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Simultaneous analysis of five molecular markers provides a well-supported phylogenetic hypothesis for the living bony-tongue fishes (Osteoglossomorpha: Teleostei)

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Abstract

Fishes of the Superorder Osteoglossomorpha (the "bonytongues") constitute a morphologically heterogeneous group of basal teleosts, including highly derived subgroups such as African electric fishes, the African butterfly fish, and Old World knifefishes. Lack of consensus among hypotheses of osteoglossomorph relationships advanced during the past 30 years may be due in part to the difficulty of identifying shared derived characters among the morphologically differentiated extant families of this group. In this study, we present a novel phylogenetic hypothesis for this group, based on the analysis of more than 4000 characters from five molecular markers (the mitochondrial cytochrome *b*, 12S and 16S rRNA genes, and the nuclear genes RAG2 and MLL). Our taxonomic sampling includes one representative of each extant non-mormyrid osteoglossomorph genus, one representative for the monophyletic family Mormyridae, and four outgroup taxa within the basal Teleostei. Maximum parsimony analysis of combined and equally weighted characters from the five molecular markers and Bayesian analysis provide a single, well-supported, hypothesis of osteoglossomorph interrelationships and show the group to be monophyletic. The tree topology is the following: (*Hiodon alosoides*, (*Pantodon buchholzi*, (((*Osteoglossum bicirrhosum*, *Scleropages* sp.), (*Arapaima gigas*, *Heterotis niloticus*)), ((*Gymnarchus niloticus*, *Ivindomyrus opdenboschi*), ((*Notopterus notopterus*, *Chitala ornata*), (*Xenomystus nigri*, *Papyrocranus afer*)))))). We compare our results with previously published phylogenetic hypotheses based on morpho-anatomical data. Additionally, we explore the consequences of the long terminal branch length for the taxon *Pantodon buchholzi* in our phylogenetic reconstruction and we use the obtained phylogenetic tree to reconstruct the evolutionary history of electroreception in the Notopteroidei.

Keywords: Osteoglossomorpha; Phylogeny; DNA; Basal teleosts; Teleostei; Long-branch attraction; Electroreception

1. Introduction

Although one of the oldest groups of extant teleost fishes, the Order Osteoglossomorpha comprises less than 1% of living teleost species. Of the five extant osteoglossomorph families, only the African family Mormyridae, containing 180 of the 199 living osteoglossomorph species, is not species-poor. Modern osteoglossomorphs are endemic to the freshwaters of tropical regions (Africa, South America, South-East Asia, New Guinea, and North Australia), with the exception of the North American family Hiodontidae. The fossil record of osteoglossomorphs, which begins in the Late Jurassic or Early Cretaceous and includes all continents save Antarctica (Bonde, 1996; Li and Wilson, 1996a; Li

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et al., 1997a,b; Shen, 1996; Taverne, 1979, 1998; Zhang, 1998; Zhang and Jin, 1999), indicates that in the past they were more widespread, and probably more speciose, than they are today. Despite the antiquity of the group, modern osteoglossomorphs are not "living fossils." They are morphologically heterogeneous and include both relatively unspecialized forms, such as the species of *Hiodon*, and others with highly derived features such as *Pantodon buchholzi* and the electric fishes of the families Mormyridae and Gymnarchidae.

More than 35 years ago Greenwood et al. (1966) defined the Superorder Osteoglossomorpha on the basis of two derived characters: (1) the parasphenoid-tongue bite apparatus and (2) the presence of "tendon bones" associated with the ventral portion of the second gill arch skeleton. Since that time, while the monophyly of the living osteoglossomorphs has been widely accepted, a number of alternative hypotheses of osteoglossomorph interrelationships based on studies of osteology and other anatomical characters have been advanced. We review only the most significant of these with respect to extant taxa here and in Fig. 1. A more complete history of osteoglossomorph systematics is available in Hilton (2003).

In the first of two recent cladistic analyses, Li and Wilson (1996b) obtained a most-parsimonious tree from analysis of 44 anatomical characters (Fig. 1C). In their hypothesis, the Notopteroidea (family Notopteridae) and the Mormyroidea (families Gymnarchidae and Mormyridae) are sister groups and form the Suborder Notopteroidei. Further, the Notopteroidei is sister to the Osteoglossoidei (family Osteoglossidae, in which *Pantodon* is the sister group to *Osteoglossum* + *Scleropages*) and these together form the Order Osteoglossiformes. Finally, the Osteoglossiformes are the sister group to the Hiodontiformes (family Hiodontidae) which together form the Superorder Osteoglossomorpha. This hypothesis is completely congruent with the non-cladistic "phylogenetic conception" of the Osteoglossomorpha proposed by Taverne (1979, 1998). The recent hypothesis of Hilton (2003), based on 65 informative characters, differs only in the placement of the notopteroids as the sister group to osteoglossoids, rather than to the mormyroids (Fig. 1D). Both of these hypotheses in turn differ in important respects from the earlier hypotheses of (in chronological order) Greenwood et al. (1966), Nelson (1968, 1969), Greenwood (1973), Patterson and Rosen (1977), Lauder and Liem (1983), and Bonde (1996) (Figs. 1A, B, and E).

Few studies to date have used molecular data for phylogenetic analysis of the Osteoglossomorpha and none of these have sampled all osteoglossomorph lineages, or included all relevant outgroups to assess osteoglossomorph monophyly. Kumazawa and Nishida (2000) employed sequences from the mitochondrial cytochrome *b* and ND2 genes to examine relationships among several osteoglossomorphs. *Hiodon* was not included in this study and most of the inter-familial relationships obtained were not well supported (Fig. 1F). Al-Mahrouki et al. (2001) isolated and sequenced preproinsulin cDNAs from several osteoglossomorphs. In a tree produced from a parsimony analysis of the sequences, *Chitala* (Notopteridae) and *Gnathonemus* (Mormyridae) are sister groups and *Osteoglossum* (Osteoglossidae) is the sister group to *Pantodon* + *Hiodon*. A close relationship between *Pantodon* and *Hiodon* has never been proposed elsewhere. The robustness of this result is difficult to interpret because no measures of internal branch support were given. These osteoglossomorph taxa appeared as a monophyletic group with respect to salmonid, lophiiform, cichlid, and cyprinid outgroups. O'Neill et al. (1998), suggested the

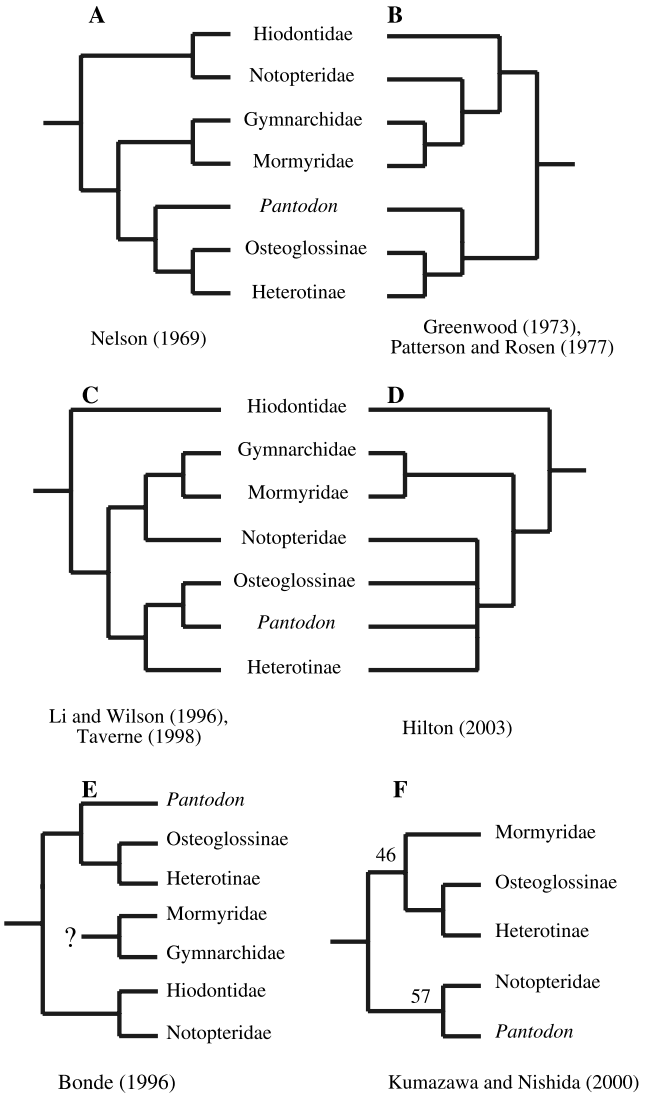

Fig. 1. Previously published phylogenetic hypotheses for the Osteoglossomorpha based on anatomical data (A–E) and molecular data (F). The hypothesis of Lauder and Liem (1983) is equivalent to tree B with the positions of *Pantodon* and Osteoglossinae interchanged.

non-monophyly of the Osteoglossomorpha upon finding a derived teleost form of GnRH in *Osteoglossum*, *Gnathonemus*, and the notopterids *Chitala* and *Xenomystus* relative to a plesiomorphic form shared by *P. buchholzi*, anguillids, and non-teleost actinopterygian fishes. Our view is that no molecular study has yet provided a complete or compelling test of osteoglossomorph monophyly or relationships.

Here, we present the first comprehensive molecular phylogenetic analysis of the living Osteoglossomorpha derived from an analysis of nucleotide characters from five different genes (the mitochondrial cytochrome *b*, 12S and 16S rRNA, and the nuclear RAG2 and MLL). In this study we include appropriate outgroup taxa to assess osteoglossomorph monophyly and interrelationships of all major osteoglossomorph lineages. We compare the phylogenetic hypothesis we obtain to those published in previous studies.

2. Materials and methods

2.1. Taxonomic sampling and molecular marker selection

Species from which we sampled DNA sequences are listed in Table 1. Voucher specimens were deposited in the fish collections of the Museum National d'Histoire Naturelle of Paris (MNHN) and the Cornell University Museum of Vertebrates (CU). Collection accession numbers are available upon request from the authors. Our sampling includes one osteoglossomorph representative of each extant non-mormyrid genus. The monophyly of the Mormyridae has been adequately demonstrated in recent molecular studies (Lavoué et al., 2000; Sullivan et al., 2000). For this reason, we sample only one taxon within this family. We selected four non-osteoglossomorph outgroups within the three clades of basal teleosts that either together, in partial

Table 1
Taxa analyzed in this study, listed following the Li and Wilson's (1996b) classification (using the sequencing convention), with GenBank accession number for each molecular marker

Species	Cyt*b*	12S	16S	RAG2	MLL
Superorder Osteoglossomorpha					
Order Hiodontiformes					
Family Hiodontidae					
Hiodon alosoides	**AY504821**	**AY504827**	**AY504835**	**AY504841**	**AY504847**
Order Osteoglossiformes					
Suborder Osteoglossoidei					
Family Osteoglossidae					
Subfamily Osteoglossinae					
Pantodon buchholzi	AF201615[a]	AF201527[a]	AF201572[a]	AF201647[a]	**AY504849**
Osteoglossum bicirrhosum	AB035238[b]	**AY504828**	**AY504830**	**AY504838**	**AY504848**
Scleropages sp.	AB035237[b]	**AY504829**	**AY504832**	**AY504840**	—
Subfamily Heterotidinae					
Heterotis niloticus	**AY504820**	**AY504831**	**AY504837**	**AY504842**	—
Arapaima gigas	AB035241[b]	**AY504824**	**AY504834**	**AY504843**	—
Suborder Notopteroidei					
Family Notopteridae					
Notopterus notopterus	**AY504822**	**AY504833**	**AY504839**	**AY504845**	**AY504852**
Chitala ornata	AF201583[a]	AF201493[a]	AF201493[a]	AF201626[a]	—
Xenomystus nigri	AF201614[a]	AF201526[a]	AF201526[a]	AF201660[a]	—
Papyrocranus afer	**AY504823**	**AY504826**	**AY504836**	**AY504844**	—
Family Mormyridae					
Ivindomyrus opdenboschi	AF201591[a]	AF201502[a]	AF201547[a]	AF201635[a]	**AY504851**
Family Gymnarchidae					
Gymnarchus niloticus	AF201586[a]	AF201496[a]	AF201541[a]	AF201629[a]	**AY504850**
Superorder Elopomorpha					
Anguilla sp.	AF006706[e]	AF266486[e]	AJ244824[g]	—	AF137221[i]
Superorder Clupeomorpha					
Engraulis sp.	AF472579[d]	AB040676[h]	AB040676[h]	**AY504846**	—
Superorder Euteleostei					
Order Ostariophysi					
Chanos chanos	**AY504825**	**AY504818**	**AY504819**	—	AF137223[i]
Order Salmoniformes					
Oncorhynchus mykiss	NC001717[c]	NC001717[c]	NC001717[c]	U31670[f]	AF137229[i]

GenBank accession numbers in bold are new reports generated for this study. References for published sequences are: [a]Sullivan et al. (2000); [b]Kumazawa and Nishida (2000); [c]Zardoya et al. (1995); [d]Jerome et al. (2003); [e]Lin et al. (2001); [f]Hansen and Kaattari (1996); [g]Bastrop et al. (2000); [h]Inoue et al. (2001); and [i]Venkatesh et al. (1999).

Scleropages sp. is a contig of *S. leichardti* (cyt*b*, 12S/16S rRNA) and *S. formosus* (RAG2). *Anguilla* sp. is a contig of *A. reinhardtii* (cyt*b*, 12S/16S rRNA) and *Anguilla* sp. (MLL). *Engraulis* sp. is a contig of *E. encrasicolus* (cyt*b*, RAG2) and *E. japonicus* (12S/16S rRNA).

combination, or singly have been proposed as the sister group of the Osteoglossomorpha (Arratia, 1997, 1998; Greenwood et al., 1966; Patterson, 1998; Patterson and Rosen, 1977): *Engraulis* sp. (Clupeomorpha), *Anguilla* sp. (Elopomorpha), *Chanos chanos* (Ostariophysi), and *Oncorhynchus mykiss* (Euteleostei). In our discussion below, we follow the classification proposed by Roberts (1992) for the Notopteridae in which two genera of Asian notopterids are recognized: *Notopterus* for the single species *N. notopterus* and *Chitala* for the other four species. For the remaining Osteoglossomorpha, we follow the classification proposed by Li and Wilson (1996b) in which *P. buchholzi* is included in the family Osteoglossidae.

Our choice of five different molecular markers (RAG2, MLL, cytochrome *b*, and rRNA 12S and 16S) was made with a view to include both protein-coding and non-coding markers of appropriate variability from both mitochondrial and nuclear sources and to exploit supplementary sequences available in the GenBank database.

2.2. Acquisition of sequences

Total genomic DNA was extracted from fresh or ethanol-preserved muscle tissue using QIAamp tissue kit (Quiagen, Valencia, CA). DNA sequences were amplified by polymerase chain reaction (PCR) on a Hybaid TouchDown thermocycler (Hybaid Limited, Teddington, Middlesex, England). The reaction mixture contained 1.25 U of AmpliTaq Gold Taq polymerase (Perkin–Elmer Applied Biosystems, Foster City, CA), 200 μM of each dNTP, 5 μl of GeneAmp Gold PCR 10× buffer (1× final), 3 mM $MgCl_2$, 50 ng of each primer, and 10–50 ng of template DNA in a final volume of 50 μl. PCR conditions included an initial denaturation step at 95 °C for 10 min, followed by 35 amplification cycles (94 °C for 1 min, a marker-dependent annealing temperature for 1 min, and 72 °C for 1.5 min) followed by a final extension step at 72 °C for 7 min. The annealing temperature used for cytochrome *b* was 42 °C, 53 °C for the RAG2 amplification, and 55 °C for the 12S, 16S rRNA, and MLL amplifications. The complete cytochrome *b* gene (1140 bp) was amplified using primers L15930 (forward): 5′-CTT-CGA-TCT-TCG-rTT-TAC-AAG-3′ and H14724 (reverse): 5′-TGA-TAT-GAA-AAA-CCA-TCG-TTG-3′. Partial rRNA 12S (about 350 bp) and 16S genes (550 bp) were amplified using primers from Palumbi (1996). These are L1067 (12S forward): 5′-AAA CTG GGA TTA GAT ACC CCA CTA T-3′, H1478 (12S reverse): 5′-GAG GGT GAC GGG CGG GCG GTG TGT-3′, L2510 (16S forward): 5′-CGC CTG TTT ATC AAA AAC AT-3′, and H3080 (16S reverse): 5′-CCG GTC TGA ACT CAG ATC ACG T-3′. Partial coding sequences of approximately 700 bp from the single-copy mixed lineage leukemia gene (MLL) were amplified using primers described by Venkatesh et al. (1999). These are MLL forward 3124: 5′-GCn-CGn-TCn-AAy-ATG-TTy-TTy-GG-3′ and MLL reverse 3376: 5′-ATr-TTn-CCr-CAr-Tcr-Tcr-CTr-TT-3′. We used two forward and two reverse primers to amplify a partial sequence of about 1300 bp from the recombination activating gene 2 (RAG2), with different combinations working best with different templates (Sullivan et al., 2000). The forward primers are F1: 5′-TTT GGr CAr AAG GGC TGG CC-3′ and F2: 5′-ArA CGC TCm TGT CCm ACT GG-3′. The reverse primers are R4: 5′-GTr GAr TAG TAG GGC TCC CA-3′ and R6: 5′-TGr TCC ArG CAG AAG TAC TTG-3′. PCR products were purified using the Promega Wizard PCR Preps DNA purification kit (Promega, Madison, WI).

We performed double-stranded cycle sequencing using dye-labeled terminators with the primers that had been used for amplification. The resulting products were analyzed on 5% polyacrylamide gels on an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA). We edited sequence chromatograms and assembled consensus sequences using the Sequencher software package (Gene Codes, Ann Arbor, MI). We deposited the 35 new DNA sequences generated for this study in GenBank database. Accession numbers are given in Table 1.

We obtained 35 new sequences for this study and utilized 20 others that we had published previously (Table 1). We were unable to produce all sequences for all taxa due to variability in the target sequences for the amplification primers. We completed our dataset to the extent possible with 16 sequences available in GenBank (see Table 1 for details). For three taxa (*Scleropages* sp., *Anguilla* sp., and *Engraulis* sp.), we were constrained to construct chimeras with sequences from different, but congeneric, species. Nine sequences are missing in our data matrix: RAG2 sequences of *C. chanos* and *Anguilla* sp., and seven MLL sequences, including six ingroup sequences (Table 1). Missing data represent 11.25% of the total dataset. However, for four genes (12S and 16S rRNA, cytochrome *b*, and RAG2) all sequences are complete for the osteoglossomorph taxa and at least one MLL sequence from each family is included.

Base composition, χ^2 tests for homogeneity of base composition across taxa, and uncorrected pairwise distance values were calculated with PAUP* version 4.0b10 (Sinauer, Sunderland, MA). As expected, we observed a strong antiguanine bias in the cytochrome *b* sequences (G = 14%), an overrepresentation of adenine in 12S/16S rRNA sequences (A = 31%), and an overrepresentation of guanine in nuclear markers RAG2 and MLL (G about 31% in both genes). The χ^2 test demonstrated significant heterogeneity in base composition across taxa only for cyt*b* and RAG2 ($P < 0.005$), but not for 12S/16S rRNA ($P = 0.95$) or MLL ($P = 0.22$).

2.3. Sequence alignment

We performed three alignments of sequences using the software Clustal X (Thompson et al., 1997) using three different gap opening/gap extension parameters of 10/5, 7/5, and 10/10. We identified by eye sites that shifted relative position in the three alignments (Gatesy et al., 1993). In our NEXUS file, we created special character sets for the ambiguously aligned positions detected in each individual molecular marker, allowing us to remove them for the subsequent phylogenetic analyses. Insertions and deletions were coded as binary characters and added to the matrix, following the method described by Barriel (1994) and implemented by the software BARCOD (http://wwwabi.snv.jussieu.fr/people/billoud/).

The cytochrome *b* alignment was trivial without insertions or deletions and contained 1140 sites. The RAG2 protein-coding sequences were also easily aligned (1290 bp plus 10 binary indel characters), with the exception of two ambiguously aligned portions that were removed from the complete dataset (51 positions). MLL alignment (745 bp plus 26 binary indel characters) was variable and we were constrained to remove 40 positions. The 12S and 16S rRNA alignments were also variable, with a succession of conserved regions (''stems'') and hyper-variable regions (''loops''). Out of these 1049 bp (plus 74 binary indel characters), 220 positions were ambiguously aligned among the three alignment settings and were excluded. Table 2 summarizes the characteristics for each individual molecular partition.

The combined matrix of the five molecular markers contains 4334 characters for 16 taxa. After exclusion of ambiguously aligned positions, the final combined matrix includes 4035 positions, of which 2171 are variable and 1537 are informative for parsimony analysis.

2.4. Phylogenetic analysis

Phylogenetic relationships were inferred by maximum parsimony (MP) using the software PAUP* version 4.0b, on the combined nucleotide dataset matrix. First, all characters were unordered and weighted equally. The most-parsimonious (MP) tree or trees were sought by using heuristic searches with starting trees obtained via stepwise addition with 1000 iterations of the random sequence addition and the ''tree bisection reconnection'' (TBR) branch-swapping option. The consistency index (CI) and retention index (RI) were calculated from the MP trees with uninformative characters excluded. Support for internal branches was estimated by calculating bootstrap proportions (Felsenstein, 1985) and the Bremer support index (Bremer, 1994). Bootstrap proportions (1000 pseudoreplicates) were calculated from heuristic searches using TBR branch swapping and 10-replicate random sequence addition in PAUP*. Bremer support indices (BSI) for each node were calculated in PAUP* and in the software TreeRot (Sorenson, 1999). To estimate potential phylogenetic signal incongruence among individual molecular markers, we further calculated the partitioned Bremer support index (PBS) at each node and for each individual partition (Gatesy et al., 1999) using PAUP* and TreeRot software. We tested three different, commonly used, relative weights for transitions (TI) and transversions (TV) to correct

Table 2
Characteristic of individual genetic partitions and phylogenetic analysis by parsimony

	12S/16S	Cyt*b*	RAG2	MLL
No. of ingroup and outgroup taxa	12 and 4	12 and 4	12 and 2	6 and 3
No. of nucleotide characters and indel characters (0/1)	1049 and 74	1140 and 0	1290 and 10	745 and 26
No. of characters after exclusion of ambiguous positions	915	1140	1249	731
No. of variable (informative) characters	372 (260)	580 (489)	741 (507)	478 (281)
No. of MP trees	1	2	3	1
Length	1018	2328	1985	1016
CI excluding uninformative positions/RI	0.428/0.447	0.386/0.292	0.539/0.499	0.617/0.362
Nodes supported by BP > 75%	8	5	9	2
Presence/absence of particular nodes in the single MP tree or in the strict consensus if several MP trees (Y, present; N, absence; —, un-applicable; in parentheses BP if >75%):				
Clade Osteoglossinae = (*Osteoglossum*, *Scleropages*)	Y (90)	Y (90)	Y (100)	—
Clade Heterodinae = (*Arapaima*, *Heterotis*)	Y (84)	Y	Y (100)	—
Clade Osteoglossidae *s.s.* = (Heterodinae, Osteoglossinae)	Y (90)	N	Y (100)	—
Clade Notopterinae = (*Notopterus*, *Chitala*)	Y (100)	Y (99)	Y (100)	—
Clade Xenomystinae = (*Xenomystus*, *Papyrocranus*)	Y (86)	Y	Y (86)	—
Clade Notopteridae = (Notopterinae, Xenomystinae)	N	N	Y (98)	—
Clade Mormyroidea = (Mormyridae, *Gymnarchus*)	N	N	Y (100)	Y (98)
Clade Notopteroidei = (Mormyroidea, Notopteridae)	N	N	Y (82)	Y
Clade ''A'' = (Notopteroidei, Osteoglossidae *s.s.*)	Y (83)	N	Y	N
Clade Osteoglossiformes = (''A,'' *Pantodon*)	Y (100)	Y (80)	N	N
Clade Osteoglossomorpha = (Osteoglossiformes, *Hiodon*)	Y (88)	N	N	Y (95)

for possible effects of substitutional saturation: TV/TI = 2, 5, and 10.

We also conducted a Bayesian phylogenetic analysis on the complete dataset with MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). This version of MrBayes allows analysis of mixed datasets containing both nucleotide and standard characters, permitting inclusion of the coded indel data. We set up this analysis such that each of four molecular partitions (RAG2, MLL, cytochrome *b*, and combined 12S + 16S) received a unique general time reversible model in which a proportion of the sites are assumed invariant and rates for remaining sites are drawn from a gamma distribution (GTR + I + Γ; Yang, 1994). To reduce the number of model parameters, we combined the partitions for the 12S and 16S genes that have similar evolutionary characteristics. We set the parameters in MrBayes as follows: "nst = 6 rates = invgamma; unlink shape = (all) pinvar = (all) statefreq = (all) revmat = (all)." The Markov chain Monte Carlo (MCMC) process included four chains (three heated and one cold) running simultaneously. Parameter values and trees were sampled every 500 generations. We compared average log likelihood values and the posterior probabilities of the trees calculated by majority rule consensus after stationarity had been reached in two separate runs to 500,000 generations, each starting from a random tree.

3. Results

3.1. Simultaneous analysis of the complete combined dataset

The simultaneous parsimony analysis of the five molecular markers provides a single most-parsimonious (MP) tree of 6396 steps when ambiguously aligned positions are excluded and 7073 steps when these are left included, with a CI (excluding uninformative characters) of 0.531 and 0.536, respectively, and a RI of 0.385 and 0.384, respectively. Within the osteoglossomorph ingroup, the branching pattern of the taxa is identical for these two trees; they differ from each other only by the relative position of *C. chanos* among the outgroups. Fig. 2 illustrates the tree obtained after exclusion of ambiguously aligned positions, with branch lengths proportional to ACCTRAN-reconstructed character state changes. In this tree, the Osteoglossomorpha form a clade, although this node receives only moderate support (BP = 67%, BSI = 5) in the parsimony analysis. Within the Osteoglossomorpha, the tree topology is completely resolved as follows: (*Hiodon alosoides*, (*P. buchholzi*, (((*Osteoglossum bicirrhosum*, *Scleropages* sp.), (*Arapaima gigas*, *Heterotis niloticus*)), ((*Gymnarchus niloticus*, *Ivindomyrus opdenboschi*), ((*N. notopterus*, *Chitala ornata*), (*Xenomystus nigri*, *Papyrocranus afer*))).

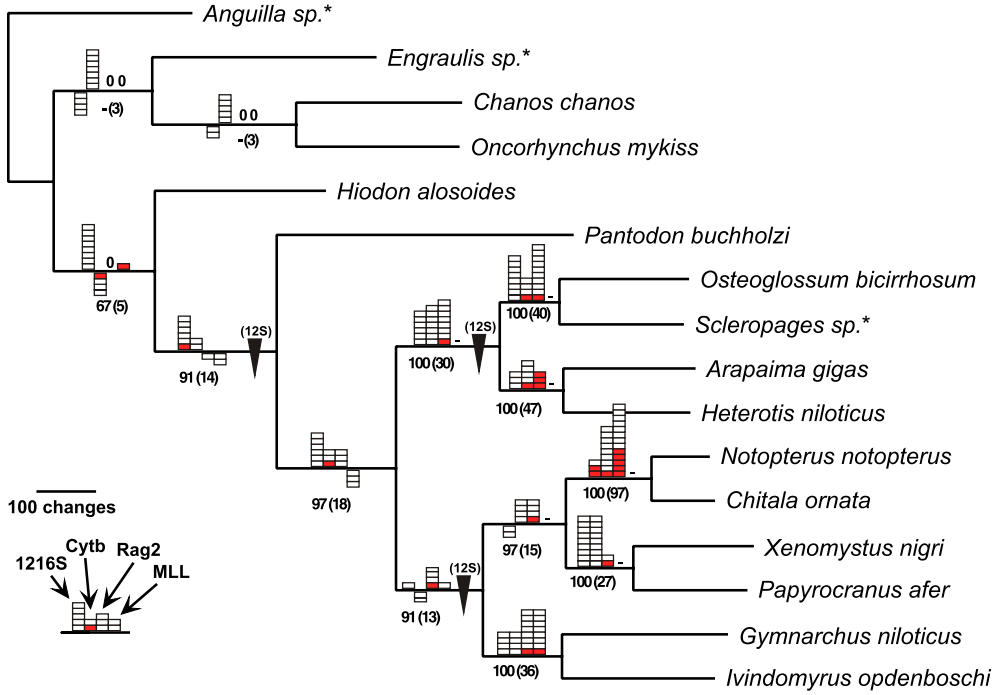

Fig. 2. Single MP tree recovered in analysis from the combined dataset of molecular markers (12S and 16S rRNA, cytochrome *b*, RAG2, and MLL): tree length = 6396, consistency index = 0.531, retention index = 0.385. Bootstrap proportions (≥ 50%) and Bremer support indices (in parentheses) are indicated below branches. Partitioned Bremer support values (PBS) for each individual dataset are indicated with columns, above branches when positive and below branches when negative. Open rectangles represent 1 step; shaded rectangles represent 10 steps. A dash indicates that PBS is not applicable for that particular partition and node. The sum of positive and negative PBS equals the indicated BSI value at each node. Informative molecular insertions from 12S rRNA, as inferred under ACCTRAN optimization, are shown by inverted triangles. Filled symbols refer to characters having CI = 1. Asterisks indicate chimeric taxa produced from combination of sequence data from congeneric species.

Each of these relationships is strongly supported by BSI values between 13 and 97 and BP values between 91 and 100%. In addition, we detected three unique nucleotide deletions (CI = 1), each of which strengthens support for three intra-osteoglossomorph clades (Fig. 2).

The decomposition of BSI into PBS indicates that moderate conflicts occur among the molecular partitions at 5 of the 10 intra-osteoglossomorph nodes. For four of them, one molecular marker provides contradictory signal to the others. For example, the 12S/16S rRNA, cytochrome *b*, and RAG2 partitions support the position of *Pantodon* as sister group of the remaining osteoglossiforms (PBS = 6, 12, and 3, respectively), while MLL is slightly incongruent (PBS = −3). At the node that groups all osteoglossomorph taxa with the exclusion of *Hiodon*, mitochondrial partitions provide strong support (PBS = +17) while nuclear partitions (PBS = −3) subtract support from the total BSI value.

The MP trees recovered in analyses in which weights had been applied to transversions over transitions via a step-matrix (TV/TS = 2, 5 or 10) were identical in topology to the unweighted analysis.

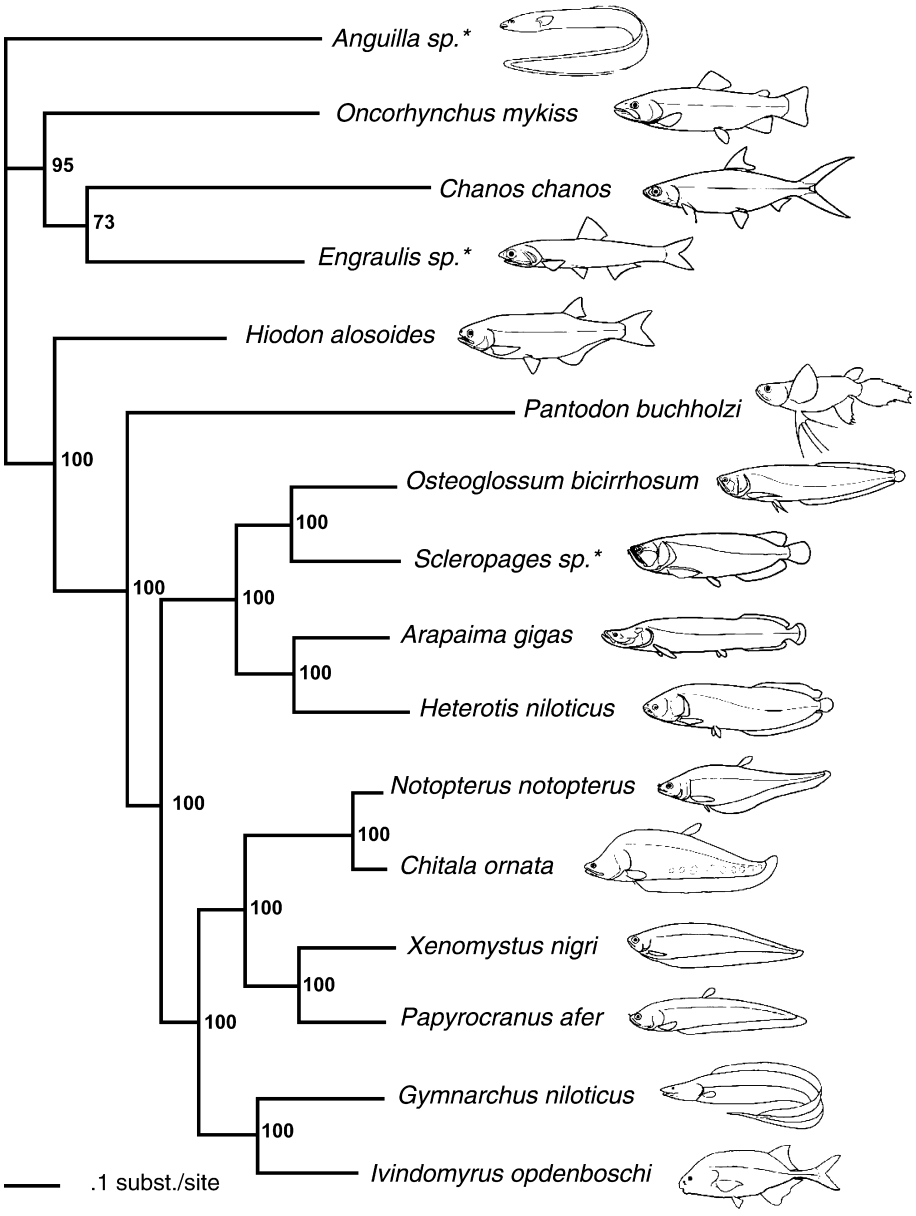

Fig. 3. The 50% majority rule consensus of 920 trees from Bayesian analysis of combined molecular partitions with ambiguously aligned sites removed and coded indels included. In this analysis, parameters for a GTR + I + Γ model of DNA substitution were independently estimated for each of four molecular partitions; mean $-\ln L = 30191.8$. All ingroup nodes are shared with the parsimony hypothesis (Fig. 2). Numbers at nodes are posterior probabilities (shown as %) and branch lengths are averaged over all 920 input trees. Asterisks indicate chimeric taxa produced from combination of sequence data from congeneric species. Ten of the fish illustrations from Nelson (1994), used by permission of John Wiley and Sons, Inc.

Bayesian analysis of the complete dataset with ambiguously aligned sites excluded produced a tree identical in ingroup topology to the parsimony tree (Fig. 3). In both independent runs beginning from different random starting trees, a plot of log likelihood against generation number indicated that stationarity of posterior probabilities had been reached at approximately 30,000 generations; trees and parameter values recorded before the 40,000th generation were eliminated from subsequent calculations. Average log likelihood scores at stationarity for the two runs were −30191.8 for the first and −30190.7 for the second. The topology of the 50% majority rule consensus tree was the same in each of the two runs and there were no notable differences in estimated branch lengths or posterior probabilities of nodes. A third run in which ambiguously aligned sites were re-included produced a consensus tree of the same topology, with the exception that *C. chanos* changed position among the outgroups. In all runs, posterior probabilities for all ingroup nodes are 1.0. In contrast to the moderate statistical support for the monophyly of the osteoglossomorph taxa found in the parsimony analysis, the posterior probability for this node is also 1.0 in all runs with MrBayes.

3.2. Analyses with no missing data and of individual data partitions

To evaluate whether the missing data in our matrix may have influenced the results of the foregoing analyses, we created a reduced matrix containing only seven taxa for which all characters are present. Included were one representative of each of the six extant osteoglossomorph lineages (Hiodontidae, Osteoglossidae, Notopteridae, Gymnarchidae, Mormyridae, and *Pantodon*), as well as one outgroup (*O. mykiss*). We re-aligned sequences and coded insertions and deletions again using BARCOD. The parsimony analysis with ambiguously aligned characters excluded provides a single tree of 3449 steps (CI = 0.741, RI = 0.354) and a single tree of 3774 steps (CI = 0.750, RI = 0.358) with these characters included. The branching pattern of both trees is identical. The topology of these two trees (not shown) is consistent with the tree topology produced from the full analysis and high BP and BSI values support internal branches. The exclusion of taxa for which some sequences are missing modified neither the major features of osteoglossomorph tree topology nor statistical support for nodes relative to the full analysis.

Additionally, we performed a parsimony analysis of each molecular partition individually (12S and 16S combined into one partition), followed by bootstrap resampling. Results are shown in Table 2. The tree topologies obtained from the four analyses differ among each other and with the topology obtained from the simultaneous analysis of the combined partitions at some nodes. Bootstrap proportions at nodes that conflict with the combined data topology are below 57%, except for the cytochrome *b* analysis in which a node grouping *H. alosoides*, *C. chanos*, *Engraulis* sp., and *O. mykiss* is supported by BP of 73%.

4. Discussion

4.1. Reliability of our phylogenetic results

The phylogenetic analysis of the combined dataset presented here provides a completely resolved and well-supported hypothesis of interrelationships for the living Osteoglossomorpha. Long-branch attraction (Felsenstein, 1978), the inclusion of incomplete taxa (those having missing data), and conflict between data partitions are three issues that could conceivably bear upon the quality of this analysis. Long branches in our tree topology are a consequence of the great antiquity and long separation of several of the extant lineages of osteoglossomorphs. Our confidence in the hypothesis presented here is bolstered by the result that Bayesian analysis that incorporates a model of sequence evolution and weighted parsimony, both of which may be less susceptible to long-branch attraction problems than unweighted parsimony (Huelsenbeck, 1995), recover the same topology. We evaluate in more detail the possible effects of the especially long *Pantodon* branch in Section 4.5 below. Incomplete taxa, including those with a large proportion of missing characters, are often non-problematic for phylogenetic analysis as long as sufficient informative characters are present in the data matrix (Wiens, 2003). We conclude from our analysis of the reduced set of taxa in which all data are present that the missing data had no significant influence on the full analysis. Conflicting signal among partitions of sequence data from independent loci can be due to different gene lineage histories or from homoplasy in the data. We explored character conflict among the partitions by PBS analysis of the combined dataset and by separate parsimony analyses of the partitions. Although we found evidence of moderate character conflict among the partitions in some cases, no single partition (or combination of them) provides strong support for an alternative topology.

4.2. Phylogenetic relationships within the Osteoglossomorpha

Our molecular data support the monophyly of the extant Osteoglossomorpha, relative to the outgroups selected. With the exception of the novel position of *P. buchholzi* (discussed in the last section below), our inter-familial phylogeny is congruent with some recent hypotheses based on osteological data. Our results show

the Hiodontiformes to occupy the basal-most position within the living Osteoglossomorpha, as the sister group to all of the others. This is in agreement with the conclusions of Taverne (1998), Li and Wilson (1996b), and Hilton (2003) and counter to those in Nelson (1972), Greenwood (1973), and Lauder and Liem (1983). Taverne (1998) and Hilton (2003) review osteological data and provide two synapomorphies that support the monophyly of living osteoglossomorphs, excluding Hiodontiformes (i.e., Order Osteoglossiformes sensu Li and Wilson (1996b)).

Within the Osteoglossiformes in our hypothesis, the family Osteoglossidae (consisting of the genera *Osteoglossum*, *Scleropages*, *Arapaima*, and *Heterotis*) is the sister group to a clade comprising the families Notopteridae, Mormyridae, and Gymnarchidae. Within the family Osteoglossidae, our results are congruent with the commonly accepted hypothesis, based on osteological data (reviewed in Hilton (2003)) and molecular data (Kumazawa and Nishida, 2000) that places *A. gigas* as the sister group of *H. niloticus* (subfamily Arapaiminae) and *Scleropages* as the sister group of *Osteoglossum* (subfamily Osteoglossinae). Our hypothesis differs from others, however, in the exclusion of *Pantodon* from a position within, or as sister group to, the Osteoglossidae.

As indicated above, the families Notopteridae, Mormyridae, and Gymnarchidae form a clade in our hypothesis. Li and Wilson (1996b) list seven derived osteological characters supporting this relationship. From this study, we add one more character: the deletion of 1 bp at the position 1400 in the 12S rRNA gene sequence. Lastly, the Mormyridae and the Gymnarchidae are sister groups and form the Superfamily Mormyroidea. The representatives of this clade share many unique derived characters, including the presence of electric organs derived from muscle, "tuberous"-type electroreceptors which function in electrolocation and communication (Hopkins, 1986), and spermatozoa that lack flagellae (Mattei et al., 1972). Interrelationships within this group have already been the subject of several recent molecular studies (Lavoué et al., 2000; Lavoué et al., 2003; Sullivan et al., 2000).

4.3. Phylogenetic relationships among the Notopteridae and insights into the evolutionary history of ampullary electroreceptors

The family Notopteridae, sometimes referred to as the "Old World knifefishes" or "featherbacks," contains four genera and eight species (Roberts, 1992). The genera *Xenomystus* (one species, *X. nigri*) and *Papyrocranus* (two species, *P. afer* and *P. congoensis*) are African, whereas the genera *Notopterus* (one species, *N. notopterus*) and *Chitala* (four species, *C. chitala*, *C. blanci*, *C. lopi*, and *C. ornata*) occur in South and Southeast Asia. All species live in freshwater, although some Asian species are known to enter brackish water environments (Roberts, 1992). All notopterids are laterally compressed and locomote by undulation of an elongate anal fin that is confluent with a greatly reduced caudal fin. The dorsal fin is reduced (or lacking in *Xenomystus*) and the pelvic fins are minute (or lacking in *Papyrocranus*). Most previous studies addressing the phylogenetic systematics of this family support the monophyly of the Notopteridae, but provide several competing hypotheses of intra-familial relationships. According to Greenwood (1963) and Li and Wilson (1996b), *Xenomystus* is the sister group of the Asian notopterids + *Papyrocranus*. Taverne (1998) suggested that the genus *Chitala* is the sister group of the remaining Notopteridae. Nelson (1969) and Forey (1997) divided the family into two clades, the first containing the Asian notopterids and the second containing the African notopterids. The incomplete fossil record of notopterids provides few insights into the relationships among these living taxa, although the recent discovery and description of †*Palaeonotopterus greenwoodi* (Cavin and Forey, 2001; Forey, 1997; Taverne, 1998; Taverne and Maisey, 1999) from the Upper Albian or Lower Cenomanian (~100 million years before present) of Morocco indicates a more ancient origin of this group than previously imagined (Bonde, 1996; Li and Wilson, 1996b; Rana, 1988). Our results support the Nelson (1969) and Forey (1997) hypotheses in grouping together the two African genera *Xenomystus* and *Papyrocranus* and the two Asian genera, *Notopterus* and *Chitala*.

Braford (1986) reviewed the ability of some, but not all, notopterids to sense low frequency electric fields. "Ampullary"-type electroreceptors in the skin and an "electrosensory lateral line lobe" (ELL) in the medulla of the brain to which the electroreceptors project are present in the two African genera *Xenomystus* and *Papyrocranus*, but are lacking in the Asian genera *Notopterus* and *Chitala*. Presumably this system functions to detect the weak bioelectric fields of prey organisms. Ampullary electroreceptors and an ELL are also present in all mormyroid species. In addition to this passive electrosensory system, mormyroid species possess a functionally separate active electrosensory system in which distortions to a high-frequency electric field generated by an electric organ in the tail of the fish are monitored by a separate set of "tuberous"-type electroreceptors in the skin. Because both the Mormyroidea and the African notopterids share this ampullary-type electroreceptors and an ELL, first Braford (1986), and then Alves-Gomes (2001) considered the possibility that these two groups could constitute a clade to the exclusion of the Asian notopterids which lack electroreception. Our results unambiguously reject this hypothesis by strongly supporting the monophyly of Notopteridae. Furthermore, our phylogenetic hypothesis can be used to reconstruct the evolution of the electroreception in the Notopteroidei (Fig. 4). There are two most-parsimonious hypotheses, each of two steps. In the first

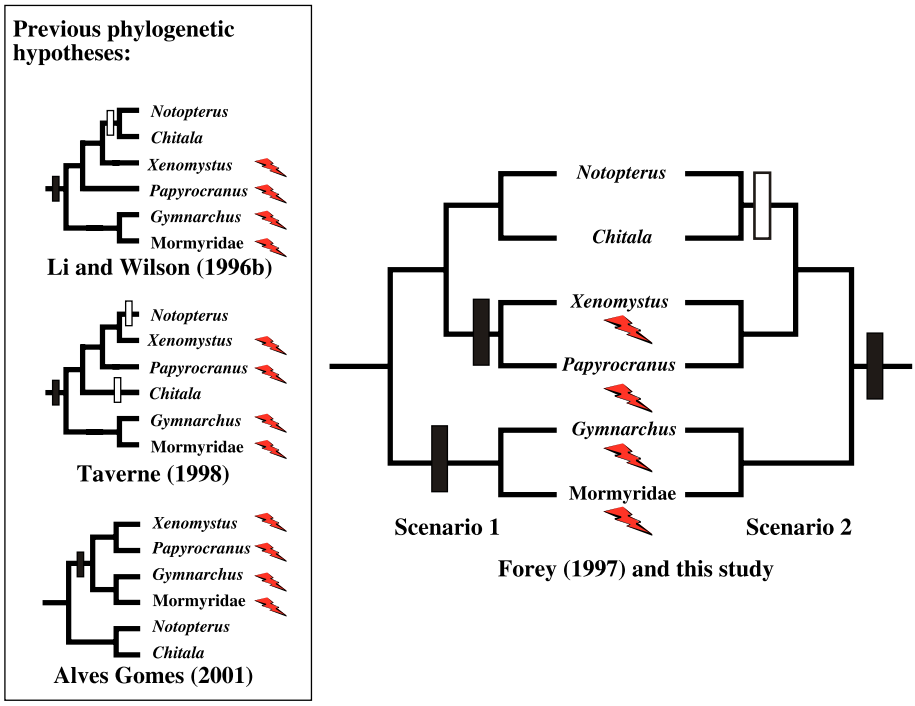

Fig. 4. Evolution of "ampullary" electroreceptors within the Notopteroidei. Earlier phylogenetic hypotheses for the Notopteroidei inconsistent with our result shown at left; the topology suggested by Forey (1997) and produced from analysis of our molecular dataset shown at right. Electroreceptive taxa indicated by lightening bolts. Black boxes represent origin of ampullary electroreceptors; open boxes represent secondary loss of ampullary electroreceptors. On our tree, unweighted parsimony reconstruction of the evolution of ampullary electroreceptors generates two most-parsimonious hypotheses, each of two steps. Possible reconstructions derived from earlier hypotheses shown at left.

scenario, electroreception evolves independently twice: in the ancestor of the Mormyroidea and in the ancestor of African Notopteridae. In the second scenario, electroreception evolves a single time in the common ancestor of the mormyroids and notopteroids, but is secondarily lost in the common ancestor of *Notopterus* and *Chitala*. We favor this latter scenario for two reasons. First, the rarity of electroreception within teleost fishes makes independent origins unlikely. Apart from the Mormyroidea and African Notopteridae, only the distantly related Siluriformes and their probable sister group, the South American Gymnotiformes, are electroreceptive. Second, the significant similarities of the structures involved in both mormyroids and the African notopterids (Braford, 1986) suggest a single origin. Why the Asian Notopteridae would have lost electroreception is open to speculation. New (1997) enumerated possible reasons for the loss of electroreception in the ancestor of Neopterygii, some of which—for instance genetic drift or a transition from freshwater to higher conductivity marine or brackish water—may apply to Asian Notopteridae.

4.4. The novel position of *P. buchholzi*

The position of *P. buchholzi* within the Osteoglossomorpha is the only phylogenetic result we obtained that conflicts with all previously proposed phylogenies for the group. This small (usually less than 13 cm) surface-dwelling insectivorous fish, popularly known in the aquarium trade as the African butterfly fish for its enlarged and patterned wing-like pectoral fins, is endemic to the Congo, Niger, and Lake Chad basins of Africa. In every parsimony and Bayesian analysis of the combined sequence data, *Pantodon* appears as the sister group of the clade (Osteoglossidae, (Notopteridae, Mormyroidea)); i.e., as the sister group of all living osteoglossomorphs excluding *Hiodon*. By contrast, anatomy-based studies have consistently placed this taxon either as sister to the family Osteoglossidae (Bonde, 1996; Greenwood, 1973; Greenwood et al., 1966; Kershaw, 1976; Nelson, 1969; Patterson and Rosen, 1977), or within the Osteoglossidae as sister group to *Osteoglossum* + *Scleropages* (Hilton, 2003; Lauder and Liem, 1983; Li and Wilson, 1996b; Taverne, 1979, 1998). While no morphology-based study has ever suggested the position for *Pantodon* obtained here, it is interesting to note that Patterson (1994), quoted in Hilton (2003), wrote: "The problematic Recent member of the group is [...] the African butterfly fish, *Pantodon*, which can be placed either with the osteoglossoids [...], with the notopteroids [...], or as the sister group of both in parsimony trees that differ in length by no more than 1%."

4.5. Testing an alternative hypothesis for Pantodon

Face value interpretation of the high support values for the sister group relationship between *Pantodon* and all other osteoglossiforms in both the parsimony and Bayesian analyses suggests that the data could be compatible with no other phylogenetic position for *Pantodon*. (Below we refer to this position for *Pantodon* as topology "O" for "obtained.") Nevertheless, the novelty of this result justifies its further scrutiny.

We observe that *Pantodon* occupies a very long branch on the combined data tree relative to other osteoglossomorph groups (Figs. 2 and 3). Furthermore, this branch is disproportionately long for each genetic partition considered separately, with the possible exception of cytochrome *b* (Fig. 5), suggesting that the rate of molecular evolution in the *Pantodon* lineage is accelerated relative to other osteoglossomorphs. Unequal rates of evolution within a study group are a known cause of inconsistency for phylogenetic reconstruction methods (Felsenstein, 1978). Could our result for *Pantodon* be a consequence of an inference artifact?

In MacClade 4.0, we examined the cost in increased tree length relative to the MP tree incurred by changing the position of *Pantodon* within the tree. Placing *Pantodon* as sister to the osteoglossine taxa (*Osteoglossum* and *Scleropages*), in agreement with Li and Wilson (1996b) and Taverne (1998), adds 38 steps to the 6396 steps of the MP tree. Placing *Pantodon* as sister to all the osteoglossids [(*Osteoglossum*, *Scleropages*)+(*Heterotis*, *Arapaima*)] in agreement with Nelson (1969), Greenwood (1973), Patterson and Rosen (1977), and Bonde (1996) adds 18 steps: a seemingly modest 0.28% increase in tree length from the MP topology. This is the least costly alternative placement for *Pantodon* according to parsimony criteria. (Below we refer to this topology as topology "A" for "alternative.") A third possible position for *Pantodon* as sister group to the notopteroids, mentioned in the Patterson quotation above, adds 26 steps (topology "N" below).

If one were to assume topology A to be the topology of the true tree, what is the probability that the analysis methods we used would incorrectly infer topology O versus correctly inferring topology A? To answer this question, we analyzed artificial datasets produced by simulating character evolution up the branches of a tree of topology A according to a ML model and base frequencies calculated from our empirical dataset.

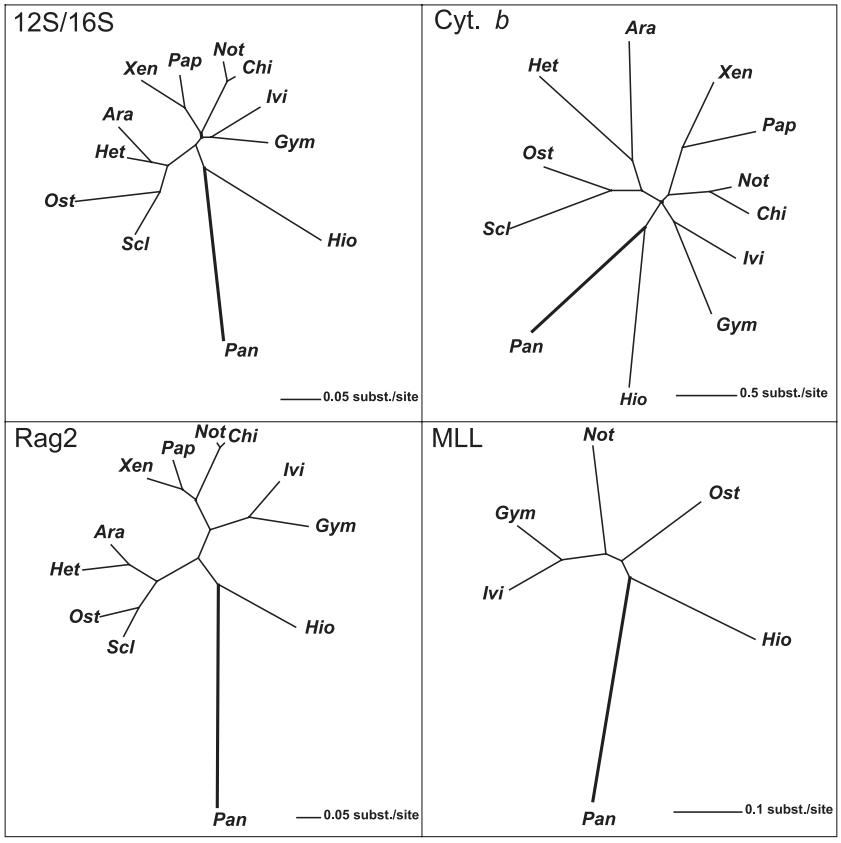

Fig. 5. Branch lengths of osteoglossomorph taxa in each of the four molecular data partitions considered separately. Topology of networks is that obtained by parsimony and Bayesian analysis of full dataset (only ingroup taxa shown). Branch lengths calculated in PAUP*4.0b10 from model parameters estimated for each partition by MrBayes 3.0 in the full analysis. Disproportionately long terminal branch for *P. buchholzi* is evident in all partitions except cytochrome *b*. Names of taxa abbreviated to first three letters.

First, we estimated a single GTR + Γ + I model for the complete combined-partition dataset (minus the ambiguously aligned data as well as the insertion/deletion characters) using MrBayes (MCMC settings as above). Then, using the parameter values obtained in this analysis, we calculated ML branch lengths for a tree of topology A from our original dataset in PAUP* (Fig. 6). From this tree and these GTR + Γ + I model parameters, we generated 1000 simulated datasets of the same size as our empirical dataset in the software package Mesquite version 1.0 (Maddison and Maddison, 2003). The Batch File Architect module of Mesquite was used to write a PAUP command file to sequentially perform unweighted parsimony analysis on each of the 1000 datasets. Even though the model tree on which the datasets were simulated was of topology A, we obtain this topology in only 16.6% of the MP trees, whereas 80% of them are of topology O. Thus, even were *Pantodon* the sister group to the osteoglossids (and branch lengths similar to those we estimated for the topology A tree in Fig. 6), we would expect unweighted parsimony analysis to place it incorrectly as sister group to all other osteoglossiforms, the position it occupies in the analysis of our empirical dataset.

Can we attribute this result of the simulation to the disproportionately long branch length for *Pantodon*? To investigate this question, we re-ran the simulation after reducing *Pantodon*'s terminal branch length by 43% in the Mesquite input tree such that this taxon's total distance from the *Pantodon* + osteoglossid common ancestor was equal to the mean distance for the osteoglossid taxa. All other branch lengths were left as before. (We note that the ML lengths of other branches in the topology A phylogram are not independent of the length of the *Pantodon* branch. Thus, were the evolutionary rate in *Pantodon* equal to the average rate for osteoglossids, not only would its terminal branch be shorter, but, given the hypothesis of topology A, the very short internal branch linking *Pantodon* to the osteoglossids might well be longer, making recovery of the model topology more likely. In this way, this test for the effects of a "shorter-branch" *Pantodon* is imperfect.) Shortening *Pantodon*'s branch changes the ratio of recovered topologies: 41% of the MP trees derived from these simulated datasets are of topology A, while 47% are of topology O. Clearly, the length of *Pantodon*'s branch by itself affects the ability of parsimony to correctly infer the topology A over topology O.

Given the finding that parsimony would be expected to infer topology O even if topology A were true, would we expect to obtain bootstrap proportions as high as those we recovered? Furthermore, would Bayesian anal-

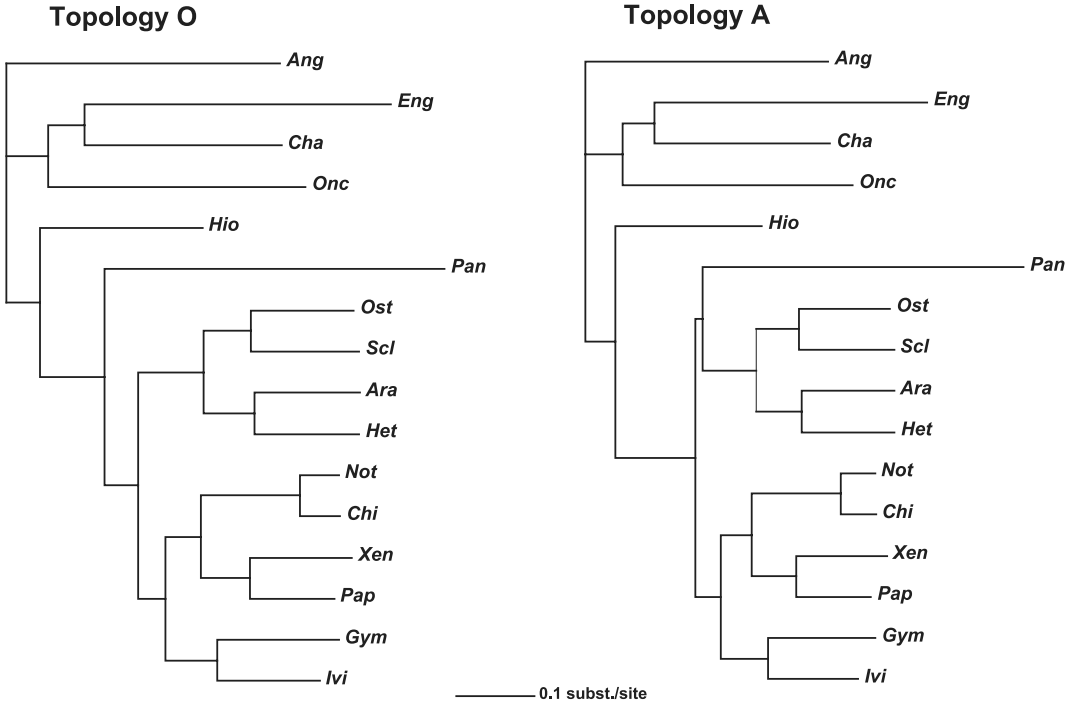

Fig. 6. Tree topologies and ML branch lengths for two alternative phylogenetic positions of *Pantodon buchholzi*. Topology O ("obtained") is that recovered by parsimony and Bayesian analysis of our dataset. Topology A ("alternative") is that consistent with several previous phylogenetic hypotheses for the group based on anatomical characters. ML branch lengths calculated in PAUP*4.0 from GTR + Γ + I model parameters estimated over the combined partition dataset in MrBayes 3.0 in the full analysis (−lnL = 32913.2 and 32899.6 for topology A and O, respectively). Topology A and its ML branch lengths used as model tree to create simulated datasets in Mesquite version 1.0 for subsequent parsimony and Bayesian analysis. Names of taxa abbreviated to first three letters.

Table 3
Parsimony and Bayesian support for three alternative positions for *Pantodon* in a sample of 11 consecutive simulated datasets out of 1000 generated in Mesquite from a model tree of topology A with ML branch lengths estimated from our empirical dataset (Fig. 6)

Sim. matrix	Topology O		Topology A		Toplogy N	
	Parsimony bootstrap	Bayesian posterior probability	Parsimony bootstrap	Bayesian posterior probability	Parsimony bootstrap	Bayesian posterior probability
986	**86**	5	14	**88**	<5	7
987	**83**	<5	17	**100**	<5	<5
988	**81**	15	18	**79**	<5	5
989	**85**	17	11	**65**	<5	18
990	**84**	<5	16	**99**	<5	<5
991	**63**	28	25	**38**	12	34
992	**86**	<5	14	**99**	<5	<5
993	**88**	<5	6	31	6	**62**
994	33	13	**58**	43	9	**44**
995	**95**	13	5	**80**	<5	7
996	**99**	18	<5	**59**	<5	23

(Topology O is the topology recovered by both parsimony and Bayesian from our empirical dataset; topology A is supported by morphological studies, see text.) For 10 out of these 11 simulated datasets, unweighted parsimony incorrectly infers a tree of topology O, while in 9 of 11 Bayesian correctly infers a tree of topology A and never favors topology O. In two cases, Bayesian prefers a third topology (“N”), albeit with weak support. Thus, while parsimony could have recovered topology O with high support values from our empirical dataset even if the real tree is of topology A, this appears unlikely for Bayesian. Parameter settings for PAUP*4.0b10 and MrBayes 3.0 described in text.

ysis also infer the wrong position in the tree for *Pantodon*, and if so, could the posterior probability for this topology be as high as those we obtained (1.0)?

We randomly chose 11 consecutive Mesquite-simulated matrices from the original set of 1000 (modeled on topology A, *Pantodon* branch length unaltered). On these we performed 1000-pseudoreplicate bootstrapping in PAUP*4.0b10 as well as a Bayesian analysis with MrBayes 3.0. Settings for these runs are identical to those reported in Section 2 for analysis of our empirical dataset. Results are given in Table 3. For 10 out of these 11, parsimony incorrectly infers a MP tree of topology O. Among these, mean bootstrap support for topology O is 85%, with a range between 63 and 99%. By contrast, Bayesian never prefers topology O and for 9 of these 11 simulated datasets correctly prefers topology A (with a mean posterior probability of 0.79 and a range of 0.59–1.0). For the two remaining cases, Bayesian inferred highest posterior probability for a third topology (topology “N”) in which *Pantodon* is the sister group to the notopteroid taxa (notopterids + *Ivindomyrus* + *Gymnarchus*), albeit with relatively low posterior probability values (0.62 and 0.44). In all 11 cases, Bayesian estimates of model parameters were very close to those used in Mesquite to simulate the data. Thus, while unweighted parsimony could have recovered topology O with high support values from our empirical dataset even if the real tree is of topology A, Bayesian analysis would be unlikely to do so.

The relative performance of parsimony and Bayesian analysis on artificial datasets in which data are simulated to fit a specified model of evolution may incompletely reflect their performance on real-world datasets such as ours. Nevertheless, in light of *Pantodon*’s long terminal branch, the agreement of the Bayesian analysis of our empirical data with the result from parsimony analysis strengthens our confidence in the phylogenetic placement of *Pantodon*. These analyses of combined sequence data from five molecular markers reject the hypothesis that *Pantodon* is an osteoglossid, or sister group to them, and instead support the hypothesis that *Pantodon* is the sister group to the osteoglossoids and notopteroids combined. Following our results, *P. buchholzi* should not be considered a member of the family Osteoglossidae, but should be placed in its own family, the Pantodontidae. The position of *Pantodon* indicated in our analysis should stimulate reappraisal of phylogenetically informative morphological characters within Recent and fossil osteoglossomorphs. In addition, the discovery of accelerated genetic evolution in the *Pantodon* lineage relative to other osteoglossomorphs deserves further exploration.

Acknowledgments

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