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Phylogenetic systematics of leaffishes (Teleostei: Polycentridae, Nandidae)

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Abstract

The Asian (nandid) and Afro-Neotropical (polycentrid) leaffishes represent two superficially similar, but historically poorly diagnosed families – a situation resulting in a convoluted systematic history. Here, and including for the first time in a molecular study all leaffish genera, we generate a hypothesis of the phylogenetic history of both groups. We analyse a multilocus molecular data set encompassing 257 acanthomorph taxa, carry out a survey and assessment of selected osteological characters for the polycentrid leaffishes and also provide a reanalysis of previously published morphological data. Our results confirm: (1) that the Polycentridae and Nandidae are only remotely related, and hence, the classic leaffishes are diphyletic; (2) that the Polycentridae is monophyletic, with new skeletal synapomorphies being congruent with molecular data in placing the enigmatic *Afronandus* – a taxon that thus far has never been included in any molecular study – as sistergroup to the remaining genera; (3) the monophyly of the Nandidae + Badidae and their inclusion into a larger monophyletic group – along with the Pristolepididae, Anabantoidei and Channoidei – comprising the Labyrinthici *sensu* Rosen & Patterson. We also review the morphological and molecular evidence for both the conflicting placement of *Pristolepis* and the putative sistergroup relationship between the labyrinth fishes (Anabantoidei) and snakeheads (Channoidei).

Key words: Ichthyology – morphology – Percomorpha – molecular systematics

Introduction

The common name 'leaffish' is given to five genera of tropical freshwater percomorph fishes: *Nandus* Valenciennes 1831 from Asia, *Afronandus* Meinken 1955 and *Polycentropsis* Boulenger 1901 from Africa, and *Polycentrus* Müller & Troschel 1849 and *Monocirrhus* Heckel 1840 from South America (Berra 2001). Leaffishes are laterally compressed predators, with a cryptic, leaf-like colouration and shape (Fig. 1); the extreme of this specialization is found in *Monocirrhus* with its petiole-mimicking chin barbel (Eigenmann and Allen 1921). Due to their disjunct distributions, relationships among the leaffishes have been of interest in the study of continental biogeography (Cracraft 1974; Lundberg 1993), but despite their low diversity – *Afronandus*, *Polycentropsis* and *Monocirrhus* are monotypic while *Polycentrus* and *Nandus* have two and six valid species respectively (Ng 2008; Coutinho and Wosiacki 2014) – their phylogenetic affiliations have been difficult to ascertain, due in part to their ostensibly convergent appearances. Consequently, the systematic history of the leaffishes was described by Berra (2001) as 'tortured' (p. 425), with genera having been classified and reclassified in several families on multiple occasions (Table 1). Arguably, the most influential study of leaffish anatomy was conducted by Liem (1970), who concluded all five genera of leaffishes comprised a single family, Nandidae. However, Liem recognized the possibility of convergent evolution in the group, and no reliable synapomorphies have so far been found to unite the Asian genus *Nandus* with the Afro-Neotropical genera.

Afro-Neotropical leaffishes: morphological and ethological data

The absence of evidence supporting Liem's Nandidae was recognized by Britz (1997), who proposed the monophyly of the Afro-Neotropical species in respect to the Asian species on the basis of egg, larval and behavioural characters (Figs 2 and 3). These data additionally suggested *Afronandus* as sistergroup to

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the remaining three polycentrids, which formed an unresolved trichotomy. The only morphological study testing these hypotheses using other suites of characters was the survey of actinopterygian dorsal gill-arch musculature undertaken by Springer and Johnson (2004). That study corroborated the monophyly of the Afro-Neotropical polycentrid genera including *Afronandus* and presented a further three putative synapomorphies for the group (Fig. 3). However, the accompanying parsimony analysis of 56 morphological characters – mainly from the same dorsal gill-arch character system – presented by Springer and Orrell (2004) recovers a paraphyletic Polycentridae, with the inclusion of a batrachoidid in the clade. The characters supporting this polycentrid–batrachoidid lineage were 'dorsal transverse muscles attaching to epibranchial four (TD on Eb4)' and 'absence of sphincter esophagi division (SOD)'. Disregarding the batrachoidid, *Polycentrus* was recovered in their 50% majority-rule consensus as sistergroup to the remaining polycentrids, with *Afronandus* then sistergroup to *Monocirrhus* and *Polycentropsis*.

Asian leaffishes and related groups: morphological and ethological data

Regarding the affinity of the Asian leaffishes, Gosline (1968) hypothesized that based on the unique – among 'higher' teleost fishes – presence of parasphenoid dentition, *Nandus* should be included in a clade comprising the Badidae, Pristolepididae, Anabantoidei and Channoidei, a group referred to but not explicitly diagnosed by Rosen and Patterson (1990) as Cuvier's Labyrinthici (Cuvier and Valenciennes 1828). Liem (1970) rejected the phylogenetic importance of this character due to the inclusion of the polycentrid genera in the Nandidae, but his non-cladistic approach was later questioned by Gosline (1985). Springer and Johnson (2004) agreed with the assessment of Rosen and Patterson (1990), but used the presence of parasphenoid dentition to define the new order Anabantomorpha; Wiley and Johnson (2010) recognized the order Anabantiformes for the anabantoids and channoids only. From herein, we refer to the fishes bearing parasphenoid dentition as the Labyrinthici, as this is the oldest name for the group (Müller 1845).

Fig. 1. Photographs of live leaffishes and selected Labyrinthici for comparison: (a) *Afronandus sheljuzhkoi* (Meinken 1954); (b) *Monocirrhus polyacanthus* Heckel 1840; (c) *Polycentropsis abbreviata* Boulenger 1901; (d) *Polycentrus schomburgkii* Müller & Troschel 1849; (e) *Badis corycaeus* Kullander and Britz 2002; (f) *Dario hysginon* Kullander and Britz 2002; (g) *Nandus nandus* (Hamilton 1822); and (h) *Pristolepis marginata* Jerdon 1849. Photographs (c) and (d) were horizontally flipped to face left.

Among this Labyrinthici group, some relationships are well supported by morphological characters: the labyrinth fishes (Anabantoidei) and snakeheads (Channoidei) both possess a modified suprabranchial (labyrinth) organ used for accessory aerial respiration (Cuvier and Valenciennes 1831; Britz 2003, 2004), while the Asian leaffish *Nandus* was shown by Kullander and Britz (2002) to be the putative sister taxon to the Badidae as indicated by a skeletal synapomorphy of the caudal fin (Fig. 4). Yet, relationships at the base of the Labyrinthici remain less clear, and this is particularly the case for *Pristolepis* Jerdon 1849, a taxon with a generalized percomorph body plan, and for which few phylogenetically informative features other than autapomorphies have so far been uncovered. Two characters are, however, likely to be of importance in inferring its position: (1) Barlow et al. (1968) observed a unique anti-parallel spawning embrace in *Badis* Bleeker 1854, a trait also found to be present in *Nandus* as well as in anabantoids and channids (Britz 1995, 1997, 2003), but lacking in *Pristolepis marginata* Jerdon 1849 (Mercy et al. 2003); and (2) a uniquely derived condition among percomorphs – an additional insertion of rectus ventralis four on the anterior region of ceratobranchial five uniting *Pristolepis*, *Badis* and *Nandus* – as

uncovered by the infrabranchial gill-arch muscle study of Datovo et al. (2014). To summarize current knowledge of the Labyrinthici, we have identified from the literature 13 morphological and behavioural characters of putative phylogenetic informativeness, and these characters are presented as a tree-based argumentation of apomorphies in Fig. 5.

Using the dorsal gill-arch data presented by Springer and Johnson (2004), the Labyrinthici were also investigated by Springer and Orrell (2004), but, despite including all relevant taxa, their parsimony analyses failed to find a monophyletic Labyrinthici, with various atheriniforms, beloniforms, mugiliforms and cyprinodontiforms nested within the group in both their strict and majority-rule consensus trees. However, some of the characters for the Labyrinthici were inadvertently coded incorrectly in this publication (V. Springer, pers. comm.): (1) character three, the absence of external levator three (LE3), should have been coded as present for the badids and nandids; and (2) character 42, the presence/absence of a bony flange on epibranchial four (Eb4), should have been coded as absent for the Channidae and present for the Pristolepididae. Hence, an opportunity is presented to test whether a reanalysed data set can recover a monophyletic Labyrinthici.

Table 1. Summary of the major contributions to the systematics of leaffishes and labyrinth fishes, as well as results from this study.

| Author	Group	Comprising
Günther (1861)	Nandidae	*Badis, Nandus, Pristolepis*
	Polycentridae	*Monocirrhus, Polycentrus*
Regan (1913)	Nandidae	*Nandus, Monocirrhus, Polycentropsis, Polycentrus*
	Pristolepididae	*Badis, Pristolepis*
Jordan (1923)	Nandidae	*Badis, Nandus, Pristolepis*
	Polycentridae	*Monocirrhus, Polycentropsis, Polycentrus*
Barlow et al. (1968)	Badidae	*Badis*
Gosline (1968)	Anabantomorpha (cf. Springer and Johnson, 2004)	Anabantoidei, Badidae, Channidae, *Nandus, Pristolepis*
Liem (1970)	Badidae	*Badis*
	Nandidae	*Afronandus, Nandus, Monocirrhus, Polycentropsis, Polycentrus*
	Pristolepididae	*Pristolepis*
Rosen and Patterson (1990)	Labyrinthici (cf. Cuvier and Valenciennes, 1828)	Anabantoidei, Badidae, Channidae, *Nandus*
Britz (1997)	Polycentridae (cf. Berra, 2001)	*Afronandus, Monocirrhus, Polycentropsis, Polycentrus*
Kullander and Britz (2002)	Badidae	*Badis, Dario*
	Nandidae	*Nandus*
	Pristolepididae	*Pristolepis*
Nelson (2006)	Nandidae (Nandinae)	*Nandus*
	Nandidae (Badinae)	*Badis, Dario*
	Nandidae (Pristolepidinae)	*Pristolepis*
	Polycentridae	*Afronandus, Monocirrhus, Polycentropsis, Polycentrus*
This study	Badidae	*Badis, Dario*
	Nandidae	*Nandus*
	Pristolepididae	*Pristolepis*
	Polycentridae	*Afronandus, Monocirrhus, Polycentropsis, Polycentrus*
	Labyrinthici	Anabantoidei, Badidae, Channidae, Nandidae, Pristolepididae
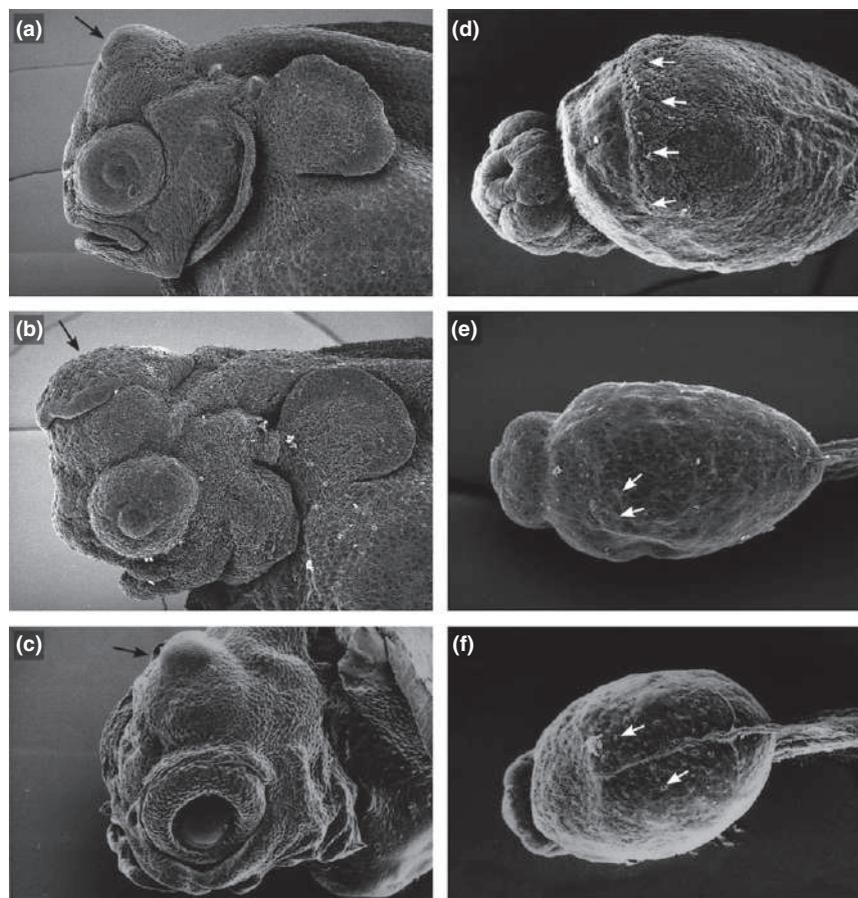
Fig. 2. Scanning electron micrographs (SEMs) showing the larval substrate attachment organs (cement glands) in Polycentridae, Badidae and Nandidae, with the addition of new data for *Dario hysginon* and *Polycentrus schomburgkii* (previous images as presented by Britz 1997). a–c shows lateral view of the location of the multicellular attachment organ in the Polycentridae (a, *Polycentrus schomburgkii*; b, *Monocirrhus polyacanthus*; c, *Polycentropsis abbreviata*). d–f shows ventral view of larval yolk sacs, highlighting the individual cement cells in Badidae [d, *Badis badis* (Hamilton 1822); e, *Dario hysginon*] and Nandidae (f, *Nandus nandus*). Note that *Dario* resembles *Nandus* in having the plesiomorphic condition of only few scattered attachment cells, which are more numerous and cluster together forming an anterior ridge on the yolk sac of *Badis*. For materials and methods, see Britz (1997).
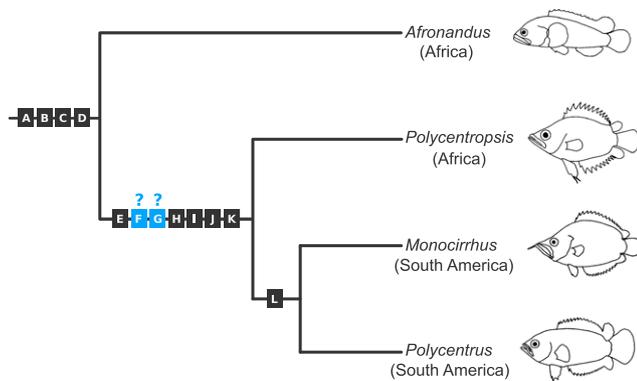
Fig. 3. Tree-based argumentation scheme for apomorphic characters of putative phylogenetic significance in the Polycentridae following Britz (1997), Springer and Johnson (2004), and this study: (A) adhesive filaments at the vegetal egg pole (Britz 1997); (B) loss of external levator three (LE3; Springer and Johnson 2004); (C) loss of sensory canals on most lateral-line scales, including all of those on posterior half of body (Springer and Johnson 2004); (D) the presence of slender ligament attaching dorsal margin of epibranchial one (Eb1) anterior to external levator one (LE1) to ventral surface of skull at or near origin of LE1 (Springer and Johnson 2004); (E) characteristic pattern of ridges on the micropylar region of the egg (Britz 1997); (F) larvae with a multicellular cement gland organ on top of the head (Britz 1997); (G) upside-down female spawning procedure (Britz 1997); (H) reduction in vertebral number (this study); (I) increase in number of anal- and dorsal-fin spines (this study); (J) loss of coronalis pore (this study); (K) full neural spine on PU2 (this study); and (L) three pterygiophores anterior to first haemal spine (this study). Blue boxes with question marks signify characters where no data are available for *Afronandus*. The figure is annotated with line drawing examples of taxa (not to scale).

Molecular phylogenetics of leaffishes and their relatives

A number of the recent large-scale molecular studies that attempted to resolve the phylogenetic relationships of the extremely speciose Percomorpha – e.g., Sparks and Smith (2004), Santini et al. (2009), Wainwright et al. (2012), Betancur-R et al. (2013a), Friedman et al. (2013), McMahan et al. (2013), Near et al. (2013) – included leaffish taxa and related groups, but also produced sometimes contradictory phylogenetic trees and resulting classifications for these taxa. In all of these studies, leaffishes appear only in the respective trees accompanying the publications, but their phylogenetic position is neither mentioned nor discussed. Both Sparks and Smith (2004) and McMahan et al. (2013) showed the African leaffish *Polycentropsis* as sistergroup to the Asian *Badis* (to the exclusion of the South American *Monocirrhus*), while Wainwright et al. (2012), Betancur-R et al. (2013a), Friedman et al. (2013) and Near et al. (2013) showed monophyly of Afro-Neotropical leaffishes (see Table S1 for taxon coverage of these studies). The tree of Santini et al. (2009) shows a paraphyletic Channidae (*Parachanna* Teugels & Daget 1984 closer to *Nandus* than *Channa* Scopoli 1777) and a polyphyletic Anabantoidei (Osphronemidae sistergroup to all other Labyrinthici). The classification of Betancur-R et al. (2013a) divided the Anabantiformes of Li et al. (2009) – Anabantidae, Channidae, Mastacembelidae, Symbranchidae [sic] and Indostomidae – into two orders (Synbranchiformes and Anabantiformes), both comprising the 'Anabantomorphariae'; their Anabantiformes were restricted to Channoidei + Anabantoidei with the Channoidei comprising Channidae + Nandidae. Near et al. (2013) presented a monophyletic group referred to as the Nandidae comprising *Pristolepis*, *Badis*, *Dario* Kullander and Britz 2002, and *Nandus*; this group was in turn sistergroup to a

clade labelled Anabantiformes (Anabantoidei + Channoidei). An update to the Betancur-R et al. (2013a) classification, published online (Betancur-R et al. 2013b; accessed 12 December 2013), increased taxon sampling to include the data from Near et al. (2013), but presented a different resulting tree from Near et al. (2013), with the Channidae being sistergroup to the Pristolepididae, Badidae and Nandidae rather than the Anabantoidei; the resulting classification of Betancur-R et al. (2013b), however, placed the newly analysed Badidae and Pristolepididae in the Anabantoidei, despite this placement being inconsistent with their new tree topology. The latest iteration of these online classifications (Betancur-R et al. 2014; accessed 2 August 2014) removed the Nandidae from the Channoidei and the Badidae and Pristolepididae from the Anabantoidei and created the Nandoidei for these three families. A summary of the Labyrinthici intrarelationships is presented in Table 2 and shows that, despite many studies, there remains significant uncertainty in their suprafamilial relationships. Key inconsistencies to be noted are the position of *Pristolepis* and the monophyly of the Anabantoidei + Channoidei, a group regarded as morphologically incontrovertible (Britz 2004; Springer and Johnson 2004; Wiley and Johnson 2010).

Study aims

Despite previous morphological studies (Britz 1997; Springer and Johnson 2004) hypothesizing two distinct families of leaffish (Afro-Neotropical Polycentridae, Asian Nandidae), and the string of recent reclassifications of Asian leaffishes and labyrinth fishes based on molecular trees (Betancur-R et al. 2013a,b, 2014), there remains to be an explicit synthesis and targeted investigation of relationships among these groups using a complete sample of data for all relevant genera (see Table S1 for a summary of sampling in previous studies). In particular, no molecular study has included the rare taxon *Afronandus sheljuzhkoi* (Meinken 1954) – a species described from the Ivory Coast in 1954 and only known from a handful of museum specimens – whose phylogenetic position is uncertain and critical to any attempt at biogeographical inference of the group (Lundberg 1993; Britz 1997). Additionally, and given that only egg, larval, behavioural and gill-arch muscle characters have previously been used to elucidate polycentrid relationships, there also remains the opportunity to investigate whether osteological characters can support the previous findings or whether they can highlight intrarelationships with a greater resolution.

Sampling all relevant genera, and including the key taxon *Afronandus*, this study aims to investigate the following: (1) the monophyly of the Polycentridae using molecular data; (2) intrarelationships of the Polycentridae using osteological characters and molecular data; (3) the monophyly of the Labyrinthici by reanalysing the amended morphological data matrix of Springer and Orrell (2004); and (4) monophyly and intrarelationships of the Labrinthici using molecular data, with a special focus on the Asian leaffish *Nandus*, the anabantoid–channoid sistergroup relationship and the phylogenetic placement of *Pristolepis*.

Materials and Methods

Overview of analyses

All computational procedures, unless otherwise stated, were conducted in the R 3.1.1 environment (R Core Team, 2014). Data matrices and trees were manipulated using the following R packages: APE 3.2 (Paradis et al. 2004), PHANGORN 1.99-12 (Schliep 2010), PHYLOCH 1.5-3 (Heibl 2013), PHYTOOLS 0.4-49 (Revell 2012) and SPIDER 1.3-0 (Brown et al. 2012). To ensure repeatability of analyses, data sets as well as commands for each software application used are provided as a Supporting Information
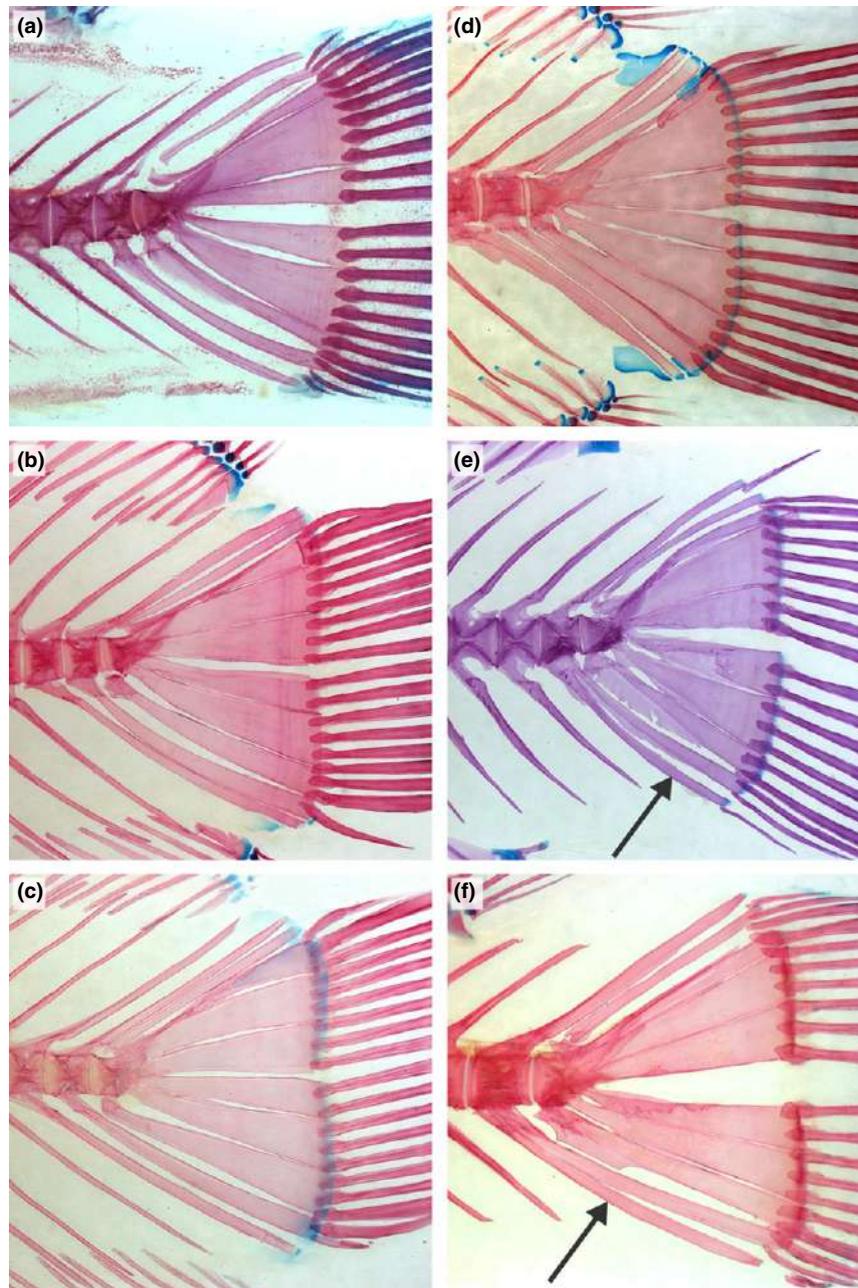
Fig. 4. Cleared and double-stained caudal skeleton of: (a) *Afronandus sheljuzhkoi* BMNH uncatalogued; (b) *Monocirrhus polyacanthus* BMNH uncatalogued; (c) *Polycentropsis abbreviata* BMNH uncatalogued; (d) *Polycentrus schomburgkii* BMNH uncatalogued; (e) *Badis siamensis* BMNH uncatalogued; and (f) *Nandus nandus* BMNH uncatalogued. Arrows indicate location of synapomorphy – distally divided haemal spine on second preural centrum – uniting the Badidae and Nandidae.

hosted at the Natural History Museum Data Portal (http://data.nhm.ac.uk) and can be accessed via the following stable URL: http://dx.doi.org/10.5519/0065571.

Osteological study and reanalysis of morphological characters

All polycentrid species including *Afronandus sheljuzhkoi* were examined for skeletal features following clearing and double staining of material for bone and cartilage (Taylor and Van Dyke 1985).

To test whether the reanalysed data set of Springer and Orrell (2004) would result in monophyletic Polycentridae and Labyrinthici clades after recoding the erroneous character states, we carried out a parsimony analysis on the amended 56 character, 169 taxon matrix using TNT (Goloboff et al. 2008). A 'new technology' search strategy was employed

(level 10 *xmult* search, 500 hits to best tree length), with the resulting shortest trees summarized into a 50% majority-rule consensus.

Taxon sampling and genetic markers

To assess the phylogenetic position of the leaffish groups, we added nucleotide sequences for representatives of all leaffish genera and putatively related groups to published percomorph data for five nuclear genes (Near et al. 2012; Wainwright et al. 2012). As well as an estimation of overall relationships among percomorphs, we also explored conflicting phylogenetic signal among the Labyrinthici in a reduced analysis including three additional mitochondrial genes.

The five single-copy nuclear exons chosen comprised *plagl2*, *sreb2*, *SH3PX3* and *zic1* (Li et al. 2007), and exon three of *rag1* (López et al.
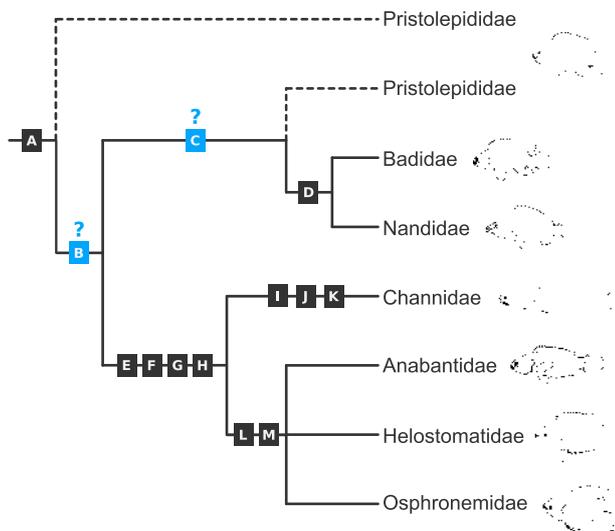
Fig. 5. Tree-based argumentation scheme for apomorphic characters of putative phylogenetic significance in the Labyrinthici: (A) teeth on the parasphenoid (Gosline 1968); (B) unique antiparallel spawning embrace (Barlow et al. 1968; Britz 1997; Mercy et al. 2003); (C) additional insertion of rectus ventralis four on the anterior region of ceratobranchial five (Datovo et al. 2014); (D) distally divided haemal spine on second preural centrum (Kullander and Britz 2002); (E) first epibranchial modified into suprabranchial organ with unique blood supply (Cuvier and Valenciennes 1831; Britz 2004); (F) basioccipital with paired articular processes forming diarthrosis with upper pharyngeal jaws (Britz 1995, 2003, 2004); (G) gas bladder extending posteriorly to parhypural (Britz 1995, 2003); (H) larvae with bilateral pair of oil vesicles as floating device (Cambray 1990; Britz 1995); (I) otic bulla for sacculith mostly contained in prootic (Lauder and Liem 1983); (J) metapterygoid with anterodorsal uncinate process approaching neurocranium (Lauder and Liem 1983) or articulating with it (Bhimachar 1932; Berg 1947); (K) an autogenous elongate bone – like a detached haemal spine – between haemal spines of PU2 and PU3, first figured and mentioned by Day (1914); (L) suprabranchial air chamber clearly separated from buccal cavity, and respiratory air confined to suprabranchial cavity (Lauder and Liem 1983); and (M) distinct foramen exoccipitale covered by tympanum-like membrane present in exoccipital (Lauder and Liem 1983). Dashed branches and blue boxes with question marks signify the conflicting characters pertaining to the position of *Pristolepis*. The figure is annotated with line drawing examples of taxa (not to scale).

2004). To select an in-group taxon set, sequence information from Near et al. (2012) and Wainwright et al. (2012) was collated into a single table (Table S2). Only percomorph fishes were retained in the table for the analysis along with eight polymixiiform, percopsiform and trachichthyoid beryciforms as out-groups. To assist filtering, the table was augmented with higher taxonomic classification (family, order) using RFISHBASE 0.0-9 (Boettiger et al. 2012). An R script was used to download sequences from GenBank according to a filter rule in the table; the rule was set to populate a semicomplete matrix of taxa for which at least three of the five genes were available. Because of the demonstrated phylogenetic utility of the *rag1* gene (Rüber et al. 2004, 2006; San Mauro et al. 2009) and the

importance of selecting informative genes (Salichos and Rokas 2013), taxa missing this gene were also excluded. A missing *rag1* sequence for *Oreochromis niloticus* (Linnaeus 1758) was added from the Ensembl genome browser (ENSONIG00000014593), and GenBank searches were conducted to fill any other gaps (accessions added to table).

Preliminary analyses showed four taxa – *Callionymus bairdi* Jordan 1888, *Perccottus glenii* Dybowski 1877, *Eleotris pisonis* (Gmelin 1789) and *Lepidogobius lepidus* (Girard 1858) – on disproportionally long branches, and as these species were not of direct interest, we decided to exclude them to minimize extreme rate variation or potentially erroneous sequences. The final data set comprised 257 taxa, with 249 of these being percomorphs.

A second, smaller analysis of the Labyrinthici was carried out using the same five nuclear genes (nDNA), but with the addition of mitochondrial (mtDNA) data from the protein-coding cytochrome *b* gene and a contiguous section of ribosomal 12S rRNA, tRNA-Val and 16S rRNA. The adrianichthyid *Oryzias latipes* (Temminck & Schlegel 1846) was used as an out-group taxon, along with the synbranchiforms *Monopterus albus* (Zuiew 1793) and *Macrognathus siamensis* (Günther 1861); these latter two were included as they are putatively closely related to the Labyrinthici according to molecular studies (Chen et al. 2003; Dettai and Lecointre 2005; Li et al. 2009; Wainwright et al. 2012; Betancur-R et al. 2013a). This data set comprised a total of 36 taxa.

DNA extraction, PCR amplification and sequencing

Tissue samples were maintained in absolute ethanol, and genomic DNA was extracted from muscle tissue or fin clips using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's protocol. PCR was carried out in 25 μl reactions using the QIAGEN Multiplex PCR Master Mix, again following the manufacturer's protocol. Primer combinations and annealing temperatures for each locus are presented in Table S3. PCR products were checked for quality and length conformity on a 1% agarose gel before being bidirectionally Sanger sequenced (for protocol see Rüber et al. 2006).

Alignment of sequence data

Sequence data were obtained from chromatograms using FINCHTV 1.4 (GEOSPIZA) and aligned together with the GenBank data using MAFFT version 7.123 (Katoh and Standly 2013). Resulting alignments were then assessed and edited manually using translated amino acids in MEGA 5.1 (Tamura et al. 2011). Alignments were trimmed at both the ends to reduce the number of potentially misinformative sites due to the increased chance of sequencing error in these regions (Stoeckle and Kerr 2012). For the ribosomal data, divergent and ambiguously aligned blocks were removed using GBLOCKS 0.91b (Castresana 2000; Talavera and Castresana 2007).

Molecular phylogenetic analyses

For the 257-taxon nDNA data set, a suitable partitioning and substitution model scheme was generated using PARTITIONFINDER 1.1.1 (Lanfear et al. 2012). The *a priori* defined data blocks for PARTITIONFINDER comprised each separate codon position for each gene (total 15 data blocks). The selected partitioning scheme comprised two partitions: first + second codon positions of all genes together and third codon positions of all genes together. Using these partitions, a maximum-likelihood (ML)

Table 2. Summary of the conflicting topologies reported from molecular studies including representatives from multiple lineages of Labyrinthici. The 'Polycentropsis' sample used by Sparks and Smith (2004) and McMahan et al. (2013) is a *Nandus*, and has been renamed as Nandidae here and marked with an asterisk (see discussion section for more details).

| Author	Topology
Betancur-R et al. (2013a)	(Anabantoidei,(Channidae,Nandidae))
Betancur-R et al. (2013b)	(Anabantoidei,(Channidae,(Pristolepididae,(Badidae,Nandidae))))
Near et al. (2013)	((Anabantoidei,Channidae),(Pristolepididae,(Badidae,Nandidae)))
McMahan et al. (2013)	((Anabantoidei,Pristolepididae),(Badidae,Nandidae*))
Santini et al. (2009)	(Osphronemidae,((*Channa*,(*Parachanna*,Nandidae)),(Helostomatidae,Anabantidae)))
Sparks and Smith (2004)	(Pristolepididae,(Anabantoidei,(Badidae,Nandidae*)))
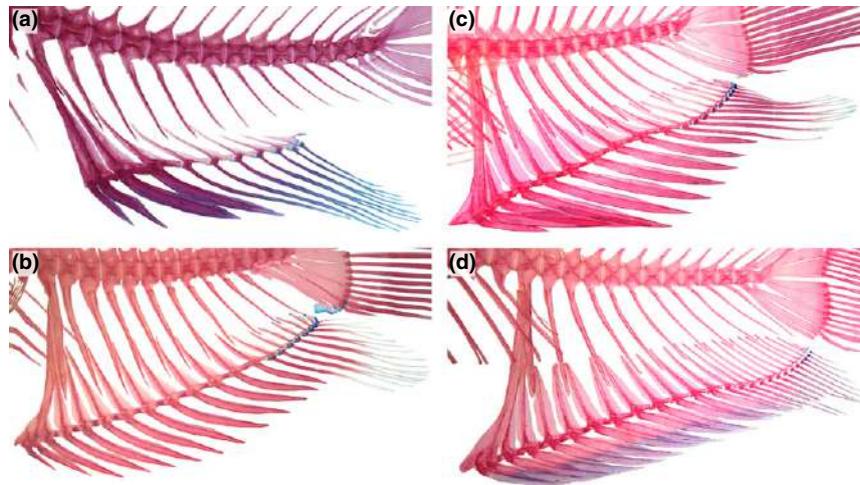
Fig. 6. Cleared and double-stained skeletons of: (a) *Afronandus sheljuzhkoi* BMNH uncatalogued; (b) *Polycentrus schomburgkii* BMNH uncatalogued; (c) *Polycentropsis abbreviata* BMNH uncatalogued; and (d) *Monocirrhus polyacanthus* BMNH uncatalogued. An increase in the number of anal-fin pterygiophores and the subsequent reduction of caudal peduncle length is evident in *Polycentropsis*, *Polycentrus* and *Monocirrhus*.

analysis was carried out from 48 random starts using the 'GTRGAMMA' model in RAXML 8.1.15 (Stamatakis 2006). Node support was estimated with 1000 replications of the 'GTRCAT' rapid bootstrapping method (Stamatakis et al. 2008). Individual gene trees for each nuclear locus were also generated using RAXML, but were not partitioned.

Bayesian analysis of the 36-taxon nDNA + mtDNA data set was conducted using MRBAYES 3.2.4 (Ronquist et al. 2012), in conjunction with the BEAGLE library (Ayres et al. 2012). Data were again partitioned after using PARTITIONFINDER, in this case, into four partitions: nDNA first + second codon positions, nDNA third codon positions, rRNAs and cyt*b* first + second codon positions and cyt*b* third codon positions. Substitution models were averaged using reversible jump MCMC (Huelsenbeck et al. 2004), and rate heterogeneity across sites was gamma distributed (four Γ categories). Four independent runs of 30 million generations (single cold chain for each run) were carried out from random starting topologies, resulting in 10 004 trees in total. Chain convergence, effective sample size (ESS) and parameter mixing was assessed using TRACER 1.5 (Rambaut and Drummond 2009) and AWTY (Nylander et al. 2008); 2004 of the trees were discarded as burn-in, and the 8000 postburnin trees were summarized using TREEANNOTATOR 1.8.1 (mean node heights).

Topology tests were carried out using Shimodaira–Hasegawa (SH) tests (Shimodaira and Hasegawa 1999) in PHANGORN, while conflicting topologies among the postburnin MRBAYES trees were visualized using the Lento plot (Lento et al. 1995) function also implemented in PHANGORN.

Results

Osteological study of Polycentridae

The osteological study revealed the following characters in *Afronandus* (Fig. 6), which are hypothesized to be plesiomorphic at the level of Polycentridae: (1) the presence of 26–27 vertebrae; (2) the presence of only 14 dorsal- and four anal-fin spines; (3) the presence of only one pterygiophore in front of first haemal spine; (4) the presence of a coronalis pore in the dorsal midline of the epiphyseal cross-connection of the supraorbital canals; and (5) reduced neural spine on preural centrum two.

In the remaining three polycentrids, the number of vertebrae is reduced to 23–24, while the number of fin spines is increased to 15 (*Polycentropsis*), 17 (*Monocirrhus*) and 18 (*Polycentrus*) in the dorsal fin, and to 10 (*Polycentropsis*) or 13 (*Monocirrhus*, *Polycentrus*) in the anal fin, resulting in a shorter caudal peduncle (Fig. 6). This higher number of dorsal and anal-fin spines and their supports, a synapomorphy of these three polycentrids, has been achieved by adding pterygiophores at the end of the

dorsal and anal fins. While the posteriormost pterygiophore of the dorsal fin is situated between neural spines 19/20 in *Afronandus*, it is located between neural spines 20/21 in *Polycentropsis* and *Monocirrhus*, and 21/22 in *Polycentrus*. The posteriormost pterygiophore of the anal fin of *Afronandus* sits between haemal spines 18/19, but between haemal spines 19/20 in *Monocirrhus* and 20/21 in *Polycentropsis* and *Polycentrus*. A further solution to the problem of fitting more pterygiophores and associated fin rays into a shorter body in *Polycentropsis*, *Polycentrus* and *Monocirrhus* has been achieved by adding more pterygiophores into the fewer interneural and interhaemal spaces, so that each space accommodates 2–3 pterygiophores in *Polycentropsis*, *Polycentrus* and *Monocirrhus* rather than the 1–2 in *Afronandus*. A third source for the higher number of the anal-fin pterygiophores and associated rays is the addition of 1–2 pterygiophores at the front end of this fin. *Afronandus* has only a single pterygiophore with two associated fin rays – one serially associated, one in supernumerary association – in front of the first haemal spine, while *Polycentropsis* has two, and *Polycentrus* and *Monocirrhus* each has three pterygiophores in front of the first haemal spine (Fig. 6).

In *Polycentropsis*, *Polycentrus* and *Monocirrhus*, the coronalis pore of the head lateral line, which sits at the junction in the dorsal midline of the left and right supraorbital canals, is absent. This means that both epiphyseal connecting canals are now confluent. This rearrangement has to do with the posterior extension of the much more elongated ascending processes of the premaxillae, which reach beyond the epiphyseal cross-connection in these three taxa compared to *Afronandus*. In the latter, the posterior tips of the ascending premaxillary processes end in front of the connecting canals and in front of the coronalis pore, representing the plesiomorphic condition. *Polycentropsis*, *Polycentrus* and *Monocirrhus* also share the presence of a full neural spine on PU2, another putative synapomorphy for these three polycentrids. A reduced neural spine on PU2 has been hypothesized to represent a primitive condition at the level of Acanthopterygii (Rosen 1973). Among *Polycentropsis*, *Polycentrus* and *Monocirrhus*, the latter two seem to be more closely related to each other than either one is to *Polycentropsis*. Characters in support of this hypothesis are the shared presence of three pterygiophores in front of the first haemal spine and the increase in the number of spines in the dorsal and anal fins compared to *Polycentropsis*, both mentioned above.
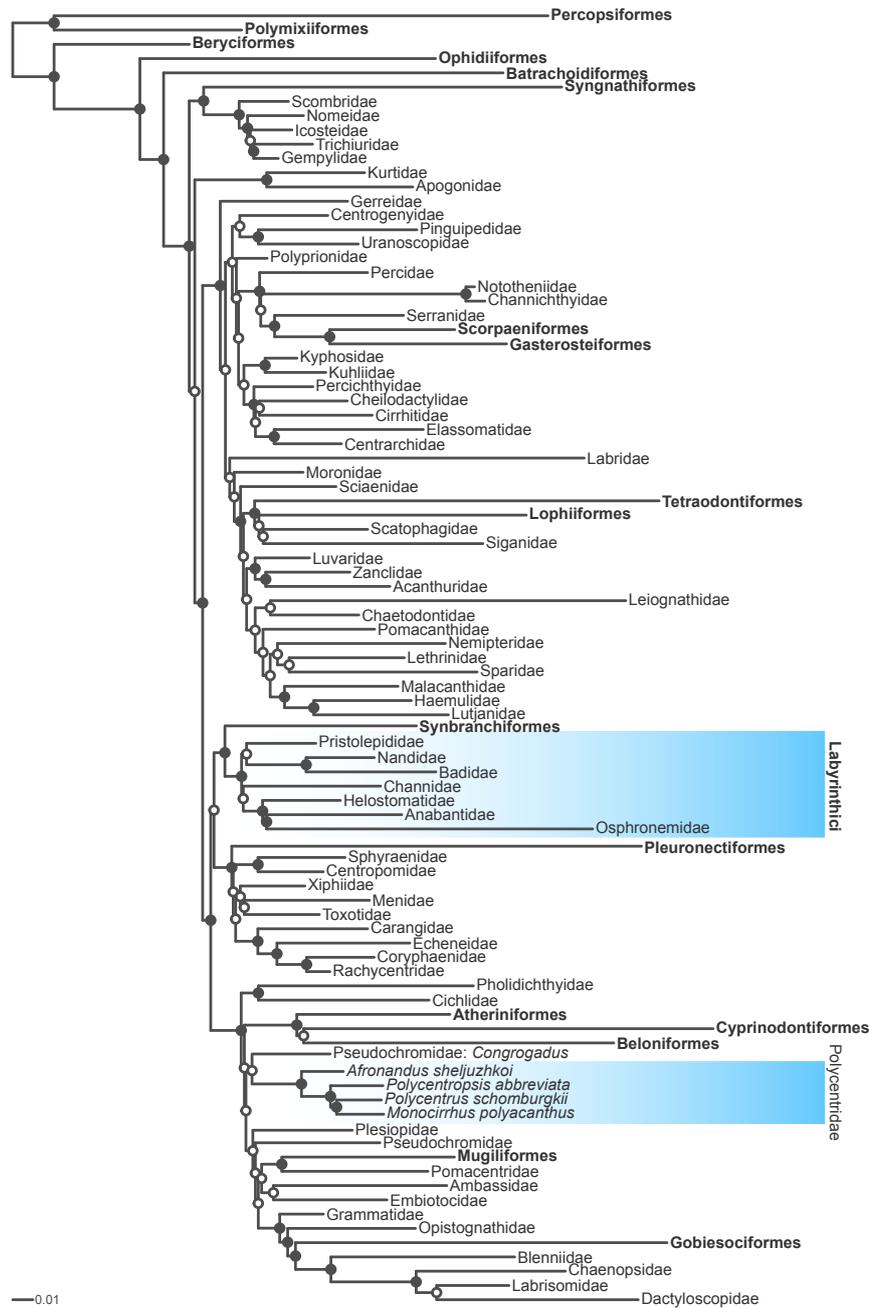
Fig. 7. RAXML tree of the 257-taxon analysis (4491 bp from five concatenated nuclear markers). Taxa of interest are highlighted in blue boxes. Nodes are coloured according to bootstrap (BS) support (filled circles = BS ≥ 0.7; unfilled circles = BS < 0.7). Terminal taxa are collapsed into orders (bold font), or families if Perciformes. Non-monophyletic orders such as Scorpaeniformes were presented as monophyletic to aid visualization. The Labridae includes the Odacidae and Scaridae.

Parsimony reanalysis of Springer and Orrell (2004)

Our parsimony reanalysis of the data set of Springer and Or-rell (2004) recovered 2637 most parsimonious trees of 604 steps (Figure S1). The Polycentridae was not monophyletic due to the inclusion of the Batrachoididae; the clade was sup-ported by five putative synapomorphies (characters 3, 6, 15, 17 and 18 of Springer and Orrell 2004). *Polycentrus* was sist-ergroup to the remaining polycentrids (plus the batrachoidid), which formed a polytomy. The Labyrinthici was monophyletic (supported by characters 40 and 55) and fully dichotomous, with the Nandidae + Badidae (character 32) sistergroup to the Pristolepididae + Channidae + Anabantidae (characters 36 and 43); the latter two air-breathing families were grouped together

to the exclusion of the Pristolepididae (characters 6, 19, 44 and 54).

Molecular data summary

For the 257-taxon nDNA analysis, we downloaded 1025 sequences from GenBank for the five genes (Table S2). In addi-tion, we provide 172 new sequences for 38 taxa (GenBank accessions KR131444-KR131615; Table S4). The final 257-taxon alignment totalled 4491 nucleotides, and individually the gene alignments comprised the following: *plagl2* (235 taxa, 666 bp, mean 646); *rag1* (257 taxa, 1341 bp, mean 1318 bp); *SH3PX3* (246 taxa, 687 bp, mean 683 bp); *sreb2* (218 taxa,
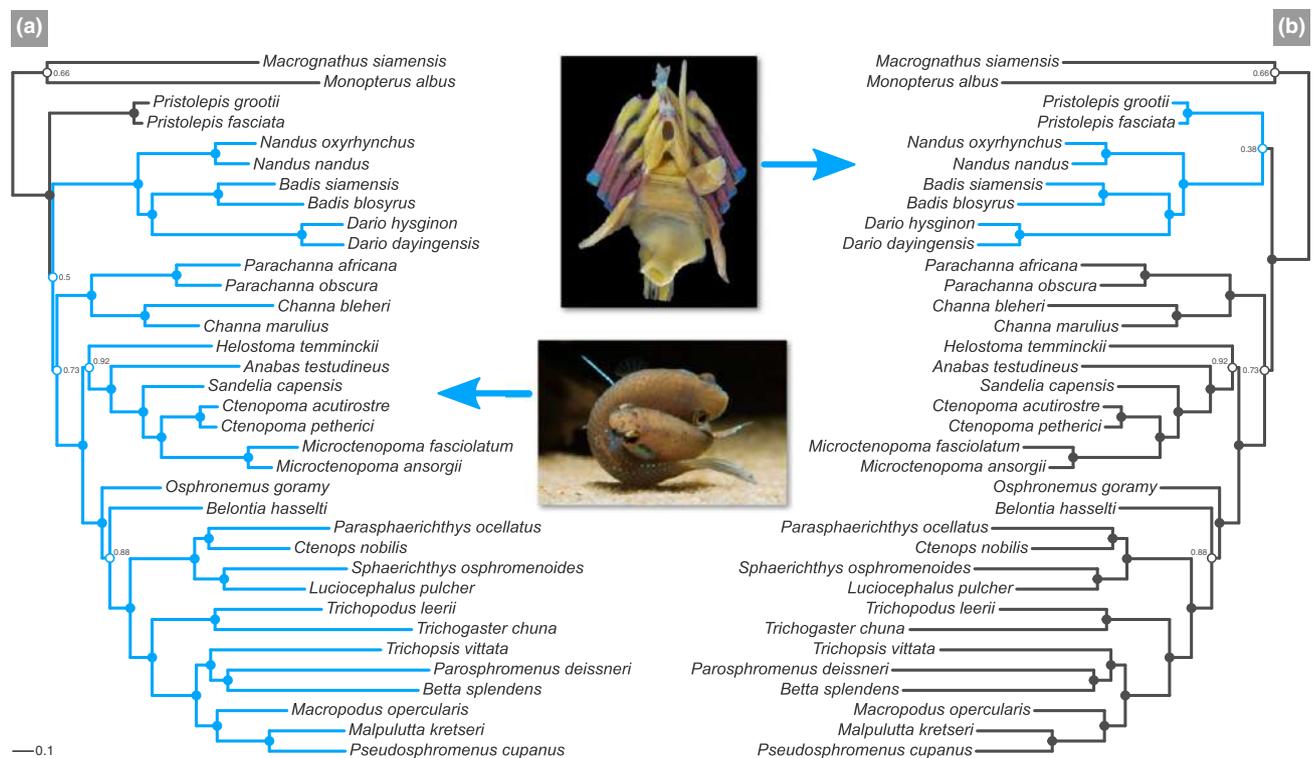
Fig. 8. Intrarelationships among the Labyrinthici inferred from the 36-taxon analysis (7619 bp of combined nDNA and mtDNA). Two summaries are provided from the 8000 postburnin MRBAYES trees: (a) the topology with highest overall Bayesian posterior probability (= 0.21) supporting the spawning embrace character uniting all Labyrinthici to the exclusion of *Pristolepis* (Britz 1997); and (b) the topology with the next highest posterior probability (= 0.16) supporting the ventral gill-arch character uniting *Pristolepis*, *Nandus*, *Badis* and *Dario* (Datovo et al. 2014). Inset images picture these characters: *Pristolepis fasciata* (Bleeker 1851) ventral gill arch above (for details on the exact location of the character please refer to Datovo et al. 2014), and spawning position of *Betta kuehnei* Schindler & Schmidt 2008 below. Nodes are coloured according to posterior probability (BPP); well-supported nodes are shown with filled circles (BPP ≥ 0.95), while poorly supported nodes (BPP < 0.95) are shown with unfilled circles (values added). The out-group *Oryzias* Jordan & Snyder 1906 is not shown.

942 bp, mean 932 bp); and *zic1* (246 taxa, 855 bp, mean 827 bp). The 36-taxon nDNA + mtDNA analysis comprised a final data set of 7619 bp after exclusion of the ambiguously aligned sites in the rRNA data.

Polycentrid relationships: molecular data

A monophyletic group containing the four polycentrids was present in the concatenated 257-taxon nDNA analysis (Fig. 7), as well as in all individual gene trees (Figures S2–S6). *Afronandus* was found to be the sistergroup of the other three polycentrids, with the South American genera monophyletic (Fig. 7). All nodes in this clade were supported by bootstrap (BS) values ≥0.90. The family was found as sistergroup to the pseudochromid *Congrogadus subducens* (Richardson 1843), but this relationship was not well supported (BS = 0.47). Overall, the polycentrid leaffishes were nested among the 'sticky egg' clade of percomorphs, that is the Ovalentaria of Smith & Near in Wainwright et al. (2012) or the Stiassnyiformes of Li et al. (2009). The full, uncollapsed ML tree is presented in Figure S7.

Labyrinthici relationships: molecular data

In the 257-taxon nDNA analysis (Fig. 7), the Labyrinthici formed a well-supported clade sistergroup to the Synbranchiformes (BS = 0.99), which in turn was the sister taxon of a diverse group comprising pleuronectiforms, carangiforms and echeneids among others. Within the Labyrinthici, two main clades were found: Pristolepididae + Nandidae + Badidae and Channoidei + Anabantoidei, although neither group was well supported (BS = 0.32 and 0.37, respectively). The Nandidae and Badidae formed a monophyletic group with high bootstrap support (BS = 1).

Notwithstanding the placements of *Helostoma* Cuvier 1829 and *Parasphaerichthys* Prashad & Mukerji 1929, the 36-taxon Bayesian analysis of nDNA + mtDNA (Fig. 8) was largely congruent with the 257-taxon ML topology (Fig. 7); the taxa with labyrinth organs (Channoidei and Anabantoidei) formed a monophyletic group (Bayesian posterior probability = 0.73), as did the Nandidae + Badidae (BPP = 1). Of note was the conflicting placement of *Pristolepis*, which was placed in the maximum clade credibility tree (Fig. 8a) as sistergroup to the rest of the Labyrinthici (BPP = 0.50). However, in agreement with the 257-taxon ML tree, the topology with the second highest probability in the posterior sample (Fig. 8b) recovered *Pristolepis* as sistergroup to the Nandidae + Badidae clade (BPP = 0.38), and the tree was not rejected by an SH test (p = 0.16). Alternative topologies also sampled in the posterior, but unable to be rejected, included Pristolepididae + Channidae + Anabantoidei (BPP = 0.03; p = 0.13) and Channidae + Nandidae + Badidae (BPP = 0.13; p = 0.29/0.47, depending on the relative position of *Pristolepis*). Split frequencies for clades of interest are visualized as a Lento plot presented in Fig. 9.
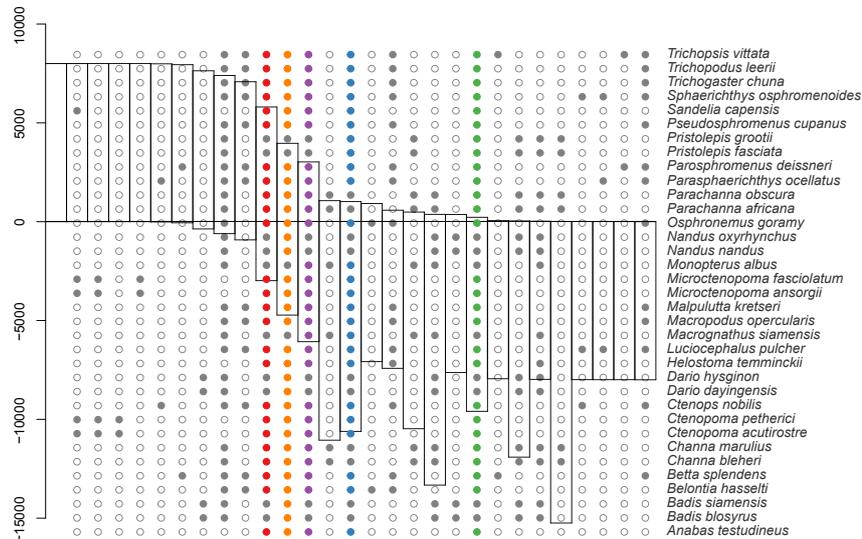
Fig. 9. Lento plot of intrarelationships among the Labyrinthici from 8000 postburnin MRBAYES trees for the 36-taxon analysis (7619 bp of combined nDNA and mtDNA). The x-axis represents each phylogenetic split, with filled circles indicating the clade composition of each split; the y-axis shows relative support (values above zero) or conflict (values below zero) for that split. Splits of interest are colour coded as follows: Channoidei + Anabantoidei (red); monophyly of Labyrinthici clade excluding Pristolepididae (orange); Pristolepididae + Nandidae + Badidae (purple); Channidae + Nandidae + Badidae (blue); and Pristolepididae + Channoidei + Anabantoidei (green). Plot does not show all splits in the posterior sample; most splits with unequivocal or negligible support were removed to aid visualization.

Discussion

Leaffish diphyly and polycentrid monophyly

The data presented here support the morphology-based hypotheses of Britz (1997) and Kullander and Britz (2002), as well as the tree topologies published in more recent molecular studies (Betancur-R et al. 2013a; Near et al. 2013), by showing that the Afro-Neotropical leaffishes are only remotely related to the Asian leaffishes therefore rejecting the monophyly of the Nandidae *sensu* Liem (1970). Despite Liem's own doubts about the monophyly of this group, the classification he proposed persists in the scientific (Sparks and Smith 2004; Froese and Pauly 2014) and popular literature (Wikipedia, 2014). The support for a monophyletic Polycentridae including *Afronandus* is compelling, with congruence between our nuclear gene analyses, morphological characters (Britz 1997; Springer and Johnson 2004; this study) and also analysis of mitochondrial DNA (Figure S8). Two previous studies, however, did not report their polycentrid taxa forming a monophyletic group: both the phylogenetic hypotheses of Sparks and Smith (2004) and McMahan et al. (2013) found *Polycentropsis* as sistergroup to *Badis*. To shed light on this contradictory result, we suggest here that the *Polycentropsis* used in these two studies is, in fact, a misidentified *Nandus*. We found that the 16S sequence deposited in GenBank (AY662705) clusters among our *Nandus* samples rather than with the polycentrids (Figure S8). Further sequences (AY662756, AY662881 and AY662805) may also have been generated from the same misidentified specimen and should therefore be used with caution in future studies. Springer and Orrell (2004) also reported the non-monophyly of the Polycentridae with a batrachoidid nested within this family in their parsimony consensus tree. Our reanalysis of the amended matrix failed to change this result, which was described by Springer & Orrell as 'improbable' and ignored by Springer and Johnson (2004), who reasserted the monophyly of the Polycentridae.

Our analyses were unable to offer a convincing hypothesis as to the exact phylogenetic placement of the polycentrid leaffishes, beyond being part of the sticky egg clade of percomorphs (Wain-

wright et al. 2012). The recent studies of Wainwright et al. (2012), Betancur-R et al. (2013a), Near et al. (2013) and Friedman et al. (2013) were also unable to resolve this problem with confidence; bootstrap support was low, and internodes were short in this part of their trees.

Polycentrid intrarelationships

While the Afro-Neotropical leaffishes of the family Polycentridae form a monophyletic unit, their intrarelationships have not been previously resolved. As discussed, Liem (1970) provided a monographic treatment of leaffish osteology and functional head anatomy, but still included the Asian genus *Nandus*. He concluded that 'the evolutionary series *Nandus–Afronandus–Polycentrus–Polycentropsis–Monocirrhus*' does not represent a 'single phylogenetic line', but that 'it reflects the best documented evolutionary pathway'. He also concluded (p. 153) that 'most of the differences are correlated with differences in the relative growth of bones within mechanical units and degrees of ossification'. Like Liem, we also found that a large number of skeletal differences among the four species are related to different shapes and sizes of bones, which are difficult to objectively evaluate and therefore in our opinion are of lesser value for phylogenetic arguments. We also found that each species has a high number of autapomorphies, often reductions, which in this case provide no clue as to their relationships.

The osteological analyses indicate *Afronandus* as sistergroup to remaining polycentrids, as shown by the following synapomorphies in the three remaining genera (also see Fig. 3): (1) the reduction in number of vertebrae; (2) increase in the number of anal-fin spines, dorsal-fin spines and associated pterygiophores; (3) loss of coronalis pore of the head lateral line; and (4) a full neural spine on PU2. In the remaining polycentrids, the two South American taxa *Polycentrus* and *Monocirrhus* appear united based on the shared presence of three pterygiophores anterior to the first haemal spine. This phylogenetic interpretation of the skeletal characters of the polycentrids is corroborated by both the egg character (Britz 1997), and the current molecular analysis (Fig. 7).

Afronandus type material

We take this opportunity to clarify some confusion surrounding the number of type specimens of *Afronandus sheljuzhkoi*. This species was described by Meinken in 1954 in a German aquarium journal (*Die Aquarien- und Terrarienzeitschrift*) as *Nandopsis sheljuzhkoi* Meinken 1954. Because *Nandopsis* Meinken was preoccupied by *Nandopsis* Gill in the family Cichlidae, Meinken (1955) erected the new genus *Afronandus* a year later in the same journal. In the original description, Meinken (1954; p. 27) mentioned that Leo Sheljuzhko sent to him towards the end of 1952, four specimens of an unknown species collected from the Ivory Coast for determination. Although Meinken (1954) provided only measurements of two specimens, the type series must have consisted of the four specimens. Meinken (1954) also indicated that the types are in the British Museum in London. The fish collection at the Natural History Museum in London holds three lots of *Afronandus* sent by Meinken and registered as BMNH 1954.12.21.1–2, 1954.12.21.3–4, and 1954.12.21.5 (Natural History Museum, 2014). While the first two lots contain alcohol specimens, the third lot comprises a cleared and alizarin stained, dissected specimen. Eschmeyer's catalogue entry for *A. sheljuzhkoi* lists only BMNH 1954.12.21.1–2 as syntypes (Eschmeyer 2014; accessed 14 September 2014). Because Meinken (1954) mentions four specimens in his paper and does not expressly exclude any of them from the description, all four specimens are syntypes. It is unclear where the fifth cleared and stained specimen came from, but Meinken mentioned that Sheljuzhko's team collected around 10 specimens, and the c&s specimen may have been one of those additional ones. Meinken (1954) gave the dorsal-fin spine number as 15 and 16 for the two larger specimens he studied in more detail. The radiographs for these two syntypes (BMNH 1954.12.21.1–2), however, show they both have only 14 dorsal-fin spines. The same count is also present in the other two syntypes (BMNH 1954.12.21.3–4) and in the c&s specimen (BMNH 1954.12.21.5).

Systematics of the Labyrinthici

We report that the Labyrinthici of Cuvier (= Anabantomorpha of Springer and Johnson 2004) comprises a well-supported monophyletic group in our molecular analysis (Fig. 7) and is diagnosed by the unique presence of parasphenoid dentition (Fig. 5). This group was also present in the trees of Wainwright et al. (2012), Betancur-R et al. (2013a) and Near et al. (2013). The parsimony reanalysis of the amended matrix of morphological characters presented by Springer and Orrell (2004) now also recovers this group as monophyletic (Figure S1), in contrast to their previous tree, indicating that the corrected and recoded characters were important in resolving this relationship (see also Britz 2006). The sistergroup relationship of Nandidae + Badidae is also unambiguously supported in both the 257-taxon and 36-taxon molecular analyses and the parsimony analysis (Figure S1); the clade is diagnosed by a bifurcating haemal spine on PU2 (Fig. 4) and single attachment cells restricted to the ventral side of the yolk sac (Fig. 2d–f).

On the basis of their topology grouping the Channidae and Nandidae together, Betancur-R et al. (2013a) reclassified the Nandidae into the Channoidei thus rejecting the morphological evidence that the air-breathing snakeheads and labyrinth fishes form a natural group (Fig. 5; Britz 2004; Springer and Johnson 2004). Betancur-R et al. (2013b) went further still, in including the Badidae and Pristolepididae in the Anabantoidei (to the exclusion of the Channidae and Nandidae). In our analysis – and in agreement with the tree presented by Near et al. (2013) – the fishes with labyrinth organs (Channoidei and Anabantoidei) formed a monophyletic group (Figs 7 and 8). Although support was low, and our molecular data cannot categorically reject the non-monophyly of the air-breathing group, the results here agree with the morphological evidence and therefore provide the most parsimonious explanation as to their relationship.

The position of *Pristolepis* is one of greater uncertainty (Fig. 5), with three plausible reconstructions: (1) *Pristolepis* sistergroup to all other Labyrinthici as suggested by an anti-parallel spawning embrace uniting the Badidae, Nandidae, Channidae and Anabantoidei, but lacking in *Pristolepis* (Barlow et al. 1968; Britz 1997; Mercy et al. 2003); (2) *Pristolepis* sistergroup to *Badis* + *Nandus* as suggested by the ventral gill-arch musculature character (Datovo et al. 2014); and (3) *Pristolepis* sistergroup to the Anabantoidei + Channoidei as suggested by our parsimony reanalysis (Figure S1; Springer and Orrell 2004). The molecular data we present (Fig. 8a) favour reconstruction (1), but not overwhelmingly so (BPP = 0.50). Reconstruction (2) comprises the same topology as reflected in the classification of Betancur-R et al. (2014) in their proposal of suborder Nandoidei comprising the Nandidae, Badidae and Pristolepididae. We found that this topology (Fig. 8b) had a marginally lower level of support in the molecular data (BPP = 0.38) and could not be rejected by our statistical tests. Reconstruction (3), although having much lower support (BPP = 0.03) could not be entirely rejected either, but given the morphological and molecular evidence, we feel that this reconstruction is the least plausible of the three options. Therefore, given this ambiguity and lack of decisive support for any single explanation of the data, we suggest that it is premature to include *Pristolepis* in a nandoid group until further work can be carried out to clarify its position. As shown by Britz (1997), egg and larval structures have provided a useful character system for leaffish phylogenetics, and the eggs and larvae of *Pristolepis* have not been examined for such characters; obtaining these data would be a valid line of future research, as would further molecular information.

The sistergroup relationship of the Labyrinthici + Synbranchiformes has been reported by several molecular studies using independent data sets (Chen et al. 2003; Li et al. 2009; Wainwright et al. 2012; Betancur-R et al. 2013a; Near et al. 2013), while possible relationships among these groups have also been proposed in the pre-molecular ichthyological literature (Rosen and Patterson 1990). Therefore, these findings may warrant further investigation in regard to morphological characters that may corroborate or reject this grouping.

Conclusions

Using data from 257 widely sampled acanthomorph taxa and new sequences from multiple nuclear and mitochondrial loci, we present an analysis supporting phylogenetic hypotheses and classification of the leaffishes after previous studies (our proposed classification of the leaffishes is presented in Table 1). Long-standing questions regarding convergent evolution and the placement of the elusive taxon *Afronandus* have been addressed, resulting in a clearer picture of this poorly understood group. We also confirm using molecular methods known relationships among the Labyrinthici clade of parasphenoid tooth bearing percomorphs, but conclude by stressing that further work is required to better understand the placement of *Pristolepis*, which remains problematic in regard to conflicting morphological and ethological evidence.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Fifty percent majority-rule consensus tree from the parsimony reanalysis of morphological data from Springer and Orrell (2004).

Figure S2. RAXML gene tree for *plagl2*.
Figure S3. RAXML gene tree for *rag1*.
Figure S4. RAXML gene tree for *SH3PX3*.
Figure S5. RAXML gene tree for *sreb2*.
Figure S6. RAXML gene tree for *zic1*.
Figure S7. Uncollapsed version of Fig. 7 (RAXML tree of the 257-taxon analysis), including all terminal taxa and bootstrap support values.
Figure S8. RAXML tree of 740 16S rRNA sequences showing the '*Polycentropsis*' GenBank sequence AY662705 from Sparks and Smith (2004) is a misidentified *Nandus*.

Table S1. Table of taxon sampling coverage of previous studies presenting phylogenies containing leaffishes and related groups.
Table S2. Table of collated GenBank accessions from Near et al. (2012) and Wainwright et al. (2012) as used in the 257-taxon nDNA analysis.
Table S3. Table of primer names, sequences and annealing temperatures for molecular markers used in this study.
Table S4. Table of GenBank accession numbers for sequences used in the 36-taxon nDNA + mtDNA analysis and including those generated in this study.