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# Molecular Phylogenetics and Evolutionary Diversification of Labyrinth Fishes (Perciformes: Anabantoidei)

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*Abstract.*— Labyrinth fishes (Perciformes: Anabantoidei) are primary freshwater fishes with a disjunct African-Asian distribution that exhibit a wide variety of morphological and behavioral traits. These intrinsic features make them particularly well suited for studying patterns and processes of evolutionary diversification. We reconstructed the first molecular-based phylogenetic hypothesis of anabantoid intrarelationships using both mitochondrial and nuclear nucleotide sequence data to address anabantoid evolution. The mitochondrial data set included the complete cytochrome *b*, partial 12S rRNA, complete tRNA Val, and partial 16S rRNA genes (3332 bp) of 57 species representing all 19 anabantoid genera. The nuclear data set included the partial RAG1 gene (1494 bp) of 21 representative species. The phylogenetic analyses of a combined (mitochondrial + nuclear) data set recovered almost fully resolved trees at the intrafamily level with different methods of phylogenetic inference. Phylogenetic relationships at this taxonomic level were compared with previous morphology-based hypotheses. In particular, the enigmatic pike-head (*Luciocephalus*) was confidently placed within the "spiral egg" clade, thus resolving the long-standing controversy on its relative phylogenetic position. The molecular phylogeny was used to study the evolution of the different forms of parental care within the suborder. Our results suggest that the evolution of breeding behavior in anabantoids is highly correlated with phylogeny, and that brood care evolved three times independently from an ancestral free spawning condition without parental care. Ancestral character state reconstructions under maximum parsimony and maximum likelihood further indicated that both bubble nesting and mouthbrooding have evolved recurrently during anabantoid evolution. The new phylogenetic framework was also used to test alternative biogeographic hypotheses that account for the disjunct African-Asian distribution. Molecular divergence time estimates support either a drift vicariance linked to the breakup of Gondwana or Late Mesozoic Early Tertiary dispersal from Africa to Asia or *vice versa*. [Ancestral character state reconstruction; breeding behavior; divergence time estimation; biogeography.]

Actinopterygii, the ray-finned fishes, evolved a remarkable variety of parental care systems among vertebrates. Approximately 19% of the 431 families of ray-finned fishes exhibit some parental care (modified from Blumer, 1982; Nelson, 1994). Differences in parental care can be related either to the sex of the caregiver (female-only, male-only, and biparental) or to the form of care (breeding behavior; e.g., substrate spawning, bubble nesting, mouthbrooding, and viviparity). These transitions from no parental care to parental care (or vice versa) and among parental care systems are responsible for major changes in life-history traits (e.g., fecundity, juvenile survival, parental investment) in fishes. Yet, little is known about the evolutionary pathways that have led to the observed diversity in forms of parental care. Phylogenetic studies are crucial to determine the number and direction of evolutionary transitions and to gain new insights into the evolution of parental care in ray-finned fishes.

Labyrinth fishes (Perciformes: Anabantoidei) show an astonishing diversity in breeding behavior that is hardly found in any other fish group, and are therefore particularly well suited to study the evolutionary diversification of reproductive modes. In anabantoids, parental care is dominant and occurs in 16 of the 19 genera (Table 1). Reproductive modes (Table 1) include free-spawning without parental care (*Anabas*, *Ctenopoma*, *Helostoma*), substrate spawning with male parental care (*Sandelia*), submerged plant nest building with male parental care (*Osphronemus*), bubble nesting with either male-parental or biparental care (*Microctenopoma*, *Belontia*, *Trichogaster*, *Colisa*, *Macropodus*, *Pseudosphromenus*, *Malpulutta*, *Parosphromenus*, *Trichopsis*, *Parasphaerichthys*, and some species of *Betta*), and mouthbrooding with either male- or female-parental care (the majority of *Betta* spp., *Ctenops*, *Sphaerichthys*, and *Luciocephalus*).

Anabantoids were already recognized as a natural assemblage in the early nineteenth century by Cuvier and Valenciennes (1831) because of the presence of a peculiar suprabranchial organ. This structure consists of a greatly modified epibranchial one that is housed in a cavity above the gills (Peters, 1853). Both the wall of the cavity and the modified epibranchial are covered with respiratory epithelium and assist in accessory air-breathing (Bader, 1937; Boake, 1865; Liem, 1980; Peters, 1978; Zograff, 1888). The suprabranchial organ is also called labyrinth organ because of its complex folding that greatly increases respiratory surface.

Anabantoids are primary freshwater fishes that inhabit large areas of Africa and southern Asia (Berra, 2001) and include roughly 140 species grouped into three families, Anabantidae (28 spp.), Helostomatidae (1 sp.), and Osphronemidae (108 spp.) (Table 1). Although a comparatively small group, anabantoids exhibit a striking variation in size, ranging from dwarfed forms such as *Parosphromenus ornaticauda*, with 19 mm standard length, to large forms such as the giant gouramies of the genus *Osphronemus*, with up to 70 cm standard length (Kottelat, 1991; Roberts, 1992). Phylogenetic relationships of anabantoids have been highly contentious. Most of the controversy is focused on the relative phylogenetic position of the enigmatic pike-head *Luciocephalus pulcher*. This highly morphologically derived teleost (Lauder and Liem, 1981) was originally included in the family

TABLE 1. Summary of specimens, PC = parental care (sex of caregiver; M = male-parental care; F = female-parental care; B = biparental care), BB = breeding behavior, ID = identification number (personal collection of first author), and GenBank accession numbers of the species used.

| Family/subfamily | Genus | Species | PC | BB | ID | GenBank | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | rRNAs + tRNA-Val | cytb | RAG1 |
| Anabantidae | | | | | | | | |
| Anabantinae | *Anabas* | *testudineus* | No | 2 | LR003 | AY763681 | AY763727 | AY763773 |
| Ctenopominae | *Ctenopoma* | *acutirostre*[1] | No | 2 | LR115 | AY763682 | AY763728 | |
| | *Ctenopoma* | *kingsleyae*[1] | No | 2 | LR107 | AY763683 | AY763729 | |
| | *Ctenopoma* | *muriei*[1] | No | 2 | LR161 | AY763684 | AY763730 | AY763774 |
| | *Ctenopoma* | *nebulosum*[1] | ? | ? | LR889 | AY763685 | AY763731 | |
| | *Ctenopoma* | *ocellatum*[1] | No | 2 | LR117 | AY763686 | AY763732 | |
| | *Ctenopoma* | *petherici*[1] | No | 2 | LR103 | AY763687 | AY763733 | AY763775 |
| | *Ctenopoma* | *nigropannosum*[2] | No | 2 | LR004 | AY763688 | AY763734 | |
| | *Ctenopoma* | *pellegrini*[2] | No | 2 | LR111 | AY763689 | AY763735 | AY763776 |
| | *Microctenopoma* | *ansorgii*[3] | Yes (M) | 0 | LR131 | AY763690 | AY763736 | |
| | *Microctenopoma* | *damasi*[3] | Yes (M) | 0 | LR893 | AY763691 | AY763737 | |
| | *Microctenopoma* | *fasciolatum*[3] | Yes (M) | 0 | LR104 | AY763692 | AY763738 | AY763777 |
| | *Microctenopoma* | *nanum*[3] | Yes (M) | 0 | LR105 | AY763693 | AY763739 | |
| | *Microctenopoma* | sp. Mai Ndombe[3] | Yes (M) | 0 | LR891 | AY763694 | AY763740 | |
| | *Sandelia* | *capensis* | Yes (M) | 3 | LR214 | AY763695 | AY763741 | AY763778 |
| Helostomatidae | *Helostoma* | *temminkii* | No | 2 | LR012 | AY763696 | AY763742 | AY763779 |
| Osphronemidae | | | | | | | | |
| Belontiinae | *Belontia* | *hasselti* | Yes (M) | 0 | LR123 | AY763697 | AY763743 | AY763780 |
| | *Belontia* | *signata* | Yes (B) | 0 | LR110 | AY763698 | AY763744 | |
| Luciocephalinae | *Colisa* | *chuna* | Yes (M) | 0 | LR099 | AF519657 | AF519696 | AF519735 |
| | *Colisa* | *fasciata* | Yes (M) | 0 | LR100 | AY763699 | AY763745 | |
| | *Colisa* | *labiosa* | Yes (M) | 0 | LR098 | AY763700 | AY763746 | |
| | *Colisa* | *lalia* | Yes (M) | 0 | LR101 | AY763701 | AY763747 | |
| | *Ctenops* | *nobilis* | Yes (M) | 1 | LR162 | AY763702 | AY763748 | AY763781 |
| | *Luciocephalus* | *pulcher* | Yes (M) | 1 | LR164 | AY763703 | AY763749 | AY763782 |
| | *Luciocephalus* | sp. | Yes (M) | 1 | LR163 | AY763704 | AY763750 | |
| | *Parasphaerichthys* | *lineatus* | Yes (M) | 0 | LR247 | AY763705 | AY763751 | |
| | *Parasphaerichthys* | *ocellatus* | ? | ? | LR140 | AY763706 | AY763752 | AY763783 |
| | *Sphaerichthys* | *acrostoma* | Yes (M) | 1 | LR895 | AY763707 | AY763753 | |
| | *Sphaerichthys* | *osphromenoides* | Yes (F) | 1 | LR088 | AY763708 | AY763754 | AY763784 |
| | *Sphaerichthys* | *selatanensis* | Yes (F) | 1 | LR168 | AY763709 | AY763755 | |
| | *Sphaerichthys* | *vaillanti* | Yes (M) | 1 | LR151 | AY763710 | AY763756 | |
| | *Trichogaster* | *leerii* | Yes (M) | 0 | LR093 | AF519656 | AF519695 | AF519734 |
| | *Trichogaster* | *microlepis* | Yes (M) | 0 | LR097 | AY763711 | AY763757 | |
| | *Trichogaster* | *pectoralis* | Yes (M) | 0 | LR094 | AY763712 | AY763758 | |
| | *Trichogaster* | *trichopterus* | Yes (M) | 0 | LR095 | AY763713 | AY763759 | |
| Macropodusinae | *Betta* | *cf. albimarginata*[4] | Yes (M) | 1 | LR076 | AF519638 | AF519677 | |
| | *Betta* | *coccina* | Yes (M) | 0 | LR036 | AF519644 | AF519683 | |
| | *Betta* | *foerschi* | Yes (M) | 1 | LR203 | AF519647 | AF519686 | |
| | *Betta* | *dimidiata* | Yes (M) | 1 | LR074 | AF519628 | AF519667 | |
| | *Betta* | *macrostoma* | Yes (M) | 1 | LR174 | AF519655 | AF519694 | |
| | *Betta* | *splendens* | Yes (M) | 0 | LR194 | AF519650 | AF519689 | AF519728 |
| | *Betta* | *unimaculata* | Yes (M) | 1 | LR056 | AF519653 | AF519692 | |
| | *Macropodus* | *opercularis* | Yes (M) | 0 | LR084 | AF519659 | AF519698 | AF519737 |
| | *Macropodus* | *spechti*[5] | Yes (M) | 0 | LR085 | AY763714 | AY763760 | |
| | *Malpulutta* | *kretseri* | Yes (M) | 0 | LR205 | AF519661 | AF519700 | AF519739 |
| | *Parosphromenus* | *anjunganensis* | Yes (M) | 0 | LR175 | AY763715 | AY763761 | |
| | *Parosphromenus* | *deissneri* | Yes (?) | 0 | LR091 | AF519662 | AF519701 | AF519740 |
| | *Parosphromenus* | *ornaticauda* | Yes (?) | 0 | LR155 | AY763716 | AY763762 | |
| | *Parosphromenus* | *paludicola* | Yes (M) | 0 | LR157 | AY763717 | AY763763 | |
| | *Pseudosphromenus* | *cupanus* | Yes (M) | 0 | LR124 | AF519660 | AF519699 | AF519738 |
| | *Pseudosphromenus* | *dayi* | Yes (M) | 0 | LR113 | AY763718 | AY763764 | |
| | *Trichopsis* | *pumila* | Yes (M) | 0 | LR087 | AY763719 | AY763765 | |
| | *Trichopsis* | *schalleri* | Yes (M) | 0 | LR086 | AY763720 | AY763766 | |
| | *Trichopsis* | *vittata* | Yes (M) | 0 | LR051 | AF519658 | AF519697 | AF519736 |
| Osphroneminae | *Osphronemus* | *exodon*[6] | ? | ? | LR892 | AY763721 | AY763767 | |
| | *Osphronemus* | *goramy* | Yes (M) | 4 | LR102 | AY763722 | AY763768 | AY763785 |
| | *Osphronemus* | *septemfasciatus*[6] | ? | ? | LR177 | AY763723 | AY763769 | |
| Outgroup | | | | | | | | |
| Channidae | *Channa* | *bleheri* | Yes (M) | 5 | LR108 | AY763724 | AY763770 | AY763786 |
| | *Channa* | *marulia* | Yes (M) | 5 | LR129 | AY763725 | AY763771 | AY763787 |
| | *Parachanna* | *obscura* | Yes (M) | 5 | LR090 | AY763726 | AY763772 | AY763788 |

Abbreviations for breeding behavior (BB): 0 = bubble nesters; 1 = mouthbrooders; 2 = free spawner; 3 = substrate spawner; 4 = builder of submerged plant nests; 5 = floating egg gaurders (the majority of snakeheads build nests by removing surrounding vegetation, and eggs are released in the water column). The buoyant eggs rise to the water surface, where they are vigorously guarded by one or both parents (Courtenay and Williams, 2004). In addition, a few snakehead species have been reported to be mouthbrooders with male parental care); ? = unknown. GenBank accession numbers AF519XXX are from Rüber et al. (2004b). 1 = *petherici* species group; 2 = *multispine* species group; and 3 = *congicum* species group according to Norris (1994); 4 = cf. *albimarginata* "Malinau"; 5 = sequences have previously been published as *M. concolor* by Rüber *et al.* (2004b). *M. concolor* is a junior synonym of *M. spechti* (Freyhof and Herder, 2002); 6 = most likely the same states as *O. goramy*. The number of species in each anabantoid genus and its distribution is as follows: *Anabas* (2 spp.; Asia), *Ctenopoma* (13 spp.; Africa), *Microctenopoma* (11 spp.; Africa), *Sandelia* (2 spp.; Africa), *Helostoma* (1 sp.; Asia), *Belontia* (2 spp.; Asia), *Colisa* (4 spp.; Asia), *Luciocephalus* (2 spp.; Asia), *Parasphaerichthys* (2 spp.; Asia), *Sphaerichthys* (4 spp.; Asia), *Trichogaster* (4 spp.; Asia), *Betta* (57 spp.; Asia), *Macropodus* (5 spp.; Asia), *Malpulutta* (1 sp.; Asia), *Parosphromenus* (18 spp.; Asia), *Pseudosphromenus* (2 spp.; Asia), *Trichopsis* (3 spp.; Asia), *Osphronemus* (4 spp.; Asia).

Esocidae (Esociformes) by Gray (1831), but subsequently considered a member of the Anabantoidei by Bleeker (1859, 1879) based on the presence of a simple labyrinth organ. Later on, Berg (1958) and Liem (1963) argued that the suprabranchial organ had evolved independently in *Luciocephalus* and the anabantoids, and therefore rejected a close relationship of the two taxa. However, it is now generally accepted that *Luciocephalus* belongs to the anabantoids based on several morphological synapomorphies (Britz, 1994, 1995; Lauder and Liem, 1983), and hence, that the labyrinth organs of both taxa are homologous.

The first phylogenetic hypothesis of anabantoid intrarelationships was proposed by Lauder and Liem (1983), who divided the suborder into five families: Luciocephalidae, Anabantidae, Helostomatidae, Osphronemidae, and Belontiidae (Fig. 1a). Lauder and Liem (1983) identified Luciocephalidae as the most basal anabantoid family, Anabantidae as sister group of Helostomatidae, and Osphronemidae as sister group of Belontiidae (Fig. 1). Britz (1994, 1995, 2001) and Britz et al. (1995) revised Lauder and Liem's hypothesis in essential aspects. Britz (1994) demonstrated convincingly that the characters listed by Lauder and Liem (1983) to support a basal position of *Luciocephalus*, and the sister group relationship between osphronemids and belontiids were erroneous. He showed that *Luciocephalus* belongs to a monophyletic group, called Osphronemidae by Kottelat and Whitten (1996), that includes *Osphronemus* and Liem's belontiids (Fig. 1b). Within Osphronemidae, Britz (1995) and Britz et al. (1995) hypothesized the monophyly of a clade comprising *Luciocephalus* and the osphronemid genera *Ctenops*, *Sphaerichthys*, and *Parasphaerichthys* (Fig. 1b). Also, Britz (1995, 2001) presented evidence for division of osphronemids into four clades, the subfamilies Osphroneminae, Belontiinae, Luciocephalinae, and Macropodusinae (name changed from Macropodinae to Macropodusinae (ICZN, 2003); Fig. 1b; Table 1).

In contrast to the wider interest in osphronemid systematics, few authors have focused on the family Anabantidae (Liem, 1963; Norris, 1994, 1995; Elsen, 1976). Three genera were traditionally recognized within the family: *Anabas*, *Sandelia*, and *Ctenopoma*. Elsen (1976) subdivided the latter genus into three clades (named *C. petherici*, *C. multispine*, and *C. congicum* species group, respectively) based on features of the swimbladder and the suprabranchial organ. The monophyly of the three groups was further confirmed based on the comparative analysis of additional morphological characters by Peters (1976) and Norris (1994). The *C. congicum* species group was subsequently erected as a new genus, *Microctenopoma*, by Norris (1995).

Primary freshwater fishes are particularly suitable for testing alternative biogeographical hypotheses because of their restricted dispersal capability across different water drainages. In this regard, anabantoids are particularly interesting because they exhibit a rather unusual

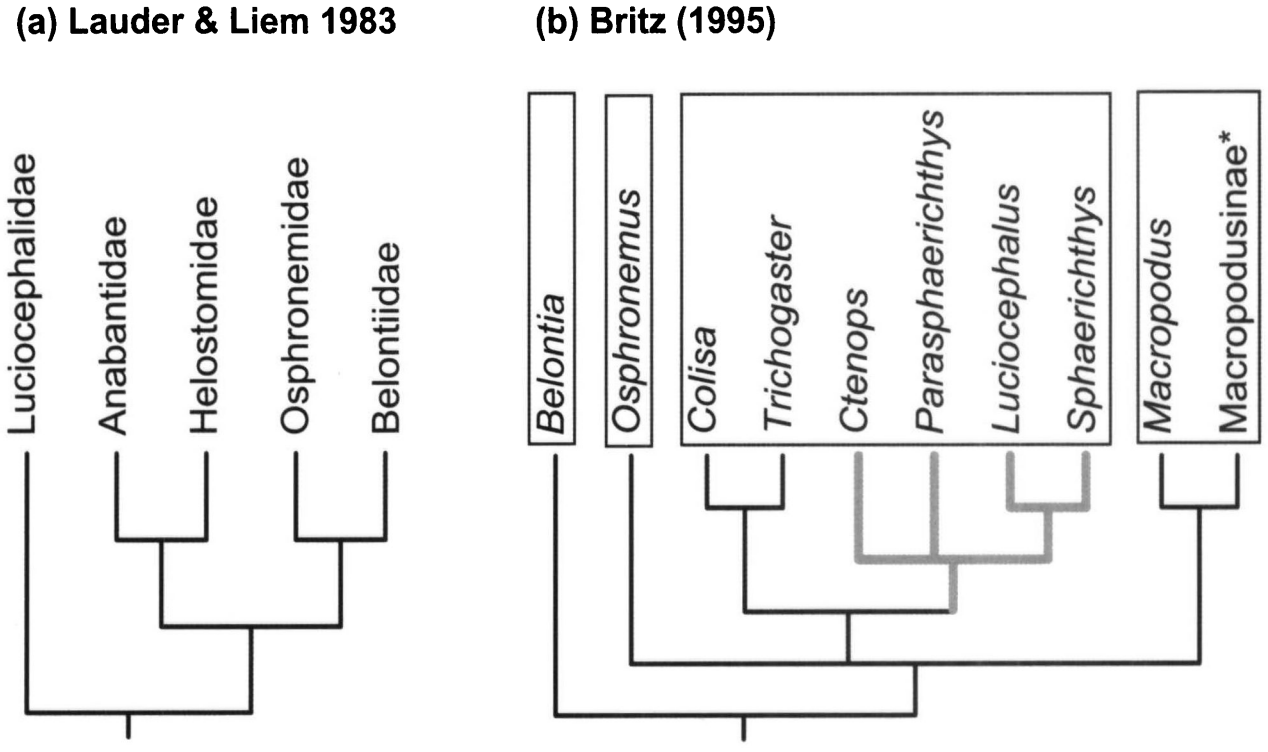


FIGURE 1. Previous morphology-based hypotheses of anabantoid intrarelationships. (a) Anabantoid intrarelationship based upon Lauder and Liem (1983). (b) Osphronemid intrarelationship based upon Britz (1995, 2001). The "spiral egg" clade of Britz (1995) is highlighted in grey and the four osphronemid subfamilies (Belontiinae, Luciocephalinae, Macropodusinae, and Osphroneminae) are shown in separate boxes (Macropodusinae* = Macropodusinae without *Macropodus*).

distribution that includes two continents. The families Osphronemidae and Helostomidae as well as the anabantid subfamily Anabantinae are restricted to southern Asia, whereas the anabantid subfamily Ctenopominae is restricted to Africa. No anabantoid is known from Madagascar. In addition to the anabantoids, few primary freshwater fishes show an exclusive African-Asian distribution (Channidae, Mastacembelidae, Schilbidae, Bagridae, Clariidae). Traditionally, a drift-vicariant scenario linked to the break up of Gondwana (165 to 121 million years ago [Mya]) in the Late Jurassic to Early Cretaceous is invoked to explain African-Asian sister group relationships (Rosen, 1978; Stiassny, 1991). However, it has been noted by several authors (e.g., Lundberg, 1993) that this drift-vicariant scenario is not compatible with the fossil record, and thus several alternative hypotheses of Late Mesozoic–Tertiary dispersal have been proposed (e.g., Chatterjee and Scotese, 1999; Sanders and Miller, 2002; Briggs, 2003a; de Queiroz, 2005).

### *Objectives*

Anabantoids are an excellent group to study evolutionary mechanisms that underlie morphological and behavioral diversification, as well as to test alternative biogeographical scenarios. However, a robust phylogenetic framework is needed to understand anabantoid evolution. Here, we determine phylogenetic relationships among labyrinth fishes based on an extensive taxon sampling, and analyzing both nuclear and mitochondrial nucleotide sequence data. We use the recovered molecular phylogeny to (1) evaluate previous morphology-based hypotheses of anabantoid intrarelationships and specifically address the controversial question of the phylogenetic position of the peculiar taxon *Luciocephalus*; (2) assess the evolutionary transitions in the forms of parental care during anabantoid evolution; and (3) test alternative biogeographical hypotheses that account for the disjunct African-Southern Asian distribution of anabantoids using molecular divergence time estimates.

## Material and Methods

### *Biological Material, DNA Isolation, and DNA Sequencing*

To assess the molecular phylogeny of the Anabantoidei, DNA samples of 57 species representing all 19 anabantoid genera were obtained (Table 1). Within a specific anabantoid genus, geographic distributions (Africa versus Asia), brood care (present versus absence), as well as breeding behavior (states 0 to 4 shown in Table 1) are constant (except for the genus *Betta*, where both bubble nesters and mouthbrooders occur). Therefore, the inclusion of taxa not sampled in the present study has no effect on our results and conclusions regarding the biogeographic history and the evolution of breeding behavior of the group. In addition, three species of the suborder Channoidei (snakeheads) were used as outgroups based upon previous morphological (Britz, 1995, 2004) and molecular evidence (Chen et al., 2003). Whole fish or fin clips were preserved in 70% to 100% ethanol, and total genomic DNA was isolated from white muscle tissue or fin clips by proteinase K/SDS digestion, phenol-chloroform extraction, and ethanol precipitation (Kocher et al., 1989).

The complete cytochrome *b* (cyt*b*) gene was amplified with two versatile primers, DonGlu F and DonThr R (Rüber et al., 2004b). A 2100-bp fragment that includes the 3′ end of the 12S rRNA, the complete tRNA-Val, and the 5′ end of the 16S rRNA was amplified by PCR amplification of three overlapping products with the primers L1091 and H1478 (Kocher et al., 1989), 16Sar and 16Sbr (Palumbi et al., 1991), and fish-12F1 and fish-16SR1 (Rüber et al., 2003). In addition, for a representative subset of the taxa (21 anabantoid species and three outgroups), approximately 1500 bp of the nuclear RAG1 gene were amplified with the primers RAG1F1 and RAG1R1 (López et al., 2004), previously called R1-2533F and R1-4090R by Rüber et al. (2004a, 2004b). All PCR amplifications were conducted in 25 $\mu$l reactions containing 75 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 0.4 mM of each dNTP, 0.4 $\mu$M of each primer, template DNA (10 to 100 ng), and Taq DNA polymerase (1 unit, Biotools, Madrid, Spain), using the following program: 1 cycle of 2 min at 94°C, 35 cyles of 60 s at 94°C, 60 s at 48–54°C, and 90 s at 72°C, and finally, 1 cycle of 5 min at 72°C. PCR products were either cloned into pGEM-T vectors (Promega, Madison, WI) and sequenced using M13 universal primers or sequenced directly after PCR purification by ethanol/sodium acetate precipitation.

Sequencing reactions were performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (V3.0) following manufacturer's instructions (Applied Biosystems, Foster City, CA) with 3.25 pmol of primer, 3 $\mu$l of Terminator Ready Reaction Mix, and 5% DMSO. The cycling profile for the sequencing reaction consisted of 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Cycle sequencing products were purified using MultiScreen plates (Millipore, Billerica, MA) and were analyzed on an ABI Prism 3700 DNA Analyzer (Applied Biosystems). Sequences specifically obtained for this study have been deposited in GenBank under the accession numbers AY763681 to AY763788. In addition, 36 sequences were obtained from Rüber et al. (2004b; see Table 1).

### *Sequence Alignment and Phylogenetic Analyses*

The cytochrome *b* and RAG1 gene nucleotide data sets were aligned by eye, whereas the 12S rRNA, tRNA-Val, and 16S rRNA gene nucleotide sequence data sets were aligned with SOAP v1.05a (Löytynoja and Milinkovitch, 2001). We generated 45 alternative CLUSTAL W (Thompson et al., 1994) alignments (gap opening penalty ranging from 7 to 15; gap extension penalty ranging from 3 to 7; both in increments of one). Unstable positions that differed between the alternative alignments were excluded (strict alignment option in SOAP), and alignments were further inspected visually. Alignments are available from TreeBASE (http://www.treebase.org) under the accession numbers S1403 and M2520 to M2523.

TABLE 2. Summary of model and model parameters obtained by the Akaike information criterion (AIC) implemented in ModelTest, v3.06 (Posada and Crandall, 1998), specification of data sets, and details on the ML, MP, ME, and BI analyses.

| | Data set | | | |
|---|---|---|---|---|
| | mtDNA60 (cyt*b*/12–16) | mtDNA24 (cyt*b*/12–16) | nucDNA24 (RAG1) | Combined24 (cyt*b*/12–16/RAG1) |
| Alignment | 3332 (1125/2207) | 3332 (1125/2207) | 1494 | 4826 (1125/2207/1494) |
| Analyzed | 2764 (1125/1639) | 2764 (1125/1639) | 1494 | 4258 (1125/1639/1494) |
| Invariant sites | 1317 (426/891) | 1490 (480/1010) | 989 | 2479 (480/1010/2479) |
| Uninformative sites | 290 (96/194) | 346 (109/237) | 187 | 533 (109/237/533) |
| Informative sites | 1157 (603/554) | 928 (536/392) | 318 | 1246 (536/392/318) |
| Model selected | GTR+I+G | GTR+I+G | TrN+I+G | GTR+I+G |
| Invariance/$\alpha$ | 0.39/0.54 | 0.37/0.41 | 0.45/0.81 | 0.42/0.54 |
| Empirical base frequency (A/C/G/T) | 0.36/0.28/0.15/0.21 | 0.33/0.28/0.19/0.20 | 0.23/0.28/0.30/0.19 | 0.28/0.27/0.23/0.22 |
| Substitution rates | | | | |
| Ts:Tv rate (MP Tv:Ts)[a] | 4.45/4:1 | 3.16/3:1 | 2.50/2:1 | 2.70/3:1 |
| A-C/A-G/A-T/C-G/C-T | 2.03/9.17/3.69/0.51/30.90 | 3.19/8.55/7.21/0.36/58.98 | 1.00/4.03/1.00/1.00/6.41 | 3.75/6.78/4.17/0.86/25.62 |
| MP: Steps (number of trees) | 21168 (2) | 9922 (1) | 1622 (1) | 11957 (1) |
| CI/RI | 0.19/0.52 | 0.34/0.28 | 0.57/0.61 | 0.37/0.36 |
| ME: score | 6.62 | 5.13 | 0.94 | 2.61 |
| ML non-clock/clock: −ln | 46,888.40/47,140.46 | 25,654.25/25,802.50 | 8350.39/8455.51 | 34,597.15/34,822.75 |
| LRT: 2$\Delta$ | $\chi^2_{58} = 504.12^{***}$ | $\chi^2_{22} = 296.50^{***}$ | $\chi^2_{22} = 210.24^{***}$ | $\chi^2_{22} = 451.20^{***}$ |
| BI mean −ln L | BI1: 46,935.43 | BI1: 25,673.65 | BI1: 8370.45 | BI1: 34,617.02 |
| BI mean −ln L | BI2: 46,792.18 | BI2: 25,602.01 | BI3: 8052.31 | BI3: 34,372.55 |
| BI mean −ln L | BI4: 46,532.20 | BI4: 25,296.64 | n/a | BI7: 33,702.17 |
| Node resolution[b] | | | | |
| BI | BI1: 43/10/2 | BI1: 13/4/2 | BI1: 12/6/1 | BI1: 14/5/0 |
| BI | BI2: 43/12/0 | BI2: 12/6/1 | BI3: 13/6/0 | BI3: 13/6/0 |
| BI | BI4: 41/13/1 | BI4: 12/7/0 | n/a | BI7: 13/6/0 |
| ML | 42/8/5 | 10/2/7 | 15/2/2 | 13/6/0 |
| ME | 44/5/6 | 11/1/7 | 14/0/5 | 17/0/2 |
| MP unweighted | 33/10/12 | 6/2/11 | 13/4/2 | 13/4/2 |
| MP weighted | 36/8/11 | 8/3/8 | 15/1/3 | 13/5/1 |

[a]Estimated from ML tree; [b]number of high (≥95% BI; ≥70% ML, ME, MP)/moderate (<95% to ≥50% BI; <70% to ≥50% ML, ME, MP )/low (<50% BI, ML, ME, MP) supported branches. *** $P \ll 0.001$.

The phylogenetic analyses were performed in two steps. First, the complete cyt*b*, 12S rRNA, tRNA-Val, and 16S rRNA nucleotide sequences of 60 taxa were combined into a single data set (henceforth referred to as the mtDNA60 data set; Table 2). Second, we performed three separate analyses of mtDNA, RAG1, and combined mtDNA+RAG1 nucleotide sequences based upon restricted 24-taxa data sets (henceforth referred to as the mtDNA24, nucDNA24, and combined24 data sets, respectively; Table 2).

We tested for base compositional biases using the $\chi^2$ test as implemented in PAUP* v4.0b10 (Swofford, 2002). Analyses were based upon the different data partitions using either all characters or only parsimony-informative sites. For the protein-coding genes (cyt*b* and RAG1), we also tested for base compositional biases in each of the three codon positions, separately.

The Akaike information criterion (AIC; Akaike, 1974) implemented in ModelTest v3.06 (Posada and Crandall, 1998) was used to determine the evolutionary model and parameter values that best fit each of the data sets (Table 2). These settings were subsequently used for maximum likelihood (ML) analyses and to estimate ML distances for minimum evolution (ME) analyses. Maximum parsimony (MP) analyses were conducted with heuristic searches (TBR branch swapping, MULTREES option effective, and 10 random stepwise additions of taxa). We conducted the parsimony analyses for each data set both with and without differential weighting of transversions (Tv) and transitions (Ts). Weights were based on empirical Ts:Tv ratios estimated from the ML trees (Table 2). Robustness of the inferred ME and MP trees were tested using nonparametric bootstrapping (Felsenstein, 1985) with 1000 pseudoreplicates. All the phylogenetic analyses mentioned above were conducted with PAUP* v4.0b10. Robustness of the inferred ML trees was tested using PHYML v2.3 (Guindon and Gascuel, 2003) with 500 pseudoreplicates.

A Bayesian inference (BI) of anabantoid phylogeny was performed with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) by Metropolis-coupled Markov chain Monte Carlo ($MC^3$) sampling for 1,000,000 generations (four simultaneous MC chains; sample frequency 100; burn-in 100,000 generations (see Results); chain temperature 0.2) under the GTR+I+G model. We used the default uniform Dirichlet distribution for the base frequencies, and default flat prior distributions for all other parameters. We plotted the −ln likelihood scores against generation time for each run to determine the number of generations needed to converge to stationarity and to evaluate the burn-in (samples obtained before the chain reached stationarity). We used PAUP* v4.0b10 to reconstruct the 50% majority-rule consensus tree of the post burn-in trees. We analyzed each of the four

data sets (mtDNA60, mtDNA24, nucDNA24, and combined24) using different data-partitioning strategies. For the mtDNA60 and the mtDNA24 data sets we analyzed three different partitions: BI1, one partition (mtDNA); BI2, two partitions (cyt*b* + RNAs); and BI4, four partitions (cyt*b* 1st + 2nd + 3rd codon positions + RNAs). For the nucDNA24 data set, two different BI analyses were conducted: BI1, one partition (RAG1); and BI3, three partitions (RAG1 1st + 2nd + 3rd codon positions). For the combined24 data set, three different analyses were performed: BI1, one partition (mtDNA + nucDNA); BI3, three partitions (cyt*b* + RNAs + RAG1); and BI7, seven partitions (cyt*b* 1st + 2nd + 3rd codon positions + RNAs + RAG1 1st + 2nd + 3rd codon positions). Model parameters were estimated independently for each of the respective data partitions using the unlink command in MrBayes v3.03. All BI analyses were run twice, starting from different random starting points, to confirm convergence and mixing. Because no significant differences between alternative runs were detected, we only present the results from one of the runs.

The effect of base compositional heterogeneity across taxa on phylogenetic inference has long been recognized as a potential problem (e.g., Lockhart et al., 1992, 1994; Mooers and Holmes, 2000; Penny et al., 1990). In order to assess the effect of base compositional biases (see Results) on phylogenetic reconstructions, we additionally performed ME analyses using log determinant distances (LogDet; Lockhart et al., 1994). This method has recently been shown to infer the correct topologies under compositional heterogeneity when other methods (MP, ML, NJ with Jukes-Cantor distances) failed to recover the true topologies (Jermiin et al., 2004).

Alternative phylogenetic hypotheses were tested with likelihood-based approaches using the Shimodaira-Hasegawa (SH; Shimodaira and Hasegawa, 1999) and the Kishino-Hasegawa (KH one-tailed; Kishino and Hasegawa, 1989) tests with 1000 RELL bootstrap replicates. The alternative hypotheses tested were either *a priori* morphology-based hypotheses or *a posteriori* hypotheses accounting for different topologies obtained during the analyses (e.g., differences between data partitions or methods of phylogeny inference).

### *Alternative Rooting of the Anabantoid Tree*

Outgroup rooting is by far the most common method to determine the root of a phylogenetic tree, although other methods (e.g., midpoint rooting, the molecular clock, or nonreversible models of substitution) have been proposed (e.g., Huelsenbeck et al., 2002; Smith, 1992). The quality of rooting provided by the outgroup criterion is generally thought to depend on the sampling strategy of the outgroup taxa (Swofford et al., 1996) and on the phylogenetic proximity of the outgroup to the ingroup (Wheeler, 1990), where distant outgroups may effectively randomize substitutions along the root. To avoid the problems caused by distantly related outgroups, we chose representatives of the sister group of anabantoids, the family Channidae (snakeheads) to root the tree (Britz, 1995, 2004; Chen et al., 2003). The accurate location of the root using the outgroup method might be hampered, however, in cases where the basal ingroup internodes are extremely short, resulting in a general lack of phylogenetic signal at the base of the ingroup. This situation is, for example, observed in lineages that underwent rapid cladogenesis early in their evolutionary history. Here, we illustrate the potential problems of accurately determining the location of the (anabantoid) root by using a Bayesian approach (Huelsenbeck et al., 2002). We determined the posterior probability distribution of the root based upon the 9000 post–burn-in trees (see Results) from the BI analyses for the three data sets mtDNA24 (BI4), nucDNA24 (BI3), and combined24 (BI7). Post–burn-in trees were filtered with PAUP* according to the following three alternative topologies resulting from different locations of the root (see Results): (1) (Helostomatidae, (Anabantidae, (remaining Anabantoidei))), (2) (Anabantidae, (remaining Anabantoidei)), and (3) (Helostomatidae, (remaining Anabantoidei)). The posterior probability distributions of the root were then mapped onto the unrooted Bayesian consensus phylograms.

### *Reconstruction of Parental Care and Parental Care Forms*

To gain insights into the evolution of the different modes of breeding behavior in anabantoids (free spawning, substrate spawning, bubble nesting, building of submerged plant nests, and mouthbrooding; see Table 1 for character state coding), we performed ancestral character state reconstructions under MP and ML. The tree used to reconstruct ancestral character states was the ML tree estimated from the mtDNA60 data set, using the backbone topology derived from the BI7 analysis of the combined24 data set as topological constraint. The constrained and unconstrained mtDNA60 topologies were not significantly different from each other according to the SH test: −ln likelihood unconstrained = 46,888.40; −ln likelihood constrained = 46,893.98; $P = 0.221$).

MacClade v4.03 (Maddison and Maddison, 2001) was used to trace the evolution of parental care and breeding behavior based on unweighted parsimony reconstruction. The assumption of equal transition probabilities, however, might not be realistic when considering gains and losses of complex characters. In such cases, unequal weighting may represent a more realistic model for the directionality of changes (Cunningham et al., 1998; Omland, 1997; see also Rüber et al., 2004b, for a case study in the fighting fish genus *Betta*). Therefore, we also applied a sensitivity analysis (Donoghue and Ackerly, 1996) for both parental care and breeding behavior. The sensitivity analysis for the breeding behavior was conducted only for the Luciocephalinae + Macropodusinae clade (bubble nesters and mouthbrooders only) because it showed several equivocal nodes under unweighted parsimony (see Results). We increased or decreased the weight of one of the two transformations (holding constant the weight of the reverse transformation) in order to find the minimum additional weight (in 0.1 increments)

needed to reverse the results obtained under the assumption of equal transition costs. We further used a randomization procedure implemented in MacClade v4.03 (Maddison and Maddison, 2001) to test whether breeding behavior in anabantoids is significantly correlated with phylogeny. To this end, we generated 1000 random trees to obtain the null distribution of the number of transitions of the character. The null distribution of the frequency of the number of transitions from the randomized trees was used to test whether the observed number of transitions was significantly more correlated with phylogeny than expected at random.

We used ML reconstructions under a continuous-time Markov model to estimate ancestral states for both parental care and breeding behavior of selected nodes using Multistate v0.8 (Pagel, 2003). Because Multistate v0.8 does not allow missing information, four species for which no information on parental care and breeding behavior is available (see Table 1) were pruned from the ML tree using TreeEdit v1.0 (Rambaut and Charleston, 2001) prior to the analyses. Under a "full" ML model, $n^2 - n$ parameters (where n is the number of states; two states for parental care, and six states for the breeding behavior as shown in Table 1) for the different transition rates need to be estimated. The accuracy of parameter estimation depends on the model complexity and on the amount of data. Mooers and Schluter (1999) showed that ML reconstruction using two character states under the "full" two-rate model may produce extremely high transition rates, resulting in many equivocal reconstructions and flat likelihood surfaces. Therefore, these authors recommended employing less complex ML models when the accuracy of parameter estimation is hindered by the amount of data. To restrict the number of parameters that need to be estimated under a "full model" for the analysis of breeding behavior, we chose equal forward/backward rates for the 15 pairwise combinations given the six states (see Table 1; $q_{xy} = q_{yx}$) in our data. We used a likelihood-ratio test (LRT; Huelsenbeck and Crandall, 1997) to evaluate whether the full model fit the data significantly better than our restricted model. The LRT was performed using a $\chi^2$ test with degrees of freedom equal to the differences in the number of parameters between the two models (df = 15). Due to the need of parameter restriction under ML, the assumption of equal forward/backward rates was relaxed only under MP using the sensitivity analyses.

We tested whether the branch length contained in our ML tree is informative about trait evolution using the scaling parameter $\kappa$. The parameter $\kappa$ defines the relationship between the lengths of individual branches and the probability that a character state changes, and thus allows stretching or compressing individual branches. In the extreme case of $\kappa = 0$, trait evolution is independent of the branch lengths (punctuational evolution), $\kappa < 1$ compresses longer branches more than shorter ones, $\kappa = 1$ indicates default gradualism, and $\kappa > 1$ stretches longer branches more than shorter ones, indicating that longer branches have contributed more to trait evolution. In order to test for the dependence of trait evolution on branch length for the breeding behavior, we used an LRT (df = 1) to compare the likelihoods under an unconstrained model ($\kappa$ = ML) and a punctuated model ($\kappa = 0$).

*Bayesian Relative Rates Test and Divergence Times Estimates*

Prior to conducting divergence times estimates for the anabantoids, we tested constancy of evolutionary rates among taxa using both an LRT based on ML trees with and without a molecular clock constraint, and a Bayesian relative rate (BRR) test (Wilcox et al., 2004). For the BRR test we obtained the posterior probability distribution of the summed branch lengths from the most recent common ancestor (MRCA) of the ingroup to each of the terminal taxa. The posterior probability distributions of the summed branch length were based upon the 9000 post–burn-in trees from the BI analyses (mtDNA24 [BI4], nucDNA24 [BI3], and combined24 [BI7], respectively). Compilation of summed branch length was conducted with Cadence v1.08 (Wilcox, 2004). We considered rates of evolution significantly different between two taxa if their 95% confidence interval of the posterior probability distribution of the summed branch length did not overlap.

In order to date major cladogenetic events we used the Kishino et al. (2001; see also Thorne and Kishino, 2002; Thorne et al., 1998) method (KTB). The KTB method is a Bayesian dating method for multilocus data that incorporates variation of rates of evolution among genes and among lineages under a relaxed molecular clock as implemented in Multidivtime v9/25/2003 (Thorne, 2003).

The anabantoid fossil record is very poor (Patterson, 1993a), and the only fossil that can be used as a calibration point is a full skeleton of *Osphronemus* from the Sangkarewang Formation in Central Sumatra (Sanders, 1934). According to recent age estimates, this formation dates back to the Early Oligocene to Late Eocene age (28.5 to 37.0 Mya; Barber et al., 2005; Humphreys et al., 1991). Because the fossil of *Osphronemus* could belong to either the crown or the stem group of the genus, we used two alternative tree nodes as calibration points: first, the MRCA of *Osphronemus* and its sister group *Belontia* (stem), and second, the MRCA of *Osphronemus* (crown). The fossil stratigraphic occurrence was translated into age constraints for minimum age estimates of anabantoids by either using the upper and lower age of the stratigraphic interval as lower and upper bounds (L = 28.5 to U = 37.0; LU constraint), or by only using the lower bound as a lower constraint (L = 28.5; L constraint).

The Bayesian dating procedure consisted of three main steps (see also Rutschmann, 2004): (1) *baseml* (PAML v3.14; Yang, 1997) was used to estimate model parameters for each gene partition separately under the F84 model of nucleotide substitution and a discrete gamma distribution with five rate categories. The F84+G was chosen following the recommendations by Wiegmann et al. (2003). (2) *estbranches* (Multidivtime v9/25/2003;

Thorne, 2003) was used to obtain ML estimates of branch lengths and the variance-covariance matrix. (3) Bayesian MCMC analyses to approximate the posterior distribution of substitution rates, divergence times, and 95% credibility intervals were conducted with *multidivtime* (Multidivtime v9/25/2003; Thorne, 2003). The Markov chain was sampled 10,000 times with 100 cycles between each sample after an initial burn-in of 100,000 cycles. To ensure convergence of the MCMC analyses, two independent runs were conducted for each data set analyzed starting from different random seeds. The mean and standard deviation of the prior distribution for the time separating the ingroup root from the present (rttm and rttmsd) was set to 74 Mya corresponding to the Upper Campanian–Lower Maastrichtian in the Late Cretaceous. This date represents the time of the earliest skeleton record for the perciforms (Patterson, 1993b). "Bigtime" in *multidivtime* was set to 150 Mya, roughly double the value of rttm. Divergence time analyses were based on the mtDNA60 (stem and crown group calibrations) and combined24 (stem group calibration only) data sets. For the mtDNA60 data set, we used the constraint ML topology employed in the reconstruction of ancestral character states (see above), whereas for the combined24 data set, we used a constrained ML tree; i.e., the ML tree under the combined24 (BI7) topology constraint. This topology was not significantly different from the unconstrained ML tree using the SH test (−ln likelihood unconstrained = 34,597.15; −ln likelihood constrained = 34,599.71; $P = 0.333$). In order to test the effect of a different "bigtime" setting on our results and conclusions we repeated all the analyses employing a "bigtime" of 300 Mya.

## RESULTS

### *Anabantoid Phylogenetics*

The mtDNA60 and mtDNA24 alignments consisted initially of 3332 positions, each. A total of 2764 positions were used for phylogenetic analyses after exclusion of sites of ambiguous homology assignment. In the nuc24 data set all 1494 aligned positions were used for phylogenetic analyses. A total of 4826 positions were aligned for the combined24 data set, and 4258 were analyzed. Specifications of the different data sets used for the phylogenetic analyses, evolutionary models applied, as well as BI, ML, ME, and MP scores are given in Table 2.

Strong base compositional bias was observed in all data sets (mtDNA60, mtDNA24, nucDNA24, and combined24) whether all positions or only parsimony-informative positions were considered (not shown). Further analyses showed that base compositional bias was apparently caused by third codon positions of cyt*b* and RAG1. In addition, rRNAs (60- and 24-taxon data set) and first codon positions of cyt*b* (24-taxon data set) showed significant deviation from stationarity when only parsimony-informative sites were considered (not shown).

For all BI analyses, plotting the −ln likelihood scores against generation time revealed that stationarity was reached at no later than 100,000 generations and the last 900,000 generations (9000 trees) were kept for all further analyses. The 50% majority-rule consensus tree recovered from the Bayesian analysis of the mtDNA60 data set employing four partitions (BI4) is depicted in Figure 2. Results of the Bayesian analyses employing different partitioning strategies (BI1 and BI2) yielded almost identical topologies (not shown). The only differences between the three Bayesian analyses were the proportions of ingroup nodes with high (≥95%), moderate (<95% to ≥50%), and low (<50%) posterior probabilities, as shown in Table 2. ML, ME, and MP (4:1 weighting or unweighted) recovered trees with almost identical topologies to that of the Bayesian tree shown in Figure 2 (see Table 2 for tree scores). Topological differences resulting from the different phylogenetic analyses after bootstrapping are indicated in Figure 2.

Phylogenetic analyses based on the mtDNA24, nucDNA24, and combined24 data sets using the Bayesian inference method (with different partitioning strategies) resulted in the phylogenetic trees shown in Figure 3a–c, respectively. Phylogenetic analyses of these three data sets under ML, ME, and MP recovered trees shown in Figure 3d–f, respectively. The different BI, ML, MP, and ME tree scores are shown in Table 2. Topological differences resulting from the distinct data partitions, and phylogenetic methods are highlighted in Figure 3. We considered the combined24 BI7 topology as our best working hypothesis for the phylogenetic relationships among the major anabantoid lineages because it is based on both nuclear and mitochondrial loci and analyzed using the maximum number of partition specific model parameters. This topology therefore was subsequently used as backbone topology for ancestral character state reconstructions and divergence time estimates. The main differences of the combined24 BI7 topology compared to alternative topologies resulting from some other analyses were (1) *Helostoma* is the sister group to anabantids (versus sister group to osphronemids); and (2) *Ctenopoma muriei* is the sister group to the remaining *Ctenopoma petherici* clade + *Microctenopoma* (versus sistergroup to *Microctenopoma*); all alternative hypotheses involved very short internodes. It is important to note that our results and conclusions regarding divergence time estimates are robust to these minor topological changes and are therefore not affected by the choice of this specific backbone topology. The alternative phylogenetic position of *Helostoma* has some effects on ancestral character state reconstruction of parental care and parental care form, which will be addressed in detail below.

Based on the significant base compositional bias observed in all data sets (mtDNA60, mtDNA24, nucDNA24, and combined24), we also conducted logDet analyses. The obtained results (data not shown) were in good agreement with phylogenetic analyses under BI, ML, ME, and MP and suggest that base compositional biases may not have significantly influenced our analyses of anabantoid intrarelationships. Ranking of species % GC content is shown in Figure 3a–c and indicates no

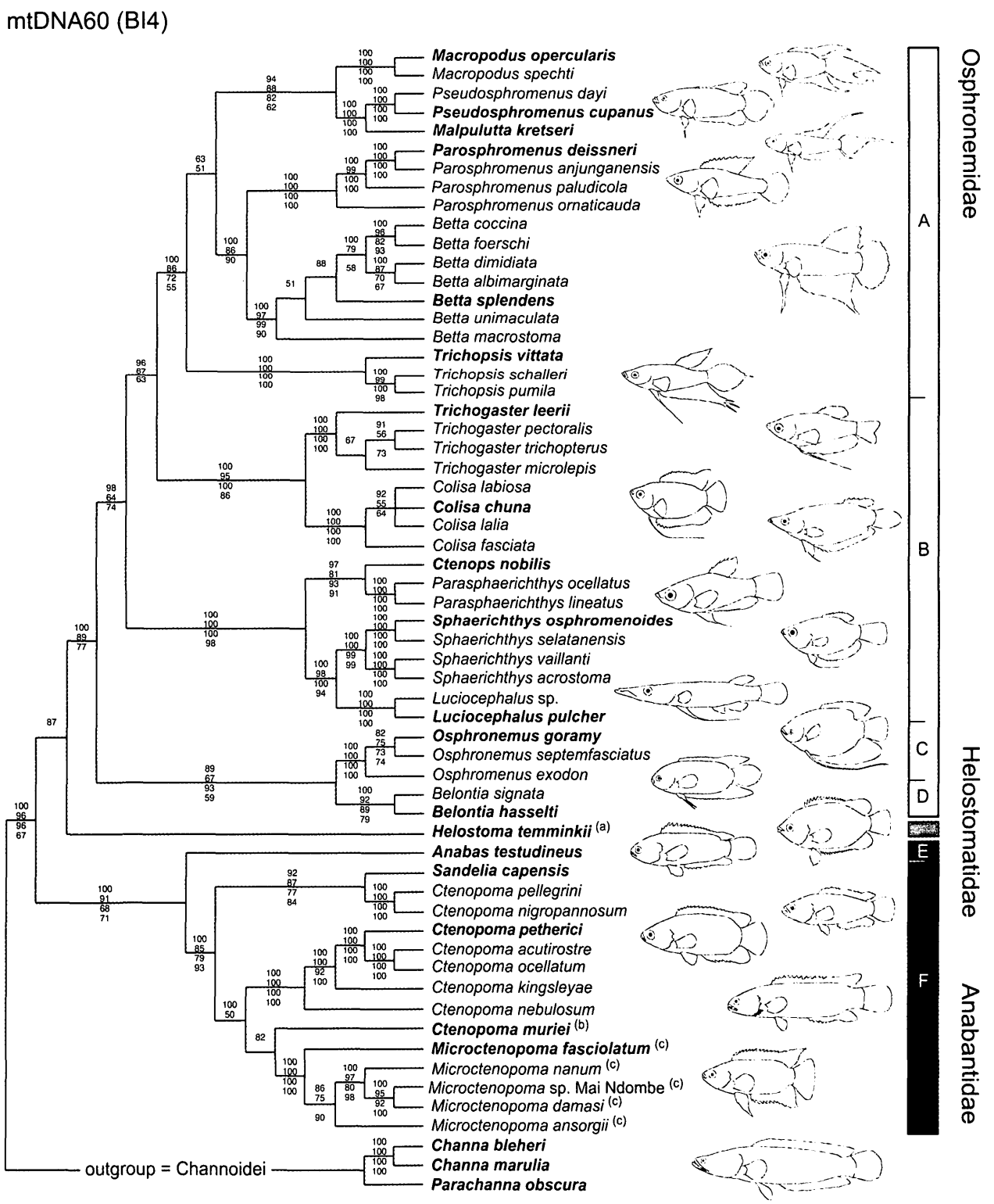


FIGURE 2. Reconstructed phylogeny of the Anabantoidei using a Bayesian phylogenetic approach based on the mtDNA60 (BI4) analysis. Three Channoidei taxa were utilized as outgroups. The topmost number above each branch refers to the Bayesian posterior probability (shown as percentage) of the node derived from 9000 MCMCMC sampled trees on the basis of the complete cytb and 12S rRNA, tRNA-Val, and 16S rRNA mitochondrial DNA nucleotide sequence data (2764 bp; mtDNA60 (BI4)). Bootstrap values (>50%; from top to bottom) for ML-Phyml, ME, and weighted MP are shown below. Subfamilies are indicated within the bars designating the three anabantoid families (see also Table 1). The Osphronemidae subfamilies are (A) Macropodisinae, (B) Luciocephalinae, (C) Osphroneminae, and (D) Belontiinae, whereas the Anabantidae subfamilies are (E) Anabantinae and (F) Ctenopominae. Taxa in bold are those used in the restricted 24-taxa data sets. Outlines are not drawn to scale. (a) *Helostoma temminkii* was resolved as sister group of the Anabantidae in the ML (57%) and ME (81%) analyses; (b) *Ctenopoma muriei* was resolved as sister group of the *Ctenopoma petherici* species group in the ME (53%) analysis; (c) *Microctenopoma* was resolved as sister group to the genera *Sandelia* and *Ctenopoma* in the ME (76%) and weighted-MP (69%) analyses.

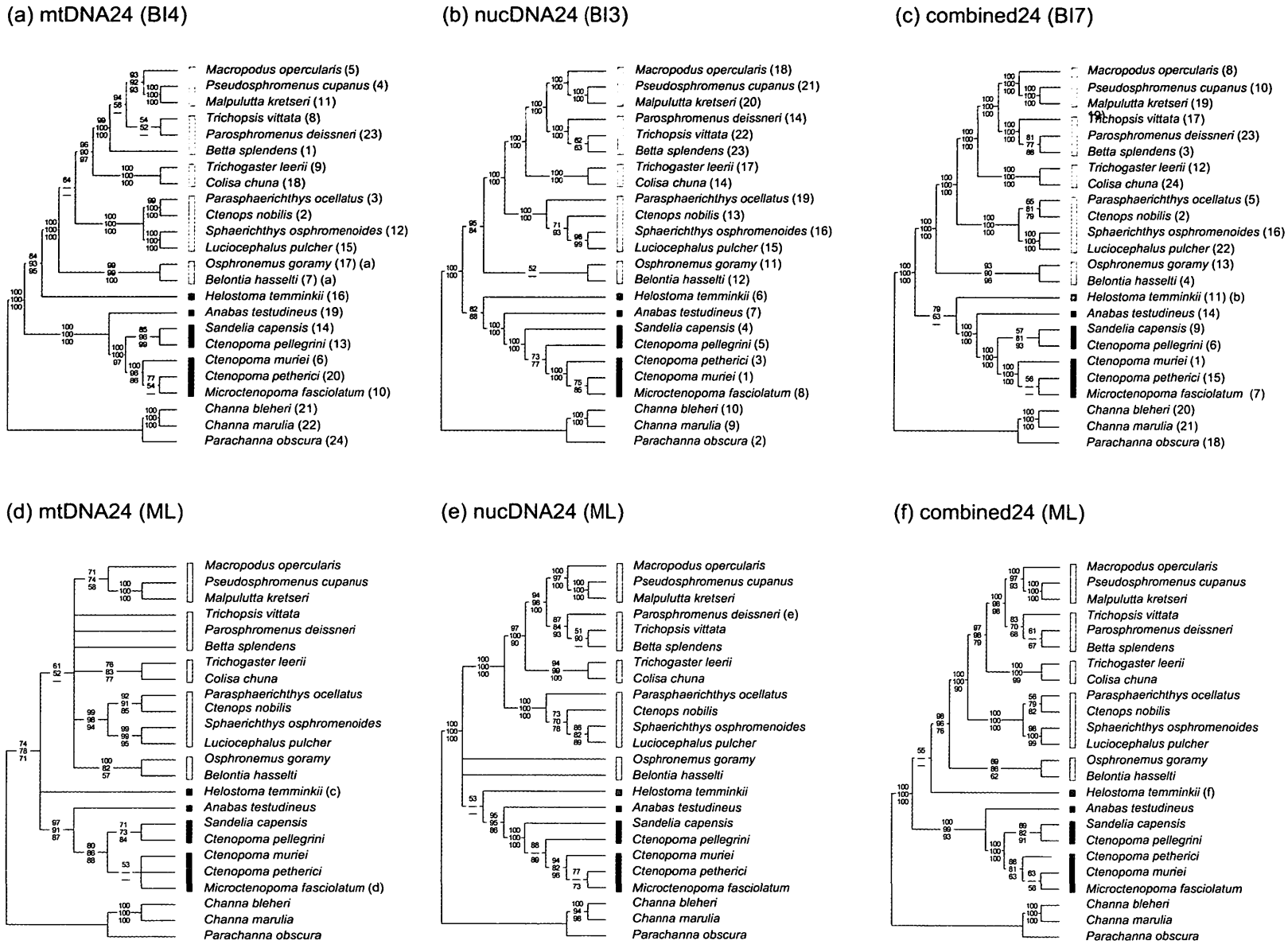


FIGURE 3. Results of the phylogenetic analyses based upon the 24-taxa data sets. (a–c) The numbers on the branches (from top to bottom) refer to the Bayesian posterior probabilities (shown as percentage; >50%) of the nodes derived from 9000 MC3-sampled trees under the different data-partitioning strategies as indicated below. (d–f) The numbers on the branches (from top to bottom) refer to the bootstrap values (>50%) for ML-Phyml, ME, and weighted MP. (a) mtDNA24 (BI4/BI2/BI1), (b) nucDNA24 (BI3/BI1), (c) combined24 (BI7/BI3/BI1), (d) mtDNA24 (ML-Phyml/ME/MP 3:1), (e) nucDNA24 (ML-Phyml/ME/MP 2:1), (f) combined24 (ML-Phyml/ME/MP 3:1). Bars to the left of species names delineate groups discussed in the text and their different shading corresponds to the three anabantoid families as indicated in Figure 2. Number in brackets behind species names in (a) to (c) indicate the ranking in % GC content (1 = lowest GC content and 24 = highest GC content). The range of values are (a) 41.6–48.4%; (b) 50.8–61.8%; (c) 46.1–52.0%. The labels (a) *Belontia* and *Osphronemus* resolved as sister group to *Trichogaster* and *Colisa* and the Macropodusinae in the BI1 analysis (68%); (b) *Helostoma* resolved as sister group to the Osphronemidae in the BI1 analysis (74%); (c) *Helostoma* resolved as sister group to Anabantidae in the ME analysis (83%); (d) *Microctenopoma* resolved as sistergroup to the remaining Ctenopominae in the weighted MP (52%); (e) *Trichopis* resolved as sister group of *Parosphronemus* and *Betta* in the weighted MP (53%); (f) *Helostoma* resolved as sister group to the Anabantidae in the ME analysis (77%).

apparent phylogenetic clustering of species according to % GC content.

The results recovered from the phylogenetic analyses of anabantoid intrarelationships as shown in Figures 2 and 3 can be summarized as follows: (1) monophyly of the Anabantidae, with Anabantinae (genus *Anabas*) resolved as sister group of the Ctenopominae; (2) the genus *Ctenopoma* was not recovered as a monophyletic group; (3) with exception of the nucDNA24 data set, *Sandelia* was consistently recovered as sister group of the *Ctenopoma multispine* species group (*C. nigropannosum* and *C. pellegrini*); (4) *Ctenopoma muriei* (traditionally assigned to the *C. petherici* species group) was either recovered as sister group of a clade comprised of the remaining members of the *C. petherici* species group plus *Microctenopoma* or as sister group to *Microctenopoma*, thus challenging the monophyly of the *C. petherici* species group; (5) the Helostomatidae (genus *Helostoma*) was either resolved as sister group of the Anabantidae, the Osphronemidae, or in a basal trichotomy with the latter two anabantoid families; (6) most of the analyses recovered the monophyly of the Osphronemidae, with the Belontiinae + Osphroneminae clade as sister group of the Luciocephalinae + Macropodusinae. However, the monophyly of the Belontiinae + Osphroneminae received moderate or low support in some of the analyses; (7) the monophyly of the Luciocephalinae was not confirmed in any of the analyses. Instead, the strongly supported "spiral egg" clade consisting of the genera *Ctenops*, *Luciocephalus*, *Parasphericthys*, and *Sphaerichthys* was consistently placed as sister group of the remaining Luciocephalinae (genera *Colisa* + *Trichogaster*) plus the Macropodusinae; (8) within the "spiral egg" clade, *Ctenops* + *Parasphericthys* were recovered as sister group to *Luciocephalus* + *Sphaerichthys* except in the nucDNA24-based analyses in which *Parasphericthys* was resolved as sister group to the remaining three genera; (9) nuclear and combined (mitochondrial + nuclear) analyses recovered a basal split within the Macropodusinae into two clades comprised of *Macropodus* + *Pseudosphromenus* + *Malpulutta*, and *Trichopsis* + *Pseudosphromenus* + *Betta*, respectively. However, phylogenetic analyses based on the mitochondrial data sets failed to recover this basal split because of a general lack of resolution among *Trichopsis*, *Pseudosphromenus*, and *Betta*.

### *Phylogenetic Hypotheses Testing*

Based upon previous morphology-based phylogenetic hypotheses of anabantoid intrarelationships, as well as on topological differences encountered in the course of our analyses, we evaluated alternative phylogenetic hypotheses using the SH and KH tests. The alternative hypotheses tested are shown in Table 3. Using the mtDNA24 data set, none of the alternative hypotheses could be rejected with any of the tests. However, the KH test clearly rejected the two morphology-based hypotheses (monophyly of the Luciocephalinae, and *Macropodus* as sister group of the remaining Macropodusinae) based on both the nucDNA24 and combined24 data sets. The sister group relationship of *Macropodus* and the remaining Macropodusinae was also rejected by the nucDNA24 data set with the SH test.

TABLE 3. Testing alternative phylogenetic hypotheses within the Anabantoidei using the Shimodaira-Hasegawa and the Kishino-Hasegawa (one-tailed) tests for the different data sets.

| Topology | mtDNA24 | nuc24 | combined24 |
|---|---|---|---|
| ((macr, pseu, malp)(paro, tric, bett)) | 25,654.631[a] | 8350.395 | 34,597.153 |
| **(macr(pseu, malp, paro, tric, bett))** | 25,659.070[a] | 8368.920*/* | 34,621.163—/* |
| ((paro(bett, tric)) | 25,656.955[a] | 8350.395 | 34,599.855 |
| ((tric (bett, paro)) | 25,654.631[a] | 8351.000 | 34,597.153 |
| ((para(spae, cten, luci)) | 25,662.304 | 8350.395 | 34,598.356 |
| ((para, cten)(spae, luci)) | 25,654.255 | 8353.755 | 34,597.153 |
| **((coli, trig)(para, spae, cten, luci))** | 25,658.815 | 8360.870—/* | 34,613.483—/* |
| ((Mac, Luc)(Osp, Bel)) | 25,655.582[b] | 8350.395 | 34,597.153 |
| (((Mac, Luc)Osp)Bel) | 25,663.494[b] | 8351.207 | 34,604.556 |
| (((Mac, Luc)Bel)Osp) | 25,659.754[b] | 8350.476 | 34,600.484 |
| ((OS, HE)AN) | 25,654.255 | 8352.378 | 34,597.153 |
| (OS(HE, AN)) | 25,658.329 | 8350.395 | 34,598.276 |
| ((sand, cpel)(cpet, cmur, micr)) | 25,654.255 | 8351.130 | 34,597.153 |
| ((sand(cpel, cpet, cmur, micr)) | 25,660.338 | 8350.395 | 34,599.977 |
| (cmur (cpet, micro)) | 25,654.278 | 8351.924 | 34,598.630 |
| (cpet (cmur, micro)) | 25,654.255 | 8350.395 | 34,597.153 |
| (micro (cmur, cpet)) | 25,655.701 | 8351.924 | 34,600.141 |

macr = *Macropodus*; pseu = *Pseudosphromenus*; malp = *Malpulutta*; paro = *Parosphromenus*; tric = *Trichopsis*; bett = *Betta*; coli = *Colisa*; trig = *Trichogaster*; para = *Parasphaerichthys*; spae = *Sphaerichthys*; cten = *Ctenops*; luci = *Luciocephalus*; sand = *Sandelia*; cpel = *Ctenopoma pellegrini*; cpet = *Ctenopoma petherici*; cmur = *Ctenopoma muriei*; micr = *Microctenopoma*; Mac = *Macropodusinae*; Luc = *Luciocephalinae*; Osp = *Osphroneminae*; Bel = *Belontiinae*; OS = *Osphronemidae*; HE = *Helostomatidae*; AN = *Anabantidae*. The −ln likelihood score of the ML hypothesis is underlined. [a]The ML topology is: (bett((tric, paro)(macro(pseu, malp))); [b]the ML topology is: ((Osp, Bel)((trig, coli)(Mac))). Alternative phylogenetic hypotheses derived from morphology are shown in bold (see also Fig. 1). x/x = SH/KH; * $P < 0.05$.

### *Anabantoid Rooting*

*Helostoma temminkii*, the only representative of the Helostomatidae, was either recovered as sister group of the Anabantidae, of the Osphronemidae, or in a basal trichotomy suggesting different rooting locations. Figure 4 shows the posterior probability distributions of the root placement mapped onto the unrooted Bayesian consensus phylograms for each data set. The posterior probabilities (in percentage) of the root location based on the mtDNA24 data set were 14.89%, 84.17%, and 0.94% for the branch leading to the Osphronemidae, Anabantidae, and Helostomidae, respectively (Fig. 4a). The corresponding values were 77.16%, 9.25%, 8.17% based on the nucDNA24 data set (Fig. 4b; 5.42% of rooting locations were located on basal osphronemid branches; data not shown). The posterior probabilities of the root location based on the combined24 data set were 79.08%, 20.67%, and 0.25%, respectively (Fig. 4c). The generally low statistical supports for the basal anabantoid nodes found in the phylogenetic analyses (none of the data sets contained the 95% credible set of the root location on a single branch) are substantiated by the very short length of

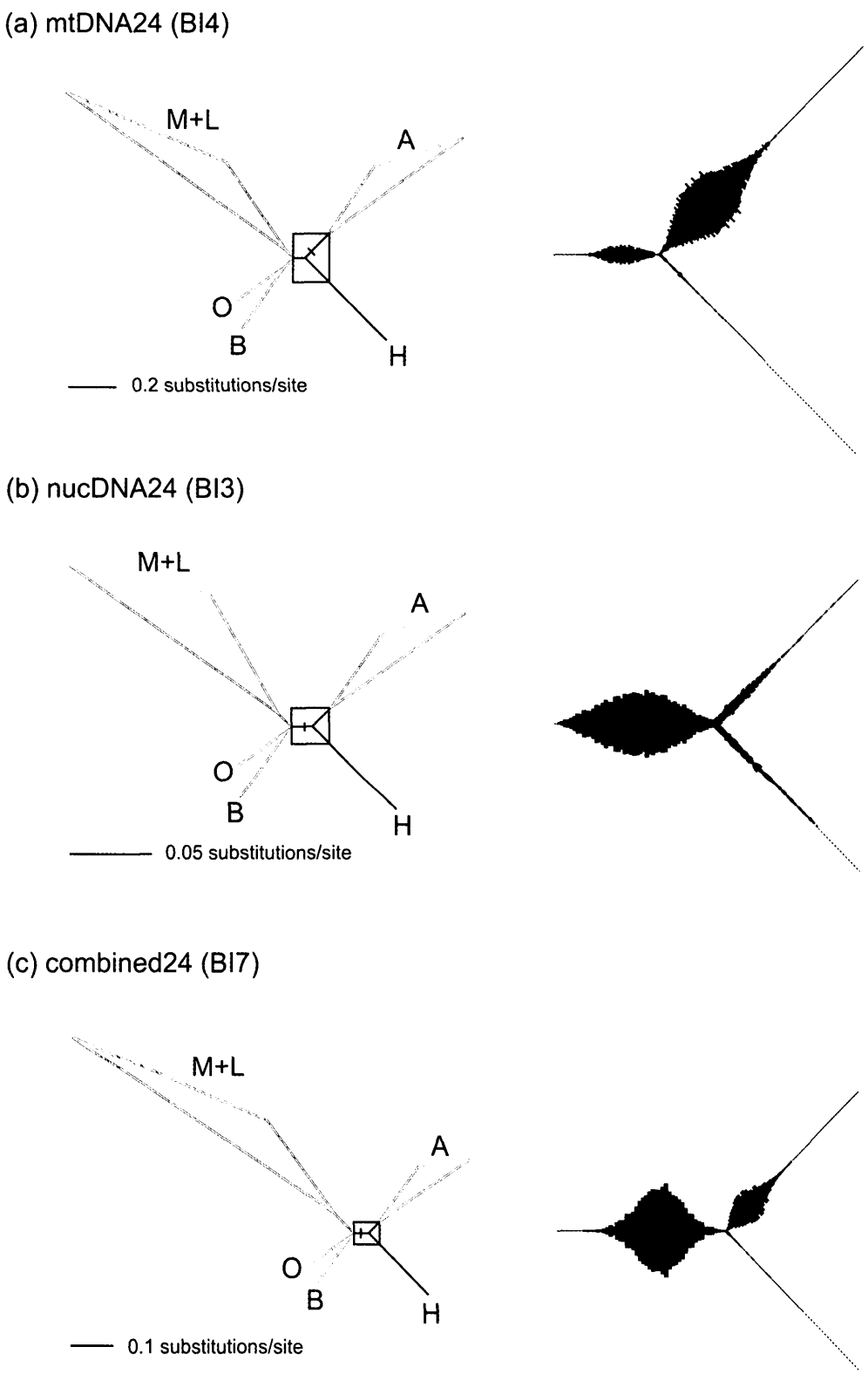


FIGURE 4. Posterior probabilities for rooting the anabantoid trees. The black box shows the area of enlargement shown on the right of each unrooted phylogram (a) mtDNA24 (BI4; interval length 0.001); (b) nucDNA24 (BI3; interval length 0.00024); (c) combined24 (BI7; interval length 0.00065); based upon 9000 MCMCMC sampled trees. The width of the branch is proportional to the posterior probability that the root is at that point. The lengths of the branches are the mean of the posterior density. (a) 14.89% (branch connecting A + H with the remaining annabantoids), 84.17% (branch leading to A), 0.94% (branch leading to H); total 100% for the three branches; (b) 77.16%, 9.25%, 8.17%; total 94.58% for the three branches; (c) 79.08%, 20.67%, 0.258%; total 100% for the three branches. For the RAG1 data set 5.42% alternative rooting positions were located on basal osphronemid branches (not shown). M + L = Macropodusinae plus Luciocephalinae; A = Anabantidae; H = Helostomatidae; B = *Belontia*; and O = *Osphronemus*.

internal branches connecting the three families and by the wide posterior probability distributions of the root location (Fig. 4).

### *Ancestral Character State Reconstruction*

The constrained mtDNA60 topology (see Material and Methods), shown in Figure 5, was used to perform character-state reconstructions for the evolution of the six reproductive modes found in anabantoids and the outgroup (see Table 1) under MP and ML. Unweighted parsimony reconstruction of the evolution of reproductive modes indicates that nine transitions (excluding the outgroup) of breeding behavior have occurred within anabantoids with either bubble nesting or free spawning as the plesiomorphic condition (Fig. 5a). An alternative topology recovered in some of the analyses with *Helostoma* as the sister group to the osphronemids (Figs. 2 and 3) resolves free spawning as the plesiomorphic condition for anabantoids (not shown). Our results indicate that both bubble nesting and mouthbrooding have evolved recurrently within anabantoids.

Character state reconstruction along some branches of the "spiral egg" clade (branches 7, 8, and 9 in Fig. 5a) was ambiguous. Delayed transformation (DELTRAN) of these equivocal reconstructions resolved these branches as bubble nesting (Fig. 5a), whereas accelerated transformation (ACCTRAN) resolved them as mouthbrooding. Using a sensitivity analysis for the parsimony character state reconstruction in the Luciocephalinae and Macropodusinae (not taking into account changes within the genus *Betta*), we found that a transition cost (t) from bubble nesting to mouthbrooding of $t > 1$ changed branches 7 and 8 (see Fig. 5a) to mouthbrooding, of $t > 2$ changed branch 1 to mouthbrooding, and of $t > 3$ changed branches 2 to 5 to mouthbrooding. A value of $t < 1$ changed branches 7 and 8 to bubble nesting, and a $t < 0.66$ changed branch 6 to bubble nesting (see Fig. 5a for details).

The distribution of character state changes with 1000 randomized trees resulted in an average (± SD) of 23.9 (±1.3) transitions in breeding behavior with a minimum of 18 transitions. The observed number of transitions (including the outgroup) in breeding behavior was ten, which is significantly smaller ($P < 0.001$) than the obtained null distribution. This suggests that transition rates are relatively low, and that the evolution of breeding behavior in anabantoids is highly correlated with phylogeny.

We estimated ancestral character states under ML incorporating branch length information. The full-unrestricted ML model with 30 transition parameters did not offer a significantly better fit compared to a restricted model with 15 transition parameters (full model $-\ln L = 28.5524$; restricted model $-\ln L = 37.7022$; $\chi^2_{15} = 18.3594$; $P = 0.2442$; no branch scaling [$\kappa = 1$]). All subsequent analyses were therefore conducted under the restricted model. ML reconstruction analyses did not reject a punctuated model of brood care evolution in the labyrinth fishes ($\kappa = 0$, $-\ln L = 39.4594$; $\kappa = 0.83$ (=ML), $-\ln L = 37.5784$; $\chi^2_1 = 3.7619$; $P = 0.0524$), indicating that the branch length contained in our ML tree might not be informative about trait evolution. The results of the ancestral character state reconstruction with the branch length scaling set to its ML value ($\kappa = 0.83$) are shown in Figure 5b. The obtained transition rates were: $q_{01} = q_{10} = 0.56$; $q_{02} = q_{20} = 0.22$; $q_{03} = q_{30} = 0.00$; $q_{04} = q_{40} = 0.14$; $q_{05} = q_{50} = 0.00$; $q_{12} = q_{21} = 0.00$; $q_{13} = q_{31} = 0.00$; $q_{14} = q_{41} = 0.00$; $q_{15} = q_{51} = 0.00$;$q_{23} = q_{32} = 0.48$; $q_{24} = q_{42} = 0.00$; $q_{25} = q_{52} = 0.41$; $q_{34} = q_{43} = 0.00$; $q_{35} = q_{53} = 0.00$; $q_{45} = q_{54} = 0.00$ (see Table 1 for description of states).

Four out of the five forms of breeding behavior in anabantoids are associated with parental care (Table 1). The outgroup (Channidae) also exhibits parental care, and unweighted parsimony reconstruction onto the topology shown in Figure 5a resolved parental care as the plesiomorphic condition for anabantoids. According to this reconstruction, parental care was lost at the base of the clade including the helostomatids and anabantids, and regained twice in *Sandelia capensis* and the genus *Microctenopoma*, respectively (not shown). However, a sensitivity analysis showed that a transition cost of $t = 0.5$ from no parental care to parental care resolved the root and the ancestral condition of ananbantoids as equivocal. A transition cost of $t < 0.5$ resulted in no parental care as the plesiomorphic condition for those two nodes, and further implies the independent evolution of parental care in the outgroup, *Microctenopoma*, *Sandelia*, and the osphronemids. Considering the alternative topology, placing *Helostoma* as the sister group to the osphronemids results in an equivocal character state reconstruction for the MRCA of anabantoids (not shown).

In contrast to the unweighted parsimony reconstruction, ML ancestral character state reconstruction onto the phylogeny shown in Figure 5b significantly supported no parental care (state 0) over parental care (state 1) as the plesiomorphic condition for anabantoids (full model $-\ln L = 9.0985$; branch scaling [$\kappa = \text{ML} = 0.78$]; support 15.84:1; significant) with three transitions from no parental care to parental care. The inferred transition rates were: $q01 = 1.84$ and $q10 = 0.00$, and are compatible with the results from the MP sensitivity analyses, indicating that a lower transition cost from no parental care to parental care than vice versa is a reasonable assumption for the evolutionary transitions between character states. Inferred support values for selected nodes are osphronemids (parental care 6.83:1; not significant), Luciocephalinae + Macropodusinae (parental care 25.99:1; significant), *Sandelia* + *Ctenopoma multispine* clade (no parental care 277.40:1; significant), *Ctenopoma petherici* clade (without *C. muriei*) + *Microctenopoma* (no parental care 3153.57:1; significant), and *Microctenopoma* (parental care 17.11:1; significant).

### *Rates of Evolution and Bayesian Relative Rate Test*

Likelihood-ratio tests with and without the molecular clock enforced clearly rejected overall constancy of rates of evolution in the anabantoids for all data sets (Table 2). Considerable variation in rates of molecular evolution

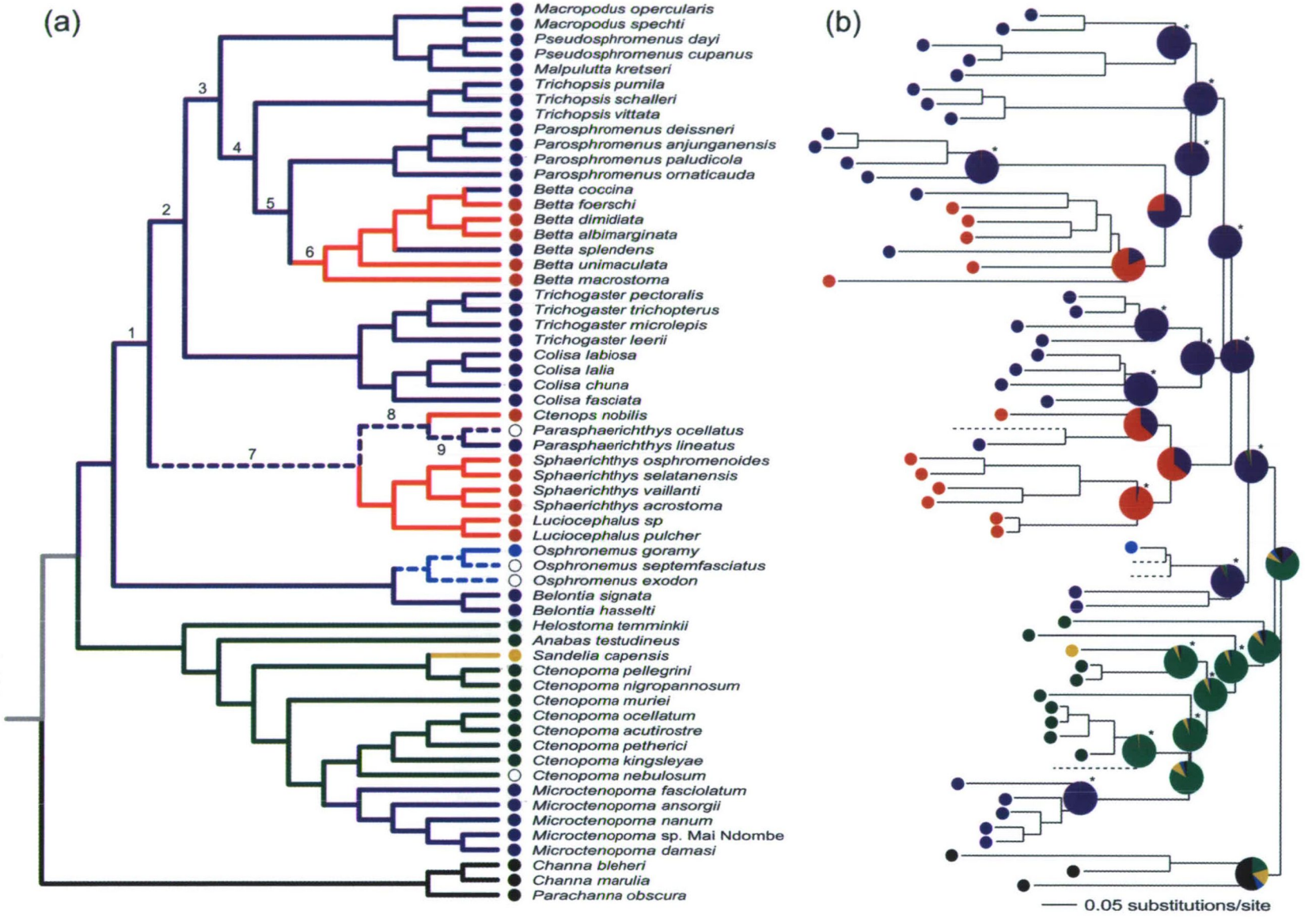


FIGURE 5. Evolution of the form of parental care in anabantoids. (a) Parsimony character state reconstruction on the constraint ML topology (see text for description of constraint). Results of the sensitivity analyses for the Osphronemidae: transition cost from bubble nesting to mouthbrooding: branches 1 + 2: t > 2.0 = red; branches 8 + 9: t > 1.0 = red; branches 3, 4, 5 + 6: t > 3.0 = red; branches 8, 9 + 10: t < 1.0 = blue; branches 7: t < 0.66 = blue. Blue = bubble nesting; cyan = submerged plant nest building; red= mouthbrooding; green = free spawning; yellow = substrate spawning; unfilled = unknown; black = floating egg gaurders (outgroup); grey = equivocal reconstruction. (b) Maximum likelihood character state reconstruction on the constraint ML tree performed using Multistate v0.8 (Pagel, 2003). Dashed lines indicate taxa that were excluded from the analyses due to missing information on parental care form. The differently colored areas in the pies represent the relative support for the four reconstructions. Asterisks indicate significantly supported reconstructions.

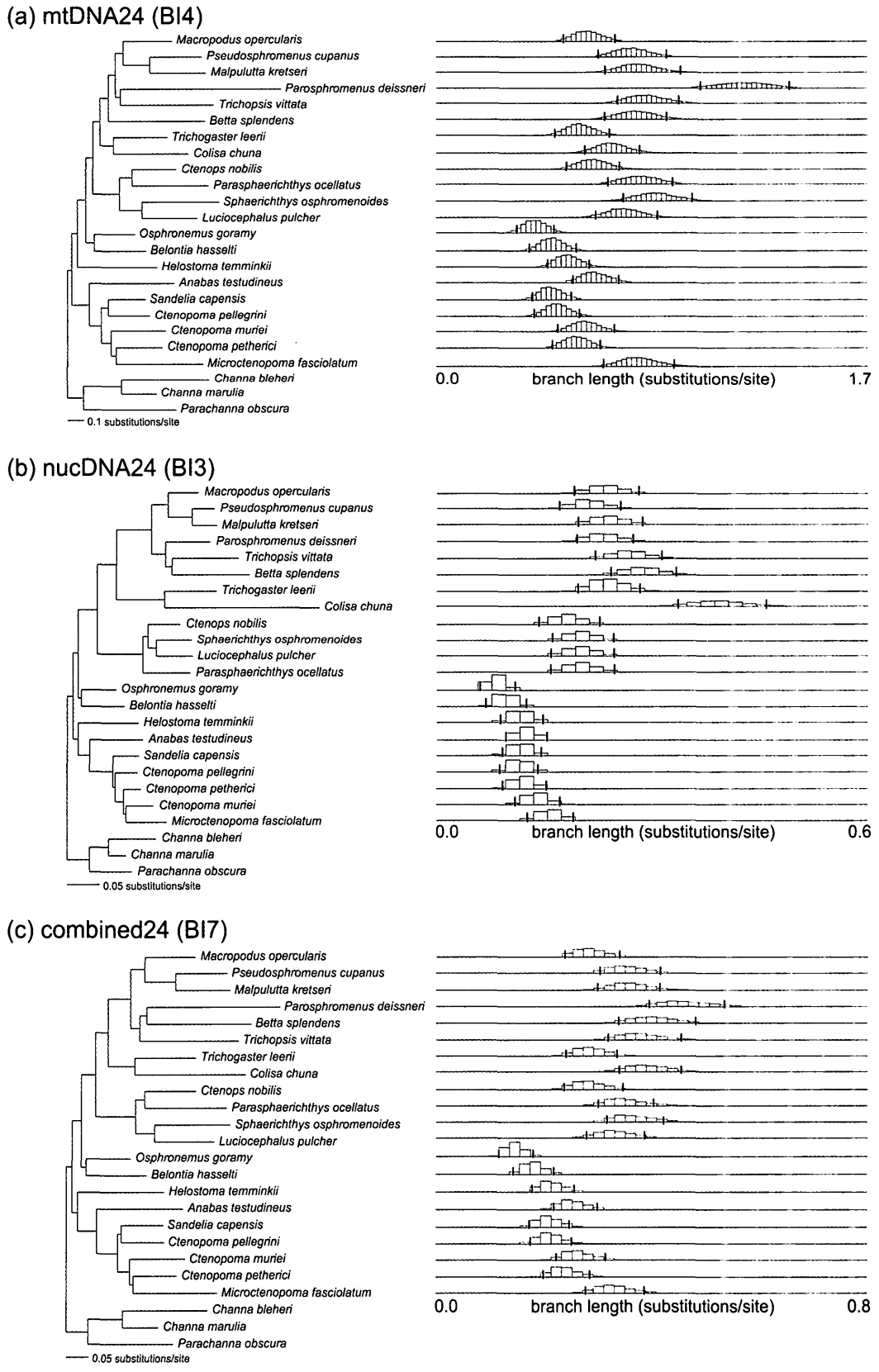


FIGURE 6. Bayesian consensus phylograms and Bayesian relative rates test based upon the (a) mtDNA24 (BI4), (b) nuc24 (BI3), (c) combined24 (BI7) analyses. Branch lengths from the most recent common ancestor (MRCA) to all tips were compiled with Cadence v1.0 based on 9000 MCMCMC sampled trees. The x-axis is shown as branch length in substitution per site and the y-axis as relative frequency. The tick marks indicate the 95% confidence intervals. Interval size for all is 0.02 substitutions per site.

among different anabantoid lineages were also indicated by the differences in branch length in the BI consensus phylograms of the mtDNA24 (BI4), nucDNA24 (BI3), and combined24 (BI7) data sets (Fig. 6). Using a Bayesian relative rate test we further examined the relative rates of molecular evolution among anabantoids. For all three data sets a general trend is apparent; the subfamilies Macropodusinae and Luciocephalinae tend to exhibit the longest branches, the Belontiinae and Osphroneminae the shortest, and the Ananabtidae intermediate branch length (Fig. 6). Several of the branch length 95% Bayesian confidence intervals did not overlap indicating significant differences in rates of molecular evolution among anabantoids in all data partitions (Fig. 6).

### *Anabantoid Divergence Time Estimates*

Both the LRT and the BRR tests rejected the null hypothesis that the mtDNA, nucDNA, and combined nucleotide sequence data were evolving with rate constancy across the anabantoids. Therefore, the use of nonclocklike methods such as KTB to estimate divergence times is justified.

First, we used the KTB method based on the mtDNA60 data set (under the topological constraints described in Material and Methods), using two data partitions (cyt*b* versus 12S rRNA–tRNA Val–16S rRNA), to estimate anabantoid divergence times. Because the rank correlation test for these two partitions rejected the null hypothesis that the two genes change rates independently ($R = 0.745$, $P < 0.000$), analyses of the mtDNA60 data set were performed using only one partition. Age estimates and their 95% confidence intervals for selected nodes for the mtDNA60– stem L, stem LU, crown L, and crown LU analyses are shown in Table 4, and the resulting chronogram and the 95% confidence intervals for selected nodes of the mtDNA60 stem LU analysis is shown in Figure 7. Depending on the analyses, the anabantoid root was located at 103.44, 91.027, 69.88, or 34.66 Mya, whereas the divergence of African and Asian groups was estimated at 87.30, 77.00, 58.91, or 26.61 Mya (Table 4).

Second, we used the combined24 data set (under the topological constraints described in Material and Methods) using two data partitions (cyt*b* versus 12S rRNA–tRNA Val–16S rRNA) to estimate anabantoid divergence times. A preliminary rank correlation analysis of the combined24 data set using three partitions (cyt*b*, 12s rRNA–tRNA Val–16S rRNA, RAG1) also indicated that the cyt*b* and RNA partition do not change rates independently ($R = 0.808$, $P = 0.002$). Final analyses were therefore performed using two partitions only (mtDNA and RAG1; rank correlation $R = 0.445$, $P = 0.158$). Root age and the African-Asian divergence were estimated at 63.43 and 52.09 Mya, respectively, for the combined24 stem L analysis, and at 37.70 and 30.83 Mya, respectively, for the combined24 stem LU analysis (Table 4).

Changing "bigtime" from 150 to 300 Mya had a negligible effect on divergence time estimates (and their 95% confidence intervals) for the mtDNA60 stem LU and mtDNA60 crown LU, and for the combined24 stem L and LU analyses (data not shown). In contrast, it considerably affected the outcomes of the mtDNA60 stem L and mtDNA60 crown L analyses. The obtained root ages were 77.13 Mya (95%: 36.69–192.00) and 120.10 Mya (95%: 68.58–231.94), respectively, whereas the African-Asian divergence was estimated at 65.09 Mya (95%: 30.40–160.44) and 101.45 Mya (95%: 57.32–196.97; see Table 4 for comparison with the results obtained under "bigtime" set to 150 Mya). These results suggest that with a single calibration point, molecular divergence time estimates employing a lower bound constraint only might be largely affected upon different "bigtime" settings.

## DISCUSSION

### *Anabantoid Phylogenetics*

*Phylogenetic position of* Luciocephalus *revisited.*—*Luciocephalus* is a highly distinctive and morphologically derived piscivorous predator with an unusual pikelike body shape, and extremely protrusible upper jaws (Lauder and Liem, 1981; Liem, 1967). The numerous autapomorphic features of *Luciocephalus* obviously hampered previous attempts to resolve its phylogenetic position. Mainly three hypotheses have been put forth to date to explain its phylogenetic position: (1) *Luciocephalus* is not closely related to anabantoids (Berg, 1958; Greenwood et al., 1966; Liem, 1963, 1967); (2) it is a close relative of the anabantoids (Bleeker, 1859, 1879; Gosline, 1968; Jordan, 1923; Weber and De Beaufort, 1922) or considered the sister group of the remaining anabantoids (Lauder and Liem, 1983); and (3) it is deeply rooted inside the derived anabantoid family Osphronemidae, forming the sister group of the chocolate gouramies, genus *Sphaerichthys* (Britz, 1994, 1995, 2001; Britz et al., 1995).

One of the most unusual characters that *Luciocephalus* shares with the osphronemid genera *Parasphaerichthys*, *Ctenops*, and *Sphaerichthys* is the presence of spiraling ridges and intermittent grooves on the egg surface that lead to the micropyle. Riehl and Kokoscha (1993) interpreted this complex pattern as a sperm-guiding system. Britz (1995) and Britz et al. (1995) proposed that this unique egg surface structure represents a synapomorphy of the four genera that defines the so-called "spiral egg" clade. Our phylogenetic analyses strongly supports the monophyly of the "spiral egg" clade, and the position of *Luciocephalus* as the sister group of *Sphaerichthys*.

*Osphronemidae intrarelationships.*—At present the family is divided into four subfamilies: Osphroneminae, Belontiinae, Luciocephalinae, and Macropodusinae (Table 1). Britz (1995) hypothesized the Belontiinae as the sister group of the remaining osphronemids (Fig. 1). In our analyses, *Belontia* was recovered as sister group of *Osphronemus* with moderately high statistical support based on the mtDNA60, mtDNA24 and combined24 data sets, respectively (Figs. 2 and 3). Phylogenetic analyses based on the nucDNA24 data set alone fail to recover basal osphronemid relationships, likely because of the slow rate of evolution of RAG1. Hence, morphological and molecular evidence yield contrasting hypotheses,

TABLE 4. Molecular divergence time estimates in Mya (with 95% confidence interval) of selected nodes based upon the KTP method. Estimates are derived from the mtDNA60 (crown- and stem-group calibration) and the combined24 (stem-group calibration) data sets. Node numbers refer to those given in Figure 7. The anabantoid root (node 1) and the node corresponding to the African-Asian divergence (node 16) are highlighted and the node used for the calibration (stem group calibration only) is underlined. L = lower and U = upper bound constraint; big time = 150 Mya.

| | (A) mtDNA60 crown-group L = 28.5 | | (B) mtDNA60 crown-group L = 28.5; U = 37.0 | | (C) mtDNA60 stem-group L = 28.5 | | (D) mtDNA60 stem-group L = 28.5; U = 37.0 | | (E) combined24 stem-group L = 28.5 | | (F) combined24 stem-group L = 28.5; U = 37.0 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Node | Mean | 95% interval | Mean | 95% interval | Mean | 95% interval | Mean | 95% interval | Mean | 95% interval | Mean | 95% interval |
| 1 | 103.44 | 67.75–145.37 | 91.27 | 64.01–126.92 | 69.88 | 36.80–136.55 | 41.66 | 34.66–49.62 | 63.43 | 33.85–130.15 | 37.70 | 32.15–43.70 |
| 2 | 91.85 | 60.76–129.95 | 80.72 | 57.71–110.67 | 61.77 | 32.7562–121.04 | 36.73 | 31.21–42.85 | 58.72 | 31.34–120.50 | 34.84 | 30.11–39.82 |
| 3 | 85.54 | 55.93–121.90 | 75.19 | 52.98–104.59 | 57.53 | 30.17–113.14 | 34.21 | 28.41–40.62 | 51.24 | 27.00–106.28 | 30.30 | 25.54–35.46 |
| 4 | 82.39 | 53.63–117.62 | 72.37 | 50.85–100.17 | 55.38 | 28.97–108.95 | 32.92 | 27.22–39.24 | 47.11 | 24.59–97.60 | 27.77 | 23.02–32.73 |
| 5 | 72.14 | 46.85–103.82 | 63.38 | 44.19–88.24 | 48.46 | 25.10–95.49 | 28.82 | 23.41–34.85 | 38.69 | 19.67–80.95 | 22.66 | 18.00–27.53 |
| 6 | 65.54 | 42.22–94.96 | 57.54 | 39.92–80.46 | 43.99 | 22.62–87.31 | 26.15 | 20.88–32.17 | 33.02 | 16.40–68.76 | 19.30 | 14.79–24.02 |
| 7 | 43.51 | 26.60–65.96 | 38.17 | 25.15–55.76 | 29.17 | 14.18–59.17 | 17.32 | 12.83–22.62 | 20.32 | 9.48–43.71 | 11.84 | 8.32–15.73 |
| 8 | 70.13 | 45.38–101.04 | 61.62 | 42.88–86.20 | 47.11 | 24.29–93.03 | 28.01 | 22.62–33.97 | 36.66 | 18.46–76.60 | 21.48 | 16.88–26.33 |
| 9 | 62.18 | 39.72–90.70 | 54.59 | 37.61–76.99 | 41.75 | 21.36–82.63 | 24.82 | 19.71–30.43 | 33.94 | 16.90–71.24 | 19.83 | 15.30–24.76 |
| 10 | 72.14 | 46.55–104.06 | 63.31 | 44.15–88.35 | 48.45 | 25.05–96.22 | 28.78 | 23.32–34.86 | 37.67 | 18.99–78.73 | 22.12 | 17.36–27.07 |
| 11 | 62.81 | 40.05–91.18 | 55.07 | 37.99–77.55 | 42.17 | 21.54–83.16 | 25.03 | 19.85–30.85 | 34.59 | 17.32–72.31 | 20.22 | 15.83–24.95 |
| 12 | 52.03 | 32.31–77.50 | 45.59 | 30.54–65.24 | 34.92 | 17.51–70.28 | 20.74 | 15.75–26.34 | 29.91 | 14.65–63.06 | 17.45 | 13.13–22.24 |
| 13 | 52.39 | 32.78–78.12 | 45.88 | 30.79–65.85 | 35.18 | 17.54–70.53 | 20.87 | 15.93–26.51 | 28.55 | 13.97–60.07 | 16.64 | 12.44–21.17 |
| 14 | 82.45 | 55.40–117.39 | 72.14 | 52.46–97.85 | 55.25 | 29.39–109.31 | 32.68 | 28.70–36.75 | 55.34 | 29.48–113.92 | 32.73 | 28.71–36.78 |
| 15 | 98.63 | 64.41–139.29 | 87.01 | 60.95–122.00 | 66.61 | 34.80–130.19 | 39.70 | 32.60–47.81 | 60.99 | 32.34–125.33 | 36.22 | 30.57–42.31 |
| 16 | 87.30 | 56.43–124.22 | 77.00 | 53.24–109.35 | 58.91 | 30.34–115.28 | 35.09 | 28.05–42.94 | 52.09 | 27.13–106.59 | 30.83 | 25.31–36.96 |
| 17 | 74.07 | 47.43–106.93 | 65.35 | 44.73–93.18 | 49.98 | 25.3320–98.41 | 29.74 | 23.27–37.06 | 40.29 | 20.42–83.57 | 23.79 | 18.62–29.45 |
| 18 | 60.37 | 37.44–89.68 | 53.14 | 35.18–77.45 | 40.72 | 20.01–80.63 | 24.18 | 18.01–31.25 | 33.77 | 16.60–70.43 | 19.88 | 14.78–25.38 |
| 19 | 67.64 | 42.93–98.44 | 59.66 | 40.58–85.88 | 45.64 | 23.06–90.18 | 27.15 | 20.90–34.32 | 34.75 | 17.19–72.66 | 20.48 | 15.52–25.95 |
| 20 | 66.31 | 41.85–96.91 | 58.48 | 39.63–84.49 | 44.73 | 22.50–88.23 | 26.61 | 20.42–33.66 | 32.24 | 15.78–67.35 | 19.00 | 14.14–24.43 |

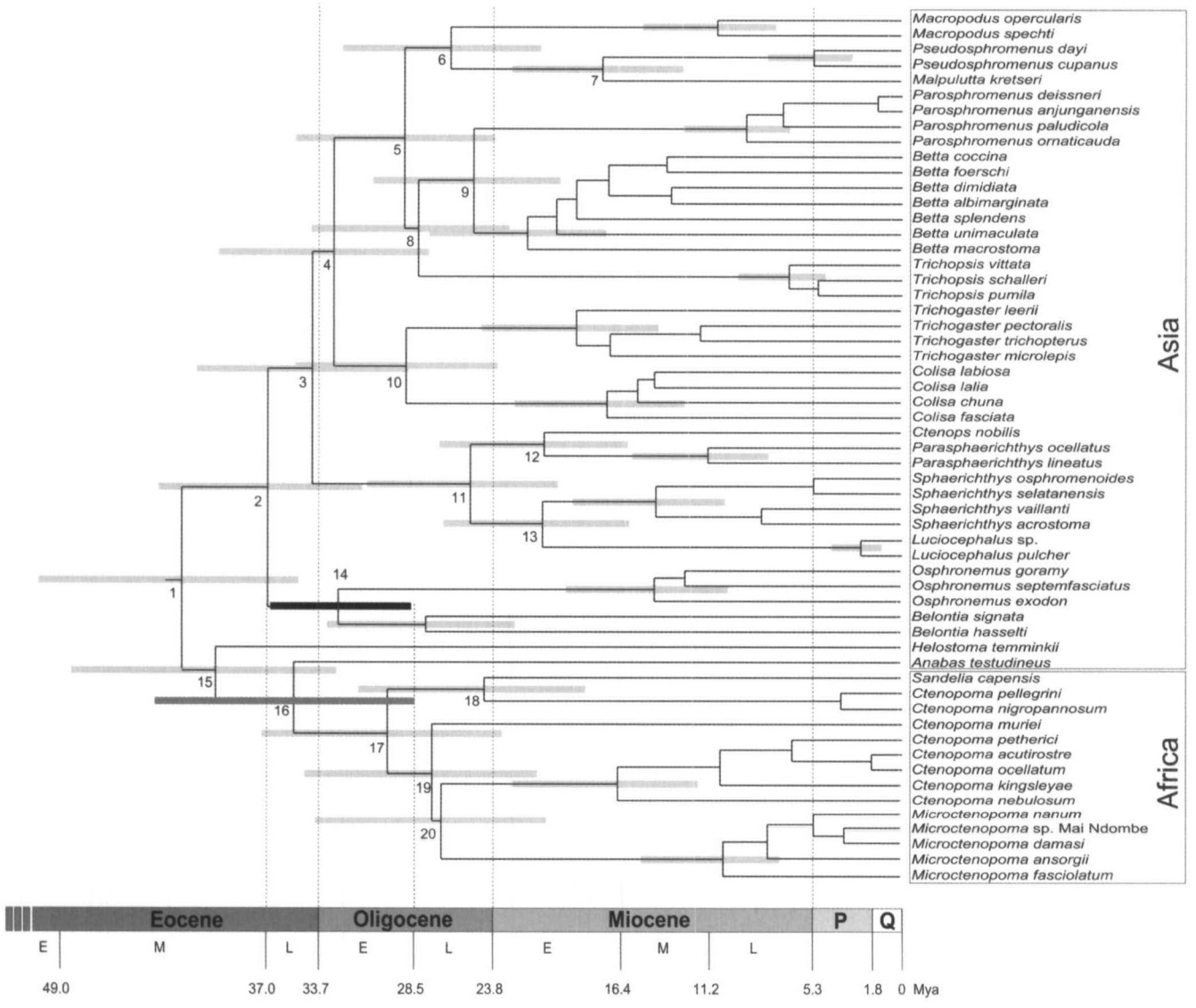


FIGURE 7. Anabantoid chronogram based on the KTB analysis of the mtDNA60 data set (stem group calibration; LU constraint). 95% confidence intervals for selected branches are shown in light grey. Black bar represents the calibration interval (stem-group calibration; Late Eocene to Early Oligocene) and the dark grey bar represents the time interval for the African-Asian divergence.

and the question on the relative phylogenetic position of Osphroneminae and Belontiinae remains open. Neither morphological nor molecular evidence is conclusive. The former was based on a single synapomorphy: the second external levator muscle of the dorsal gill arches is modified to a thin but extensive muscle layer entirely covering the posterior part of the suprabranchial chamber in all osphronemids except *Belontia*, which shows an unmodified muscle, the plesiomorphic condition shared with *Helostoma* and anabantids (Britz, 1995), and the latter is based on relatively short internodes.

According to Britz (1995; 2001), Luciocephalinae comprise the "spiral egg" clade plus *Trichogaster* + *Colisa* (Fig. 1) This is based on the following synapomorphies: (1) loss of the first branchiostegal ray; (2) presence of a median posterior process of the basioccipital that extends beneath the first vertebra and to which Baudelot's ligament is attached; this character is unique among anabantoids. In contrast, in our analyses *Trichogaster* and *Colisa* were consistently placed as the sister group of the Macropodusinae. Moreover, the morphology-based hypothesis was rejected by the KH test based on the nucDNA24 and combined24 data sets.

Among Macropodusinae, *Macropodus* is unique in showing the following hypothesized plesiomorphic character states: the presence of an oil globule in the egg and oil vesicles in the early larvae, the absence of wartlike larval attachment cells on the head, and the absence of the wrinkled egg surface. The other genera, *Pseudosphromenus*, *Malpulutta*, *Trichopsis*, *Parosphromenus*, and *Betta* have reduced the egg's oil globule and larval oil vesicles, and possess wartlike

attachment cells and a wrinkled egg surface (Britz, 2001; Britz and Cambray, 2001; Gilch, 1957; Vierke, 1975, 1991). Therefore, the genus *Macropodus* has commonly been considered the sister taxon of all other Macropodusinae (Britz, 1995, 2001; Vierke, 1975). In contrast, our phylogenetic analyses consistently recovered a monophyletic clade comprising *Macropodus* as the sister group of *Pseudosphromenus* + *Malpulutta*. Moreover, KH and SH tests based on nuclear data rejected the morphological hypothesis. Phylogenetic relationships among the four main lineages of Macropodusinae, i.e., *Macropodus* + *Pseudosphromenus* + *Malpulutta*, *Parosphromenus*, *Betta*, and *Trichopsis* could not be resolved based on mitochondrial evidence (mtDNA60 and mtDNA24; Figs. 2 and 3). However, a sister group relationship of *Parosphromenus*, with *Betta*, or *Trichopsis* was suggested based on the nucDNA24 and combined24 data sets (Figs. 2 and 3; see also Rüber et al. [2004b]).

*Anabantidae intrarelationships.*—On the basis of a number of morphological characters, Anabantidae have been divided into five clades: the genera *Anabas*, *Sandelia*, *Microctenopoma*, and the *Ctenopoma petherici* and *C. multispine* species groups (Table 1; Elsen, 1976; Norris, 1994, 1995; Peters, 1976). The only morphology-based phylogeny available for the family could not resolve phylogenetic relationships among the five clades (Norris, 1994: 396, fig. 210). Our phylogenetic analyses consistently recovered the Asian *Anabas* as sister group to the African anabantids. According to our results, the genus *Ctenopoma* (*multispine* and *petherici* species groups) is not monophyletic. The *C. multispine* species group was generally identified as the sister group of *Sandelia* (except in the nucDNA24-based phylogenies where *Sandelia* was consistently recovered as the sister group to all remaining African anabantids). The monophyly of *Sandelia*, a genus that is restricted to the Cape region of South Africa, remains a matter of debate because the two species (*S. capensis* and the highly endangered *S. bainsii*) of the genus differ significantly in several characters (Cambray, 1990, 1997, 2004). The monophyly of *Ctenopoma* is also challenged by the relative position of *C. muriei*. This species belongs to the *C. petherici* species group (Table 1), but in our phylogenetic analyses is recovered either as the sister group of *Microctenopoma* or as sister group of *Microctenopoma* and the remaining members of the *C. petherici* species group, although with low statistical support (Figs. 2 and 3). Elsen (1976) classified *C. muriei* along with the *Microctenopoma* clade, whereas Norris and Douglas (1992) thought that its affinities lie with the *C. petherici* species group.

*Phylogenetic position of* Helostoma *and the root of the anabantoid tree.*—The Southeast Asian kissing gouramy, *Helostoma temminkii*, is a highly specialized microphagous filter-feeder (Böker, 1937; Meyer, 1904; Monod, 1949; Seitz, 1937) and widely known for its kissing behavior during antagonistic interactions between males. Based on morphological evidence, Lauder and Liem (1983) considered *Helostoma* to be the sister group of the family Anabantidae. Our phylogenetic analyses were unable to resolve the relative position of the Helostomatidae with respect to the other two anabantoid families due to the wide posterior probability distributions of the root locations and short internal branches connecting the Osphronemidae, Anabantidae, and Helostomatidae in the mtDNA24, nucDNA24, and combined24 data sets (Fig. 4). Nevertheless, our combined24 analyses (BI7, BI4, ME) tentatively indicate an osphronemid sistergroup relationship to helostomatids plus anabantids as the currently best working hypotheses. Clearly, further morphology- and molecular-based studies are needed to rigorously test basal anabantoid intrarelationships.

### *Evolution of Parental Care and Parental Care Forms*

Only the representatives of the three genera *Helostoma*, *Anabas*, and *Ctenopoma* do not exhibit parental care (Table 1). Tracing the evolution of parental care using unweighted parsimony onto our molecular phylogeny in Figure 5 revealed that parental care was the plesiomorphic condition in anabantoids and that parental care was lost once along the branch leading to the clade including the helostomatids and anabantids and regained twice in *Sandelia capensis* and the genus *Microctenopoma* (not shown). We are not aware of any reported case indicating the loss of parental care in fishes, although evolutionary transitions from no parental care to parental and from one form of parental care to another are rather common (Blumer, 1982; Goodwin et al., 1998; Rüber et al., 2004b). Therefore, it seems more reasonable to assume that no parental care was the plesiomorphic condition in anabantoids and that parental care evolved three times independently (Osphronemidae, *Sandelia*, and *Microctenopoma* as well as once in the outgroup Channidae). This scenario is actually favored by the results of our MP sensitivity analysis and ML resolving nonparental care as the plesiomorphic condition in anabantoids. It should be noted that the alternative placement of *Helostoma* as the sistergroup of osphronemids changes the unweighted parsimony ancestral character state reconstruction for the MRCA from "parental care present" to "ambiguous."

The ancestral condition of the form of breeding behavior is equivocal under parsimony character state reconstruction using the tree shown in Figure 5. However, nonparental care, as we argue above, is the likely ancestral condition and therefore we consider free spawning to be the plesiomorphic condition for anabantoids, which is also indicated by our ML ancestral character state reconstruction (although not significant). Free spawning is also obtained as the plesiomorphic character state for anabantoids using the alternative topology with *Helostoma* as the sister group to osphronemids. In the phylogeny illustrated in Figure 5, one transition from free spawning to substrate spawning was observed (*Sandelia*). Bubble nesting seems to have evolved at least twice independently (not considering the genus *Betta*) within anabantoids, at the base of *Microctenopoma* and osphronemids, respectively. The fighting fish genus *Betta* consists of both bubble nesters and mouthbrooders and our analyses indicate mouthbrooding as the plesiomorphic condition (Fig. 5), a result also obtained by

Rüber et al. (2004b). However, based on an extensive analysis of the evolution of parental care form, Rüber et al. (2004b) favored an evolutionary scenario implying recurrent origin of mouthbrooding in this genus and hence bubble nesting as the plesiomorphic condition. This conclusion was based upon an MP sensitivity analysis and ML ancestral character state reconstruction as well as considering differences in phenotypic and behavioral traits between bubble nesters and mouthbrooders, thus accounting for a hypothesized transformation bias from mouthbrooders to bubble nesters and *vice versa* (see Rüber et al., 2004b, for more details).

The spiral egg clade (*Ctenops*, *Parasphaerichthys*, *Sphaerichthys*, and *Luciocephalus*) also includes several mouthbrooding taxa. *Luciocephalus* is a highly specialized mouthbrooder, with a brooding period of up to 4 weeks (Britz, 1994), during which the brooding male does not feed. Although recent morphological data strongly support the monophyly of the "spiral egg clade" (Britz, 1995; Britz et al., 1995), these authors were unable to resolve the phylogenetic position of the genus *Parasphaerichthys* among the four genera. Our data set is also ambiguous in the placement of the genus *Parasphaerichthys* within the "spiral egg" clade. It was either recovered as sister group to *Ctenops* (mtDNA60, mtDNA24, and combined24 analyses) or as the sister group of the remaining three genera (nucDNA24 analyses). Both KH and SH were unable to reject alternative placements of *Parasphaerichthys* (Table 3). At least one of the two species in the genus, *P. lineatus*, differs from the other three taxa, which are all mouthbrooders, in exhibiting the more plesiomorphic bubble-nesting behavior (Britz and Kottelat, 2002; Freyhof, 2002); the reproductive behavior of *P. ocellatus* still remains unknown. A sister-group relationship of *Parasphaerichthys* with the other three genera would therefore be in better congruence with its more plesiomorphic reproductive mode.

### *Differences in Rates of Molecular Evolution*

We found significant differences in branch length among labyrinth fishes. For all gene fragments analyzed, the subfamilies Macropodusinae and Luciocephalinae tended to exhibit longer branches than the Belontiinae, the Osphroneminae, and the Anabantidae (Fig. 6). Although variation in rates of nucleotide substitution have been correlated with a variety of factors (e.g., body size, DNA repair efficiency, environmental temperature), metabolic rate or generation time are generally thought to be the prime factors affecting the speed of molecular evolution (e.g., Gillooly et al., 2005; Martin and Palumbi, 1993; Rand, 1994). However, distinguishing between these two hypotheses has been difficult, because of the presumed covariance between generation time, metabolic rate, body size, and temperature (see references in Gillooly et al. [2005]). Recently, Gillooly et al. (2005) presented a model that is able to explain heterogeneity in rates of nucleotide substitution in different genes and taxa, by accounting for the effects of body size and temperature on metabolic rate. Although mean annual ambient temperatures are not available for all taxa studied here, labyrinth fishes with their marked differences in rates of molecular evolution will certainly be an ideal group to test predictions from the Gillooly et al. (2005) model once these data become available.

### *The Fossil Record and the Origin of Labyrinthfishes*

The anabantoid fossil record that can be used for the calibration of the lineage divergence times is scarce. The only known articulated anabantoid fossil is *Osphronemus goramy* (Patterson, 1993a; Sanders, 1934). It was found in the Sangkarewang Formation (Central Sumatra), also known as the "mergel stage" (e.g., Musper, 1930). This formation is well known due to the rich freshwater fossils including osteoglossiforms (*Scleropages*, *Notopterus*, *Musperia*†), siluriforms (*Pangasius*), cypriniforms (*Rasbora*, *Thynnichthys*, *Puntius*), gasterosteiforms (*Protosyngnathus*†), and perciforms (*Osphronemus*, *Toxodes*). Since its discovery, the age of the Sangkarewang Formation has been a matter of debate. Previous age estimates for this formation range from the Late Cretaceous to the Miocene (see review in Sanders [1934], who considered an Early Eocene age as most acceptable). Probably due to this uncertainty of the age of the Sangkarewang Formation, Patterson (1993a), in his review on the teleost fossil record, stated that "the Eocene age of the fish shales at Pandang is still dubious" without giving any further references.

The Sangkarewang Formation is part of the Ombilin basin, a Tertiary sedimentary and structural basin located in the Pandang Highlands in Central Sumatra. Recent geological studies in the Ombilin basin have shown that tectonic activity in the early Tertiary resulted in the development of an intramontane basin that was transformed into a freshwater lake. Subsequently, increased sedimentation formed a flood plain of meandering rivers and in the Early Miocene the whole region subsided and a short period of marine transgression took place followed by tectonic uplifts resulting in the current geological picture. On the basis of palynological data for the Sangkarewang Formation, Humphreys et al. (1991) and Barber et al. (2005) give a Late Eocene to Early Oligocene (28.5 to 37.0 Mya) age (but see Koesoemadinata and Matasak, 1981, who give a Paleocene age).

At the time the fossil *Osphronemus* was described, only one living *Osphronemus* species was known (*O. goramy*), whereas currently four species are recognized. It is not possible to assign the fossil *Osphronemus* to any extant species with certainty, nor is it possible to assign it without doubt to the crown group *Osphronemus*. Therefore, we used two different age estimates based on different assignments of the fossil *Osphronemus* to either the crown or stem group. Due to the incomplete nature of the fossil record, fossil calibrations can only provide minimum ages and therefore will tend to underestimate lineage divergence times (Benton and Ayala, 2003). This implies that the timing of early anabantoid cladogenesis may be considerably older than the current fossil-based estimates indicate. However, it is important to note that the crown group calibration rendered

exceedingly old divergence time estimates for the Anabantoidei, a highly advanced perciform suborder. An Early Paleocene to Cretaceous origin of anabantoids would be clearly in disagreement with the known fossil record.

The skeletal fossil record indicates that acanthomorphs (highly derived teleosts including perciforms) do not appear until the Late Cretaceous (Cenomanian; ca. 99.0 to 93.5 Mya) and the first skeletal fossil record of a questionable perciform is from the Late Cretaceous (Late Campanian–Early Maastrichtian; ca. 74 Mya). No unquestionable perciform has yet been recorded by Cretaceous skeletal remains implying an age of 65 Mya or younger for this group. The higher acanthomorph fossil record is characterized by the explosive occurrence of several perciform lineages at the Paleocene-Eocene boundary (Patterson, 1993a, 1993b). A general lack of Early Cretaceous to Middle Paleocene freshwater fossils, presumably due the paucity of fossil bearing freshwater rocks (Grande and Cavender, 1991; Lundberg, 1998), as well as a marked gap in the acanthomorph fossil record between the Late Campanian (ca. 75 Mya) and the Late Paleocene (ca. 55 Mya; Patterson, 1993a, 1993b), however, may reflect our incomplete understanding on the origin and diversification of higher acanthomorphs. Although fish otoliths provide consistently older age estimates than those based on complete skeletons (Nolf, 1993; Patterson, 1993a, 1993b), their utility is hindered by uncertainty in taxonomic assignments (Patterson, 1993a, 1993b). Further studies employing molecular based divergence time estimates on different perciform groups that exhibit a more complete fossil record than anabantoids will clearly help to further our understanding on the origin of this speciose clade.

### *Anabantoid African-Asian Biogeography*

Many freshwater fish distribution patterns have previously been explained by continental drift vicariance associated with the breakup of Gondwana. This event took place in the Late Jurassic/Early Cretaceous with the separation of the Madagascan and Indian continent from Africa at 165 to 121 Mya, the separation of Africa and South America at 101 to 86 Mya BP, and the separation of Madagascar and India at 88 to 63 Mya BP (Pitman et al., 1993; Rabinowitz et al., 1983; Storey, 1995; Storey et al., 1995).

However, it has been noted on several occasions that traditional biogeographic hypotheses of an Early Cretaceous origin of freshwater fish clades are not compatible with the fossil record of percomorphs (Bellwood and Wainwright, 2002; Lundberg, 1993; Patterson, 1993a, 1993b), hence challenging the continental drift vicariance hypothesis. Due to these discrepancies over the origin of perciform clades, it has been proposed that Late Mesozoic–Tertiary hypotheses of dispersal may better explain the origin of most extant freshwater fish groups than a Jurassic/Cretaceous vicariance (Briggs, 2003a, 2003b). Alternative scenarios that have been put forth include (1) Late Mesozoic dispersal from Africa to Asia or *vice versa* via land bridges between Africa, India, and Eurasia. This model assumes a different longitudinal position for India during its Northeastern journey (Briggs, 2003a; Chatterjee and Scotese, 1999); (2) Dispersal from Africa to Asia or *vice versa* through the Middle East facilitated by the closure of the Tethys Sea in the Early Miocene (ca. 20 to 18 Mya; (Sanders and Miller, 2002); (3) Cenozoic trans-ocean dispersal (e.g., de Queiroz, 2005). Nonetheless, the relative role that drift vicariance and dispersal have played in shaping trans ocean biogeographical patterns remains controversial (e.g., Sparks and Smith, 2005; de Queiroz, 2005).

Anabantoids show a disjunctive Southeast Asian-African distribution that might be indicative of a restricted Gondwana distribution. With both the nuclear and combined data sets, a basal split of anabantoids into osphronemids versus helostomids and anabantids was favored (Fig. 4). The former two families are exclusively found in Southeast Asia, whereas the latter family is found on both continents, with *Anabas* (Anabantinae) from Southeast Asia as the sister group of the remaining anabantids (Ctenopominae) which occur in Africa. Divergence time estimates of an Asian-African separation ranged from 87.30 to 30.83 Mya depending on the analyses (see Table 4; not including the results obtained with "bigtime" = 300 Myr). Both stem-group and crown-group calibrations indicate an older divergence of African and Asian lineages than predicted by Tertiary dispersal hypothesis from Africa to Asia, or *vice versa*, through the Middle East, facilitated by the closure of the Tethys Sea in the Early Miocene. Stem group calibration places the biogeophraphic split within labyrinth fishes at the Eocene–Oligocene boundary, whereas the crown group calibration indicates a Early–Late Cretaceous to Paleocene divergence. The oldest divergence time estimate based upon the crown group calibration of 87.3 Mya (95%: 56.43–124.22 Mya) is close to the suggested divergence of the Madagascan and Indian continent from Africa at 165 to 121 Mya. On the other hand, Late Mesozoic dispersal from Africa to Asia or *vice versa* via land bridges between Africa, India, and Eurasia cannot be ruled out. Chatterjee and Scotese (1999) pointed out that the Late Mesozoic–Early Cenozoic history is still poorly known and thus allows for wide speculations regarding biotic exchanges via land bridges. Given the poor fossil record of anabantoids, as well as the uncertainty in assigning the fossil *Osphronemus* to either the stem or crown group, it seems premature to draw any firm conclusion regarding anabantoid African-Asian biogeography. Nevertheless, anabantoids are confined to freshwaters with generally low ion content. The ecological preferences of labyrinth fishes, likely a plesiomorphic condition, therefore render unlikely any dispersal through marine habitats.

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