide sequence data were used with the Tamura-Nei substitution model (Tamura and Nei 1993). Parameters for the transition/transversion ratio and pyrimidine/purine transition ratio were estimated to be 4.62 and 0.79, respectively, with PUZZLE, version 4.0.2 (Strimmer and von Haeseler 1996). For the amino acid sequence data, we used the mtREV24 substitution model (Adachi and Hasegawa 1996*b*) with the amino acid frequency of the data set. Bootstrap probabilities for MP and NJ analyses were obtained from 300 replications, while those for ML analyses are local bootstrap probabilities (bootstrap values given to a node by fixing relationships in other parts of the tree) from 1,000 replications using the RELL method (Adachi and Hasegawa 1996*a*).

[a] Only the relationship among osteoglossids, notopterids (+*Pantodon*), and mormyrids varied from method to method. Underlined and parenthesized values are, respectively, bootstrap probabilities for osteoglossid-notopterid and notopterid-mormyrid clades supported by the corresponding methods.

fragments purified with the QIAquick PCR purification kit (QIAGEN) were subjected to dye-terminator sequencing with the Applied Biosystems 373A DNA sequencer. Complete nucleotide sequences of the genes were unambiguously determined by reading both strands. The nucleotide sequence data reported in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB035221–AB035246.

Phylogenetic Analyses

Sequence alignment was conducted by eye with translated amino acid sequences of the osteoglossiforms as well as other taxa: the carp (Chang, Huang, and Lo 1994), the loach (Tzeng et al. 1992), the trout (Zardoya, Garrido-Pertierra, and Bautista 1995), African and neotropical cichlids (Kumazawa, Yamaguchi, and Nishida 1999), *Amia calva* (Kumazawa, Yamaguchi, and Nishida 1999), the coelacanth (Zardoya and Meyer 1997), sharks (Cao et al. 1998; Delarbre et al. 1998; Rasmussen and Arnason 1999*a*), and a ray (Rasmussen and Arnason 1999*b*). The alignment will appear in the EMBL database with the accession numbers ds43644 (ND2) and ds43645 (cytb). Amino acid sequences of the two genes were concatenated for phylogenetic analyses after gap sites were removed (723 alignable sites in total). Sharks and a ray were used as an outgroup.

Phylogenetic analyses using either nucleotide or amino acid sequences were conducted by three different methods: the maximum-parsimony (MP) method with PAUP, version 4.0b2 (Swofford 1999), the neighbor-joining (NJ) method (Saitou and Nei 1987) with njboot in Takezaki's Lintre package (Takezaki, Rzhetsky, and Nei 1995), and the maximum-likelihood (ML) method with MOLPHY, version 2.3 (Adachi and Hasegawa 1996*a*). Detailed conditions for each analytical method are described in table 2. In this study, all pairwise distances used in the NJ analyses were corrected using a gamma parameter representing the extent of rate heterogeneity over sites. The importance of incorporating this parameter into deep-branch phylogenetic analyses has been widely recognized in previous literature that dealt with broad taxonomic groups (see, e.g., Kumazawa and Nishida 1999, 2000; Mindell et al. 1999; Takezaki and Gojobori 1999).

Divergence Time Estimation

Estimation of divergence times was based on gamma-corrected ML or Poisson distances of the ND2/cytb amino acid sequences. These gamma-corrected distances among mammals (birds in part) were shown to correct multiple substitutions at the same sites most effectively even for divergences of a few hundred million years ago or 0.5–1.0 substitutions per site in the pairwise distance (Kumazawa, Yamaguchi, and Nishida 1999; Kumazawa and Nishida 2000). Gamma-corrected ML distances were obtained with PAML, version 2.0 (Yang 1999), using a gamma parameter ($\alpha = 0.44$) estimated from the data set, whereas gamma-corrected Poisson distances averaged among possible species pairs at a node were obtained with tpcv in Takezaki's Lintre package (Takezaki, Rzhetsky, and Nei 1995).

The clock of bony fishes was calibrated as in our previous study (Kumazawa, Yamaguchi, and Nishida 1999), with minor modifications. First, we previously used eight sharks as chondrichthyan species, but here we used three sharks and a ray. This change had negligible effect on the calibration but saved computational

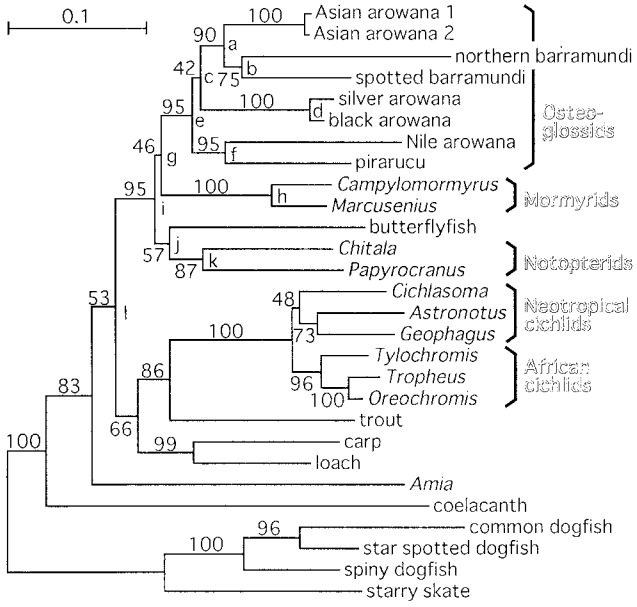

FIG. 1.—A neighbor-joining tree constructed from concatenated amino acid sequences of ND2 and cytb genes using sharks and a ray as an outgroup. The gamma-corrected Poisson distance was used with njboot in Takezaki's Lintre package (Takezaki, Rzhetsky, and Nei 1995). Bootstrap probabilities from 300 replications are shown on the corresponding branches. Nodes a–l are specified for reference to tables 2 and 3.

time for the phylogenetic analyses. Second, we previously used PUZZLE, version 4.0 (Strimmer and von Haeseler 1996), for estimating gamma-corrected ML distances (Kumazawa, Yamaguchi, and Nishida 1999). However, these distances later turned out to be slightly overestimated due to errors in the mtREV24 matrix of PUZZLE 4.0, as noted in PUZZLE's online manual (http://www.tree-puzzle.de/manual.html). In this study, we thus used PAML 2.0 (Yang 1999) for estimating gamma-corrected ML distances and confirmed that they were consistent with the values obtained using PUZZLE, version 4.0.2, in which the mtREV error was fixed.

Results

Molecular Phylogeny Using Two Mitochondrial Proteins

Among 723 amino acid sites of the ND2/cytb sequence data, 334 sites were constant among the 28 taxa used in figure 1, but the remaining 389 variable sites included 309 parsimony-informative ones. Two individuals of the Asian arowana differed from each other at five amino acid sites, which represents the sequence polymorphism within species.

We built MP, NJ, and ML trees from both nucleotide and amino acid sequences of the ND2/cytb sequence data. Figure 1 shows an NJ tree using the amino acid sequences, and results from the other methods are summarized in table 2. All of these molecular trees indicated the same osteoglossiform phylogeny, except for the topological relationship at node g. The robustness of the remaining topological relationships against methodological changes, as well as high bootstrap probabilities on several internal nodes, lend support to the reliability of the obtained molecular phylogeny in general.

Phylogenetic Relationship Within Osteoglossiformes

The molecular trees (fig. 1 and table 2) support monophyly of the osteoglossiform species with high bootstrap values in relation to the other teleostean groups used in this study (see node i). Consistent with current ichthyological classification based on morphology (Nelson 1994, pp. 1–5), the osteoglossiforms branched off basally from teleosts before the divergence of ostariophysans (carp and loach) from the lineage leading to protacanthopterygians (trout) and acanthopterygians (cichlids). Within the order Osteoglossiformes, monophyly of the osteoglossid species was supported with high bootstrap probabilities at node e. However, the relationship among the osteoglossids, notopterids, and mormyrids remained unresolved in our molecular analyses, because different tree-building methods supported different clustering patterns among them (table 2).

The molecular trees consistently showed an unexpected phylogenetic affinity of the butterflyfish with the notopterids, although bootstrap support at node j varied considerably depending on the method used (table 2). Morphological studies suggested that this species could be the sister group of either one (osteoglossines) or both (osteoglossines and heterotidines) osteoglossid subfamilies (Taverne 1979; Lauder and Liem 1983; Nelson 1994, pp. 90–97; Bonde 1996; Li and Wilson 1996). If this untraditional relationship really is the case, a number of synapomorphies defining the suborders Osteoglossoidei and Notopteroidei (see, e.g., Lauder and Liem 1983; Li and Wilson 1996) need to be reconsidered. Thus, although the phylogenetic affinity of the butterflyfish to the notopterids is currently the most straightforward interpretation of the molecular data, it will need to be further evaluated in the future. Within the family Osteoglossidae, two distinct lineages representing the osteoglossines and heterotidines were recognized, albeit with weaker bootstrap support for the osteoglossine monophyly. This is in agreement with previous morphological studies (Lauder and Liem 1983; Bonde 1996; Li and Wilson 1996). The Osteoglossinae and the Heterotidinae have distinct morphological characteristics with regard to, e.g., the presence or absence of mandibular barbels and the number of branchiostegal rays (Nelson 1994, pp. 90–97).

As for the topological relationships among the five osteoglossine species, the molecular trees were in good agreement with the traditional classification (Nelson 1994, pp. 90–97). Two species of Osteoglossum make a sister clade to the Scleropages species, among which two morphologically more similar species in Australasia form a sister clade to the Asian species. Moderately high (nodes a and b) to very strong (node d) bootstrap support was obtained. In spite of these topological consistencies with the traditional classification, the molecular phylogeny had an unexpected feature. The divergences among three Scleropages species (nodes a and b) were much deeper than expected from the fact that they were

classified within the same genus (see fig. 1). This became conspicuous by comparison with the depth of divergences between mormyrid genera and among cichlid genera. The three Scleropages species may thus be more appropriately classified into different genera, at least from the molecular standpoint. In contrast, the divergence between two Osteoglossum species is reasonably shallow in light of their classificatory status. These observations, in turn, highlight the exceptionally high morphological conservation among the Scleropages species. Further support for this argument was obtained by quantitative evaluation of the depth of each divergence point (see below). Of course, we are aware that taxonomic ranks should not be determined from molecular divergence values only. Further study should be expected to scrutinize our proposition to revise the classificatory status of the genus Scleropages.

Rate Constancy Test, Clock Calibration, and Divergence Time Estimation

In order to use molecular sequence data as the molecular clock, the homogeneity of modes and rates of sequence evolution among lineages must be carefully examined. PUZZLE 4.0.2 (Strimmer and von Haeseler 1996) uses a chi-square analysis to test at the 5% level whether the amino acid composition of each taxon is identical to the average composition among all taxa. We performed this test using the ND2/cytb amino acid sequences and confirmed that no taxon had a significantly deviated amino acid composition (data not shown).

Relative-rate tests (the two-cluster test; Takezaki, Rzhetsky, and Nei 1995) were then conducted using the gamma-corrected amino-Poisson distances among the 28 taxa. Since sharks and a ray were used as an outgroup, evolutionary rates were compared between clusters created by all nonchondrichthyan nodes of the tree shown in figure 1. The test showed, with a 99% significance level, that the lineage leading to the northern barramundi may have experienced a significantly accelerated molecular evolution compared with that leading to the spotted barramundi after their divergence at node b (data not shown). This rate inequality was not found between clusters at any other internal node. We thus excluded the northern barramundi from subsequent analyses. After that, the clock hypothesis held firm for all of the internal nodes.

When the significance level was lowered to 95%, external lineages leading to the Nile arowana and to the pirarucu were also found to possibly have different evolutionary rates. However, no other clusters created by any internal node, including those for cichlids, were found to have different rates (data not shown). We retained both of the heterotidines for subsequent analyses, allowing a somewhat unreliable estimation of the divergence time at node f. However, this did not affect the reliability of estimated times at the other internal nodes. Removal of either of the Nile arowana or the pirarucu had a negligible effect on them (data not shown). Taken together, these results indicate that there is no significant rate difference between the osteoglossiforms and other groups of fishes, which can justify the use of the clock calibrated using nonosteoglossiform fishes for estimating divergence times among the osteoglossiforms.

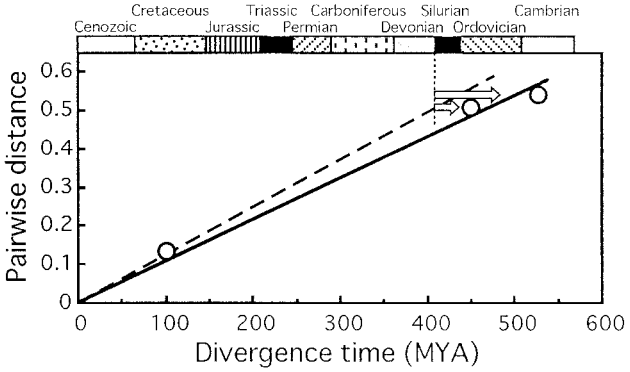

FIG. 2.—Calibration of the molecular clock. ND2/cytb amino acid sequences of the 28 taxa (see fig. 1) were used. Gamma-corrected ML distances averaged among the corresponding species pairs were plotted against estimated divergence times. Plotted data represent the divergence of African and Neotropical cichlids at the time of the continental breakup of the African and South American landmasses (100 MYA), sarcopterygian versus actinopterygian divergence at 450 MYA and chondrichthyan versus osteichthyan divergence at 528 MYA. Recent molecular (e.g., Streelman et al. 1998; Farias et al. 1999; unpublished data) and morphological (Stiassny 1991) studies indicate that the African and Neotropical cichlids are monophyletic relative to each other and that the Indian-Malagasy species make an outgroup of the African + Neotropical clade, strongly supporting the vicariant divergence of the continental cichlid clades on the Gondwanaland breakup (see Kumazawa, Yamaguchi, and Nishida [1999] for more detailed discussion). The latter two divergence times were from independent molecular time estimates using multiple nuclear gene sequences (Kumar and Hedges 1998). The earliest fossil records for sarcopterygians and actinopterygians were, respectively, from the Lower Devonian and the Upper Silurian (Benton 1993, pp. 611–613, 657–659), indicating that they diverged from each other in the Silurian (409–439 MYA) or earlier (see open arrows in the figure) and that the molecular time estimate for the divergence (450 MYA) is not considerably overestimated, if it is overestimated at all. Together with the earliest chondrichthyan fossils from the Middle Devonian (Benton 1993, pp. 593–595), these paleontological records also suggest Silurian or earlier divergence between chondrichthyans and osteichthyans. A regression line through the origin ($R^2 = 0.997$) was obtained for the molecular clock of this study (5.4×10^{-4} substitutions/site/Myr). Note that even if the calibrations were made as consistently as possible with the minimum divergence times from the fossils (see the broken line), the clock rate would increase by only 13%, and this would not change our basic arguments about the historical biogeography of osteoglossiforms.

For the time estimation, we used gamma-corrected ML and Poisson distances of the ND2/cytb amino acid sequences that have been shown to correct multiple substitutions most effectively (see *Materials and Methods*). Figure 2 shows that the clock for bony fishes can be calibrated consistently using the reasonable assumption of continental vicariance of cichlids and two external calibration points based on reliable time estimates from independent molecular and/or paleontological evidence. A regression line through the origin (5.4×10^{-4} substitutions/site/Myr) was used as the clock to infer divergence times among the osteoglossiform taxa (table 3). Similar divergence times were obtained using two independent distance measurements, i.e., gamma-corrected ML and Poisson distances.

Table 3
Divergence Times Among Osteoglossiforms Estimated in this Study

Node in Figure 1	Pairs[a]	Time (MYA)	
		Γ-ML[b]	Γ-Poisson[c]
a	2	135	138 ± 18
b[d]	—	—	—
c	6	171	172 ± 19
d	1	26	26 ± 6
e	10	223	221 ± 21
f	1	201	210 ± 25
g	14	241	242 ± 23
h	1	76	79 ± 12
i	27	259	261 ± 22
j	2	252	253 ± 26
k	1	185	188 ± 23
l	108	341	335 ± 28

[a] Number of species pairs used for the time estimation.

[b] Divergence times estimated with gamma-corrected maximum-likelihood distances and the rate obtained in figure 2 (5.4×10^{-4} substitutions/site/Myr).

[c] Divergence times estimated with gamma-corrected Poisson distances and a rate obtained from similar calibration as in figure 2 (5.2×10^{-4} substitutions/site/Myr). Means of divergence times among the corresponding species pairs are shown with one standard error.

[d] Northern barramundi was excluded after the rate constancy test (see text).

Discussion

Historical Biogeography

Geological evidence (Smith, Smith, and Funnell 1994; the Plates Project 1998) shows that all continents remained united as the supercontinent Pangea during Triassic times (208–245 MYA). Terrestrial faunas were similar worldwide, and there were probably few geographical barriers that hindered their dispersal on Pangea (see, e.g., Briggs 1995, pp. 61–65). In the middle Jurassic (157–178 MYA), Pangea was split into Laurasia and Gondwanaland, which were further fragmented into smaller landmasses: Eurasia, North America, and Greenland from the former, and Africa, South America, Australia, Antarctica, Madagascar, and India from the latter (Smith, Smith, and Funnell 1994; the Plates Project 1998). Plate tectonics has continuously reshaped these landmasses to the present arrangement.

Although extant osteoglossiforms inhabit terrestrial regions mostly of Gondwanian origin, fossil evidence suggests their once worldwide distribution (Lundberg 1993; Bonde 1996; Li and Wilson 1996). This is consistent with our molecular evidence suggesting that not only did the order Osteoglossiformes originate before Pangea began to be fragmented, but also its diversification into individual (sub)families (see values at nodes e, g, i, and j in table 3). On the other hand, the estimated divergence time between two osteoglossine genera (172 ± 19 MYA at node c, table 3) overlaps the period when Pangea separated into Laurasia and Gondwanaland (Smith, Smith, and Funnell 1994; the Plates Project 1998). It thus seems possible that the two genera originated and evolved primarily on Gondwanaland. The divergence time between black and silver arowanas at node d (26 ± 6 MYA) was small enough to deduce that they evolved within the isolated South American landmass.

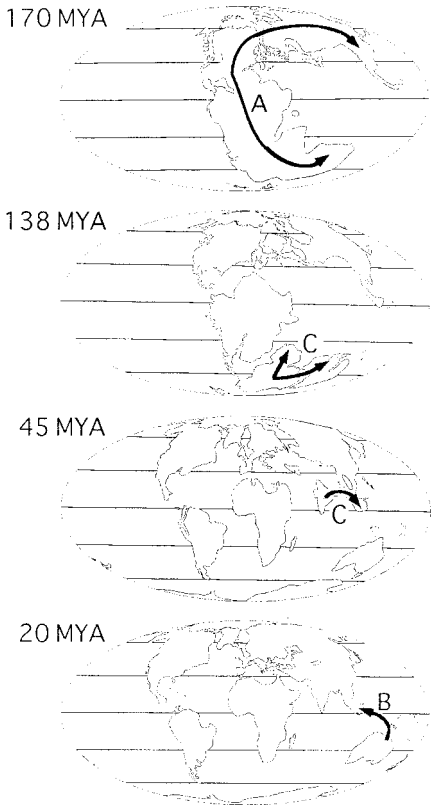

Fig. 3.—Models of the origin and migrational pathway of the Asian arowana. Paleogeographical maps at approximately 170, 138, 45, and 20 MYA (Smith, Smith, and Funnell 1994) are shown on which three models (A–C) are indicated by arrows. The three models are (A) early-middle Jurassic (157–208 MYA) origin and migration via Laurasia, (B) recent (e.g., Miocene, 5–23 MYA) origin and migration across Wallace's Line, and (C) early Cretaceous (112–146 MYA) origin and migration on the Indian subcontinent.

Origin and Migrational Pathway of the Asian Arowana

As discussed above, the genus Scleropages possibly originated and evolved on Gondwanaland after the breakup of Pangea. However, one of its members, the Asian arowana, now inhabits a part of Eurasia. How can this be explained? Figure 3 illustrates three models for this zoogeographically interesting question, and we discuss the validity of each model in light of the molecular and geological evidence.

Model A of figure 3 shows that an ancestor of the Asian arowana originated when Laurasia and Gondwanaland were still connected and that it dispersed via freshwater habitats from Gondwanaland to Laurasia (Bănărescu 1995, pp. 1349–1354). However, the estimated divergence time between the Asian and Australasian arowanas (138 ± 18 MYA; table 3) makes this model unlikely. Geological evidence shows that Laurasia and Gondwanaland had been almost completely separated by 160 MYA (Smith, Smith, and Funnell 1994; the Plates Project 1998), implying that freshwater fishes could not have migrated between the two supercontinents after 160 MYA. In addition, if this scenario were the case, it would be reasonable to propose that ancestors of the genus Scleropages inhabited regions connecting the two

supercontinents (i.e., Africa, North America, and South America). However, no fossil records for Scleropages have been found from these regions, and the habitats of extant Scleropages species (Southeast Asia and Australasia) correspond to the two eastern extremities of Laurasia and Gondwanaland.

Model B of figure 3 shows that the Asian arowana arose relatively recently via the transmarine dispersal of an Australasian arowana after plate tectonics moved Australia and New Guinea into the proximity of Southeast Asia (Bănărescu 1995, pp. 1349–1354, 1400). If this model were the case, the divergence time between the Asian and Australasian arowanas would be quite recent (<23 MYA). However, the molecular time estimate (138 ± 18 MYA) was much older (table 3), making this model unlikely. Another argument against the dispersal of a Scleropages species in this model comes from the fact that Southeast Asia and Australasia belong to two distinct zoogeographical regions bordered by Wallace's Line (Bănărescu 1995, pp. 1349–1354) and that the freshwater ichthyofauna was considered to be one of the most conspicuous indices discriminating the two zoogeographical regions (Briggs 1987, pp. 45–55). Some authors (see, e.g., Lundberg 1993; Briggs 1995, pp. 290–292) suggested the transmarine dispersal of euryhaline Scleropages populations by pointing out the existence of osteoglossid fossils found in saltwater beds (i.e., some of the Late Cretaceous to Eocene phareodontines, reviewed in Bonde [1996]). However, this is not a straightforward idea. Extant Scleropages species are highly adapted to freshwater, and their fossils are found only in freshwater beds (Sanders 1934).

Model C of figure 3 is a new model that is consistent with the molecular evidence. It assumes that ancestors of the Asian and Australasian arowanas diverged in the eastern margin of Gondwanaland during the Early Cretaceous (112–146 MYA) and that the former was transported northward across the Tethys Sea (the paleo-Indian Ocean) on the Indian subcontinent. During the Jurassic, India-Madagascar and Australia were connected through Antarctica in the eastern margin of Gondwanaland (Smith, Smith, and Funnell 1994; the Plates Project 1998). India-Madagascar was separated from Gondwanaland 120–130 MYA (Smith, Smith, and Funnell 1994; the Plates Project 1998) or somewhat more recently (Krause et al. 1997), whereas Australia remained close to Antarctica during the Cretaceous and even the Early Tertiary. The estimated divergence time between the Asian and Australasian arowanas (138 ± 18 MYA) is close to or slightly larger than the probable time of the India-Madagascan separation from Gondwanaland, which is consistent with the idea that the Asian arowana originated on a part of Gondwanaland and was carried by the Indian subcontinent. Moreover, model C can naturally explain the peculiar localization of extant Scleropages species, and there is no need to invoke a saltwater adaptation of this primary freshwater fish group.

There are some fossil records of Scleropages which should be considered in evaluating the migrational history of the Asian arowana. Freshwater beds of Eocene times (35–57 MYA) in central Sumatra yielded fossils that could be attributed to the genus Scleropages (Sanders 1934). The Indian subcontinent became connected to Eurasia by the late Early Eocene (Jaeger, Courtillot, and Tapponnier 1989; Metcalfe 1999). Thus, the fossilized Scleropages in central Sumatra could have come from India through terrestrial freshwater habitats soon after their disembarkation from the Indian subcontinent.

Another intriguing possibility is that the Asian arowana was carried by smaller terranes (continental blocks) (e.g., the Sikuleh, Natal, and Bengkulu) that drifted from the Australian part of Gondwanaland in the Late Jurassic (146–157 MYA) and were annexed to Southeast Asia in the Late Cretaceous (65–112 MYA) (reviewed in Metcalfe 1999). These three terranes actually accreted to Sumatra, the place where the fossilized Scleropages was found (Sanders 1934). Given the Late Jurassic separation of these terranes, the estimated divergence time between the Scleropages species (138 ± 18 MYA) may appear somewhat late to support this explanation. However, because the history of Gondwanian landmasses in Southeast Asia has not fully been revealed (Metcalfe 1999), we do not exclude the possibility that model C holds with one such terrane rather than with the Indian subcontinent.

Molecular Clocks of Bony Fishes

Molecular clocks of bony fishes have been studied using a variety of taxa, genes, and assumptions (see, e.g., Martin and Palumbi 1993; Ortí et al. 1994; Murphy and Collier 1996; Bermingham, McCafferty, and Martin 1997; Penzo et al. 1998; Zardoya and Doadrio 1999). Since most of these clocks are based on nucleotide sequences of relatively closely related taxa, little is known as to whether these clocks can be reliably extrapolated for divergences well over 100 MYA in time. For these old divergences, it seems reasonable to use amino acid sequences or nucleotide sequences without third codon positions, which are prone to be saturated quickly (Kocher et al. 1995).

In this study, we thus used amino acid sequences of two mitochondrial protein genes. In spite of the general idea that quickly evolving mitochondrial sequences are not suited for dating deep divergences, gamma-corrected distances from the mitochondrial protein sequences were shown to correct multiple substitutions efficiently (Kumazawa and Nishida 2000). The rate of the molecular clock for bony fishes using these distances was found to be nearly the same as or slightly faster than that for sharks but about three times as slow as that for mammals (Kumazawa, Yamaguchi, and Nishida 1999). This profile for the rate difference between fishes and mammals is consistent with previous work using transversion substitutions at fourfold-degenerate sites (Martin, Naylor, and Palumbi 1992) and restriction fragment length polymorphisms (Martin and Palumbi 1993).

Long Lack of Fossil Records?

Our molecular evidence suggested a Paleozoic origin of the order Osteoglossiformes (table 3), which is

considerably older than the first osteoglossiform fossil record in the Late Jurassic (Benton 1993, p. 624). Although the molecular data did not resolve the relationship among the osteoglossids, notopterids, and mormyrids, these groups are likely to have diverged during Permian-Triassic times (table 3). Fossil records for the three families and some supposedly related extinct groups have been found from the Cretaceous or later (Benton 1993, p. 624; Bonde 1996). Thus, there appears to be a time gap between molecular and fossil evidence. We interpret this apparent discrepancy to be indicative of the paucity of osteoglossiform fossil records rather than the inferiority of our molecular time estimates.

In this respect, it should be emphasized that our estimates were based on a reasonably calibrated molecular clock using well-corrected distances (Kumazawa, Yamaguchi, and Nishida 1999; Kumazawa and Nishida 2000; fig. 2) and that the rate constancy test was carefully carried out. Another argument is that the clock rate is consistent with the fossil evidence for the divergence point between sarcopterygians and actinopterygians (see the legend of fig. 2). Finally, in order to reconcile the molecular and paleontological time estimates on the osteoglossiforms, the clock rate would have to be roughly twice that of figure 2. However, this would elevate the fish rate to close to the mammalian one and cause clear inconsistency with previous work (Thomas and Beckenbach 1989; Martin, Naylor, and Palumbi 1992; Adachi, Cao, and Hasegawa 1993; Martin and Palumbi 1993).

Fossils of bony fishes are not necessarily considered well preserved in general. Of 425 teleostean families, 181 (43%) are completely lacking in their fossil record, and 58 (24%) of the remaining 244 families having recognizable fossil records occur with only otoliths (Benton 1993, pp. 621–622). We thus suspect that there is a long unrecorded history for the osteoglossiforms in the Mesozoic. A similar lack of fossil records has also been suggested for perciform families (Kumazawa, Yamaguchi, and Nishida 1999), some teleostean orders (Kumazawa, Yamaguchi, and Nishida 1999), and mammalian and avian orders (Janke et al. 1994; Hedges et al. 1996; Cooper and Penny 1997; Janke, Xu, and Arnason 1997; Kumar and Hedges 1998; Waddell et al. 1999). The history of vertebrates based on the paleontological evidence has not been substantially changed in its broadscale pattern since the 19th century (Benton 1998). However, these lines of molecular studies may cast a doubt on the accuracy of the well-accepted vertebrate histories or, at least, they may call for the histories to be questioned and reexamined by multidisciplinary approaches, including molecular evolutionary ones.

Conclusions

Freshwater zoogeography in general has been investigated primarily on the basis of faunal comparison using both extant and extinct species (Bănărescu 1990, pp. 11–47). Although such comparisons suggested an evolutionary affinity of the Asian arowana to the Australasian ones, definite evolutionary models which are consistent with both the geological and physiological (freshwater-adapted) conditions could not be envisaged due to the lack of corroborative evidence from molecules or fossils (for relevant discussions, see, e.g., Bănărescu 1990, p. 159; 1995, pp. 1349–1354, 1397; Taki 1993, pp. 117–130). The present study provided strong molecular evidence to propose a novel evolutionary model for the Asian arowana. More generally, it demonstrated that molecular data can effectively combine with paleogeographical (or paleontological) information to gain new zoogeographical insights. Previous paleontological studies suggested that the latest Cretaceous-Paleocene Indian fauna and flora which survived extensive volcanic activities was almost completely replaced by the diverse and relatively advanced biota of tropical Asia upon the India-Asia collision (see, e.g., Briggs 1987, pp. 123–137; Prasad and Khajuria 1995). The present study may thus provide the rare corroborative evidence to support a hypothesis that India or smaller continental blocks could serve as a cradles to convey Gondwanian freshwater faunas.

At present, the time estimates in table 3 are based on only two protein genes and a few calibration points and thus may be considered approximate estimations. However, due to the difficulties in the clock calibration for deep-branch osteichthyan groups as outlined earlier, even a rough framework of their divergence times has not been established by molecular approaches. We consider that our time estimates should be scrutinized in the future with more sequences or with other calibrations and revised if necessary. Nevertheless, our conclusion drawn herein about the origin and migrational pathway of the Asian arowana seems robust, because the three possible models of figure 3 propose quite different divergence times between the Asian and Australasian arowanas.

Since the Asian arowana is highly valued as a noble aquarium fish in Asian countries, it has been threatened with extinction in its native localities due to overfishing (or illegal fishing) and trading (Goh and Chua 1999, pp. 9–24). Given the premium that this species points to the dynamic plate tectonics across the paleo-Indian Ocean, more attention than ever should be paid to its conservation.

Acknowledgments

We are grateful to KK Istana Arowana and Messrs. E. Tajima and C. P. Jacoby for providing fish tissues. We thank Drs. M. Yamaguchi, Y. Yabumoto, T. Ueno, T. Kon, R. Nomura, and K. Tamaki and members of the Geobiology laboratory of Nagoya University for providing useful information and discussion on the results reported herein. This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan to Y.K. (grants 09214102 and 12640680) and M.N. (grants 10660189 and 12460083).

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MASAMI HASEGAWA, reviewing editor

Accepted July 25, 2000