

Multigene phylogeny of cyprinodontiform fishes suggests continental radiations and a rogue taxon position of *Pantanodon*

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Abstract

We studied phylogenetic relationships among major clades in the tooth carps (Cyprinodontiformes) based on a concatenated DNA sequence alignment of two mitochondrial and three nuclear gene segments, totalling 2553 bp, in 66 ingroup terminals. The inferred tree supports monophyly of the major tooth carp subgroups, aplocheiloids and cyprinodontoids, and of several aplocheiloid subclades corresponding to the well-established families (Aplocheilidae, Nothobranchiidae, Rivulidae), each of which is restricted to major continental settings (India-Madagascar, Africa, South America). Contrary to previous molecular studies, our tree supports a sister-group relationship of the aplocheilids and nothobranchiids, rather than a nothobranchiid-rivulid clade. Within cyprinodontoids, the phylogeny matched more closely continent-scale distribution than current classification, suggesting that the delimitation of the families Cyprinodontidae, Poeciliidae, and Valenciidae is in need of revision. The East African *Pantanodon stuhlmanni* did not show close relationships with any other taxon in our analysis, suggesting that the phylogenetic position and classification of this rogue taxon is in need of further study.

Key words

Tooth carps; Cyprinodontiformes; *Pantanodon*; phylogeny; phylogeography; Madagascar.

Introduction

Tooth carps, order Cyprinodontiformes, comprise killifishes, live bearers, as well as several allied forms. According to current classifications (COSTA, 2012; HUBER, 2006; FROESE & PAULY, 2014) this group contains over 1100 species in ca. 125 genera and ten families, allocated to two suborders, Aplocheiloidei and Cyprinodontoidei (PARENTI, 1981). Cyprinodontiforms occur mainly in tropical regions of Africa, South America, Madagascar and South Asia, as well as some temperate waters in North America and Eurasia, and are part of the acantomorph radiation (NEAR *et al.*, 2013). They comprise many prominent aquarium fishes and established or emerging

model species in evolutionary, developmental, toxicological, and ageing research such as the guppy, swordtail, mummichog, and turquoise killifish (e.g., ATZ, 1986; REZNICK *et al.*, 2008; JONES *et al.*, 2013; SCHARTL, 2014). Numerous molecular studies have addressed the phylogeny of particular subclades of cyprinodontiforms (e.g., MURPHY & COLLIER, 1997; MURPHY *et al.*, 1998, 1999; HRBEK & MEYER, 2003; DOADRIO & DOMINGUEZ, 2004; WEBB *et al.*, 2004; HRBEK *et al.*, 2005, 2007; AGNÈSE *et al.*, 2006; COLLIER *et al.*, 2009; JONES *et al.*, 2013; SEDLÁČEK *et al.*, 2014). However, the interrelationships among deep cyprinodontiform clades are

largely unassessed from a molecular perspective, and the current higher-level classification of tooth carps is predominantly based on a limited number of eminent in-depth morphological studies (PARENTI, 1981; COSTA, 1998, 2011; HERTWIG, 2008). As an exception, SETIAMARGA *et al.* (2008), based on complete mitochondrial genome sequences, placed cyprinodontiforms in the Atherinomorpha clade, along with medakas, flying fishes, and silversides, and found evidence for monophyly of cyprinodontiforms and of the two suborders, aplocheiloids and cyprinodontoids.

Understanding cyprinodontiform phylogeny has the potential to inform studies on the evolution of annualism and live bearing, and on the biogeographic origins of these fishes. Traditionally the origins of tooth carps, especially those in the Aplocheiloidei, are interpreted as being a consequence of ancient vicariance (e.g., MURPHY & COLLIER, 1997; SPARKS & SMITH, 2005; SAMONDS *et al.*, 2012; COSTA, 2013), in particular because their cladogenesis largely reflects the breakup of the Gondwana supercontinent in deep Mesozoic times, with the Indian *Aplocheilus* considered being sister to the Malagasy-Seychellean *Pachypanchax*, and the South American Rivulidae sister to the African Nothobranchiidae (MURPHY & COLLIER, 1997; SPARKS & SMITH, 2005). The vicariance hypothesis for aplocheiloid origins however requires confirmation as it conflicts with clade ages recovered in several studies (e.g., CROTTINI *et al.*, 2012; NEAR *et al.*, 2012, 2013; BROUGHTON *et al.*, 2013) that place the origin of the entire cyprinodontiform clade into the latest Mesozoic or early Cenozoic, similar to that of cichlids (VENCES *et al.*, 2001; FRIEDMAN *et al.*, 2013). Particularly relevant for this aspect of cyprinodontiform biogeography are the endemic tooth carps occurring on Madagascar, the fourth largest island of the world. This island has been separated from all other landmasses since the Mesozoic and is characterized by a unique and highly endemic biota, yet many of its radiations appear to have originated after its isolation (YODER & NOWAK, 2006; SAMONDS *et al.*, 2012). Madagascar is inhabited by two native genera of cyprinodontiforms: the genus *Pachypanchax* with currently six Malagasy and one Seychellean species; and the genus *Pantanodon*, with one described and one undescribed species known from Madagascar, and one species occurring in Eastern Africa (SPARKS, 2003; LOISELLE, 2006). So far, no molecular data are available for *Pantanodon*, and only one Malagasy species of *Pachypanchax* has been included in molecular phylogenies (MURPHY & COLLIER, 1997; CROTTINI *et al.*, 2012).

As a first step to improve the understanding of higher-level cyprinodontiform relationships, we newly determined a data set of two mitochondrial and three nuclear genes for a set of 66 cyprinodontiform terminals. Our data set spans seven of the ten currently accepted families and includes the enigmatic *Pantanodon*. By highlighting various unsolved questions and taxa that merit further phylogenetic study, we anticipate our study inform and facilitate future systematic revisions of tooth carps.

Materials and Methods

If not indicated as wild caught by precise collecting locations (Table 1), samples were from aquarium strains. Voucher specimens of the majority of specimens were preserved, labeled with provisional numbers (ZCMV – Miguel Vences Zoological Collection) and will be deposited in the Zoologische Staatssammlung München, Germany. Tissue samples were preserved in pure ethanol. DNA was sampled from fin clips of the preserved vouchers, or from eggs. Total genomic DNA was extracted from tissue or swab samples using Proteinase K (10mg/ml) digestion followed by a standard salt-extraction protocol (BRUFORD *et al.*, 1992).

Two markers of the mitochondrial and three markers of the nuclear genome were targeted: Segments of the mitochondrial genes cytochrome oxidase subunit I (COX1) and 16S rRNA were amplified, respectively, with the primers COI-Chmf4 (TYTCWACWAAAYCAYA AAGAYATCGG) and COI-Chmr4 (ACYTCRGGRTG RCCRAARAATCA) of CHE *et al.* (2012), and 16SAr-L (CGCCTGTTTATCAAAAACAT) and 16SBr-H (CCGGTCTGAACTCAGATCACGT) of PALUMBI *et al.* (1991). Segments of the nuclear genes for recombination activating protein 1 (RAG1), brain super conserved receptor (SREB2) and glycosyltransferase (GLYT), were amplified, respectively, with primers L2891_RAG1ex3 (AAGGAGTGYTGYGATGGCATGGG) and H3405_RAG1ex3 (GCNGAGACTCCTTTGACTCTGTC) of NEAR *et al.* (2012), and newly developed primers Rag1-Pachyp-F1 (TGAAAARGCTGTTTCGCTTCT), SREB2-Pachyp-F1 (CAyrCTrACCTGCAAAGTGA), SREB2-Pachyp-R1 (CCCATARTGCCARGAAGAAA), GLYT-Pachyp-F2 (CTGAATGAAsCCGAGCTrTmATGG), GLYT-Pachyp-R1 (CATGGGATCTGCCAAGAGAC).

Polymerase chain reactions were performed in a final volume of 10 µl using 0.3 µM of each primer, 0.25 mM of dNTPs, 0.4 U GoTaq and 1.25 × Reaction Buffer (Promega).

PCR products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (SAP) or Antarctic Phosphatase (AP) according to the manufacturer's instructions (NEB). Purified PCR templates were directly sequenced using dye-labeled dideoxy terminator chemistry on an ABI 3130 automated DNA sequencer (Applied Biosystems). We checked chromatograms and corrected errors manually in CodonCode Aligner 3.5.6 (CodonCode Corporation). Newly obtained sequences were submitted to GenBank (accession numbers: KJ844613-KJ844868). Sequences of outgroup taxa were taken from Genbank, and largely correspond to those determined by NEAR *et al.* (2012). We used a representative of the Gobiiformes (*Perccottus*) as outgroup and included a series of atheriniform, beloniform and perciform species as hierarchical outgroups.

We used MEGA 5 (TAMURA *et al.*, 2011) to align protein-coding sequences (COX1, RAG1, GLYT, SREB2) manually and the non-coding 16S using the MUSCLE

algorithm. The 16S alignment was subsequently processed with Gblocks 0.91b software (CASTRESANA, 2000) with a less stringent 50% threshold for the definition of reliable flanking positions and the remaining parameters at default settings, to exclude ambiguously aligned sections.

The AICc criterion implemented in PartitionFinder 1.0.1 software (LANFEAR *et al.*, 2012) was applied to infer the best-fitting model of molecular evolution and partition scheme for phylogenetic analysis of our data (Supplementary Table S1). Partitioned Bayesian inference (BI) phylogenetic analysis of the concatenated DNA sequences was carried out with MrBayes 3.2 (RONQUIST *et al.*, 2012). The analysis was run for 60 million generations and chain mixing and stationarity verified using Tracer 1.5 software (RAMBAUT & DRUMMOND, 2007). A conservative burn-in of 25% was applied and a majority-rule consensus tree constructed from the remaining trees. The analysis was repeated with the same settings after excluding *Pantanodon stuhlmanni* given its conflicting placement in single-gene trees (Supplementary Figures S1-S5) which were constructed in MEGA 5 under the Maximum Likelihood optimality criterion.

Results and Discussion

The concatenated alignment of 16S COX1, RAG1, SREB2 and GLYT sequences, after exclusion of hyper-variable 16S sections suggested by Gblocks, consisted of 2553 bp and 76 terminals (996 bp mtDNA and 1557 bp nDNA).

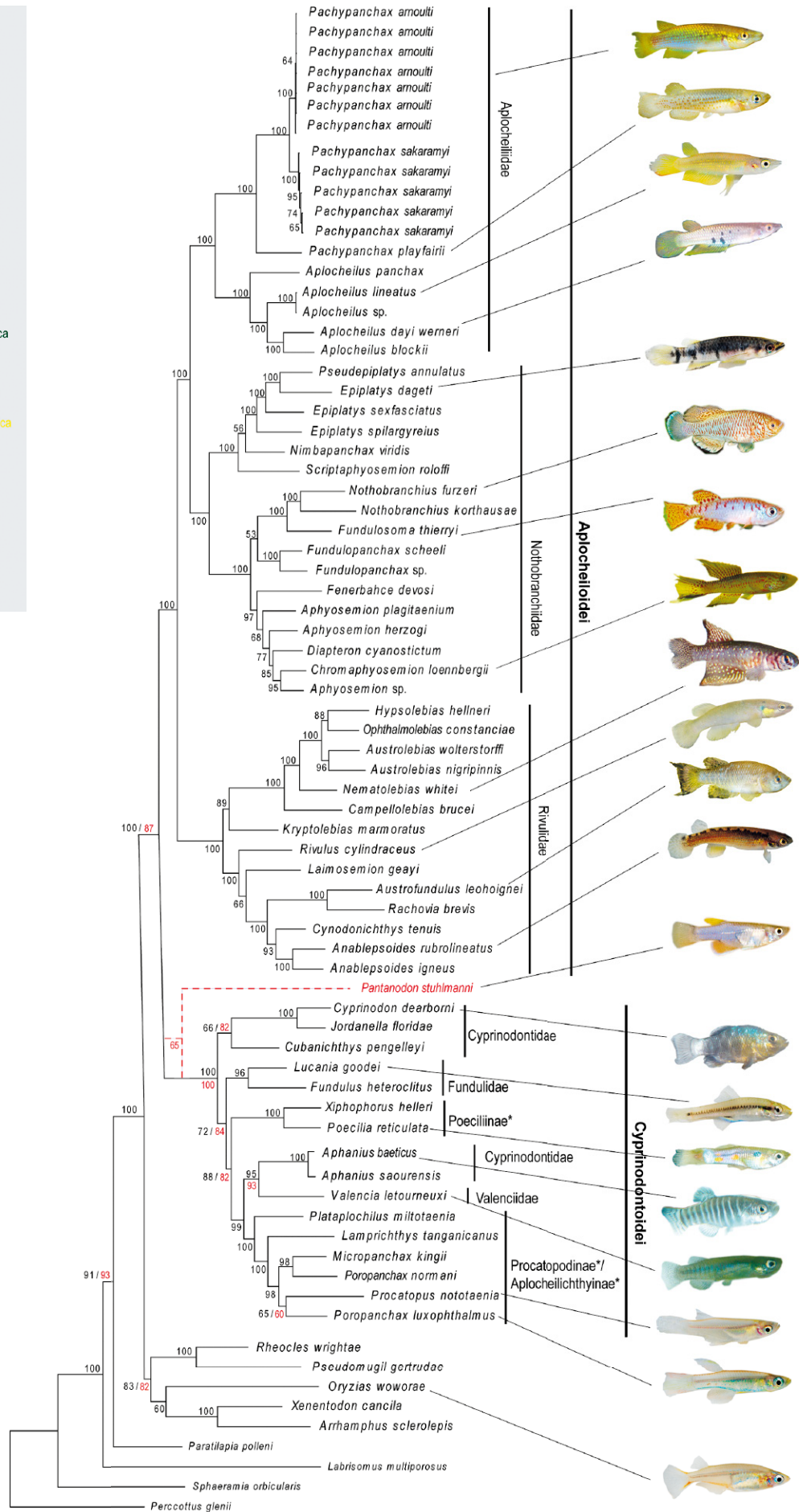
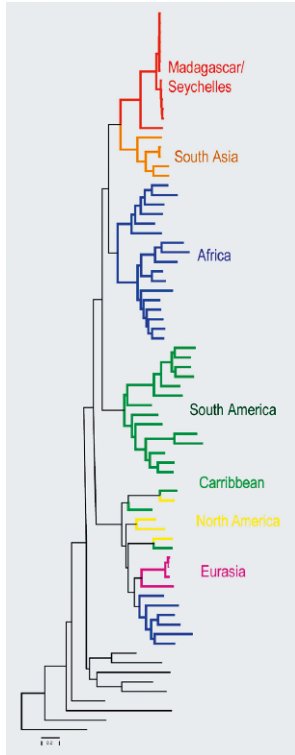
Bayesian Inference analysis excluding *Pantanodon stuhlmanni* (Fig. 1) resulted in high posterior probabilities of almost all deep nodes in the phylogeny: cyprinodontiforms, aplocheiloids, cyprinodontoids, as well as the families Aplocheilidae, Nothobranchiidae, and Rivulidae all received maximum support (PP=100%). Within aplocheiloids, the Indo-Malagasy aplocheilids and the African nothobranchiids were sister groups with maximum support. Within the cyprinodontoids, families and interfamilial relationships were less unambiguously resolved. The Palearctic genus *Aphanius* was placed outside the family Cyprinodontidae and instead was sister to the equally Palearctic genus *Valencia* (the sole representative of Valenciidae) with marginal support (95%), and this Palearctic clade was sister to egg-laying African poeciliids (lampeyes). Nearctic and Neotropical cyprinodontids, fundulids and poeciliids instead were placed successively as sister groups of the Palearctic/African clade. A few relationships within more shallow clades are worth mentioning as well: (i) In the aplocheiloid clade, the included Malagasy terminals together formed the sister group of the Seychellean *P. playfairii*, and the differentiation among the two included Malagasy species was rather low. According to an unpublished molecular data set of R. D. Randrianiaina with full taxon coverage (pers. comm. in 2014), the two populations included here are among

the most divergent within Madagascar, so that monophyly of the entire Malagasy clade vs. the single Seychellean species is likely. (ii) Within nothobranchiids, representatives of *Diapteron* and *Chromaphyosemion* were placed within *Aphyosemion*; if confirmed by future studies, then the taxonomy of these genera is in need of revision.

Inclusion of *Pantanodon stuhlmanni* in the analysis led to an identical topology, with *Pantanodon* in an unsupported position sister to the cyprinodontoids (red in Fig. 1). However inclusion of this taxon reduced posterior probabilities of several nodes, of which certainly the basal node of cyprinodontiforms is the most relevant: monophyly of tooth carps was not significantly supported any more (87%) after inclusion of *Pantanodon*. In all single gene trees (Supplementary Materials) *Pantanodon* is placed outside of cyprinodontiform clade, although typically without strong support. We therefore conclude that the phylogenetic position of this enigmatic taxon remains largely unresolved, but certainly it is not a close relative to the African lampeyes classified within the poeciliids (subfamilies Aplocheilichthyidae and Procatopodinae). This taxon appears to fit well in the definition of a “rogue” taxon which in various single gene analyses is placed in varying positions of the phylogeny and blurs the phylogenetic signal in a combined analysis of all markers (e.g., SANDERSON & SHAFFER, 2002). COSTA (2012) based on morphological characters placed *Pantanodon*, including two fossil species from the Oligocene and Miocene of Europe, within the Poeciliidae. Unfortunately we have not been able to obtain tissue samples of Malagasy species of *Pantanodon*. Of the two Malagasy species, one (*P. madagascariensis*) is probably extinct, and a second, undescribed species appears to be restricted to a very small swamp area on the southern east coast of the island (SPARKS, 2003). Whether these fishes are really related to the African *P. stuhlmanni* requires confirmation, and their molecular study is all the more a priority given the intriguingly isolated position of the African species.

We are aware that the phylogenetic hypothesis presented herein needs to be seen with some caution. Many samples in our analysis were supplied from aquarium strains, thus without reliable locality information and with unverified species-level identification. This for instance might be relevant, in our tree, for the topology within nothobranchiids such as non-monophyly of *Aphyosemion* relative to *Diapteron* and *Chromaphyosemion*. However, we here focus on relationships among genera and major clades and at this level the identification of all of our samples is reliable. Our discussion of biogeography and higher classification therefore remains valid. Taxa from the families Goodeidae, Profundulidae and Anablepidae (all three from suborder Cyprinodontoidae) are missing from our data set, but at least the position of goodeids is not expected to yield large surprises as SETIAMARGA *et al.* (2008) found *Xenotoca eiseni*, a Central American representative of this family, highly supported within Cyprinodontoidae (and sister to a Neotropical poeciliid).

Because the nuclear genes could not be sequenced for all taxa due to amplification failure, our final align-



ment contained a proportion of ca. 22% missing data. However, all higher taxa (families and subfamilies) had the two mitochondrial genes sequenced from at least one representative (Table 1), and all but one (Valenciidae) had at least two of the three nuclear genes sequenced. Given the overall congruence of single-gene trees we are confident that our preferred phylogeny (Fig. 1) has not been influenced by missing-data artefacts.

We have refrained from reconstructing a timetree of tooth carps from our data because comprehensively assessing the precise placement of available fossils at particular nodes was beyond the scope of this first study. However, despite the absence of reliable dates of origin for the major clades, a number of biogeographical conclusions can be drawn. With aplocheiloids, our tree strongly supports a sister group relationship of the Indo-Malagasy and the African clade (i.e., Aplocheilidae and Nothobranchiidae). This contrasts with previous molecular reconstructions (MURPHY & COLLIER, 1997) which placed the African and South American radiations sister to each other. Within cyprinodontoids it is remarkable that the recovered topology (Fig. 1), despite low support of many nodes, more closely matches continent-scale geographic distribution than current classification. American taxa from both North and South America, including Caribbean islands, are placed paraphyletically at the basis of cyprinodontoids, while an Old World clade (Palearctis plus Africa) is strongly supported.

Our preliminary study highlights a series of promising fields for further research. First, the family-level classification of cyprinodontoids clearly is in need of revision; this requires including additional genera, especially those assigned to the Goodeidae, Profundulidae and Anablepidae which are missing from our data set. Second, inclusion of sequences from Malagasy *Pantanodon*, and a wider outgroup sampling will be helpful to determine the true phylogenetic position of *P. stuhlmanni*, a species that might merit inclusion in a distinct family.

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References

- AGNÈSE, J.F., ZENTZ, F., LEGROS, O. & SELLOS, D. (2006): Phylogenetic relationships and phylogeography of the killifish species of the subgenus *Chromaphyosemion* (Radda, 1971) in West Africa, inferred from mitochondrial DNA sequences. – *Molecular Phylogenetics and Evolution*, **40**: 332–346.
- ATZ, J.W. (1986): *Fundulus heteroclitus* in the laboratory: a history. – *American Zoologist*, **26**: 111–120.
- BROUGHTON, R.E., BETANCUR-R., R., LI, C., ARRATIA, G. & ORTÍ, G. (2013): Multi-locus phylogenetic analysis reveals the pattern and tempo of bony fish evolution. – *PLoS Currents Tree of Life*.
- BRUFORD, M.W., HANOTTE, O., BROOKFIELD, J.F.Y., BURKE, T. (1992): Single-locus and multilocus DNA fingerprinting, in: HOELZEL, A.R. (Ed.), *Molecular Genetic Analysis of Populations: a Practical Approach*. IRL Press, Oxford, pp. 225–270.
- CASTRESANA, J. (2000): Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. – *Molecular Biology and Evolution*, **17**: 540–552.
- CHE, J., CHEN, H.M., JIN, J.Q., JOANG, K., YUAN, Z.-Y., MURPHY, R.W. & ZHANG, Y.-P. (2012): Universal COI primers for DNA barcoding amphibians. – *Molecular Ecology Resources*, **12**: 247–258.
- COLLIER, G.E., MURPHY, W.J. & ESPINOZA, M. (2009): Phylogeography of the genus *Epiplatys* (Aplocheiloidea: Cyprinodontiformes). – *Molecular Phylogenetics and Evolution*, **50**: 190–196.
- COSTA, W.J.E.M. (1998): Phylogeny and classification of Rivulidae revisited: origin and evolution of annualism and miniaturization in rivulid fishes (Cyprinodontiformes: Aplocheiloidei). – *Journal of Comparative Biology*, **3**: 33–92.
- COSTA, W.J.E.M. (2011): Comparative morphology, phylogenetic relationships, and historical biogeography of plesiolebiasine seasonal killifishes (Teleostei: Cyprinodontiformes: Rivulidae). – *Zoological Journal of the Linnean Society*, **162**: 131–148.
- COSTA, W.J.E.M. (2012): Oligocene killifishes (Teleostei: Cyprinodontiformes) from southern France: relationships, taxonomic position, and evidence of internal fertilization. – *Vertebrate Zoology*, **62**: 371–386.
- COSTA, W.J.E.M. (2013): Historical biogeography of aplocheiloid killifishes (Teleostei: Cyprinodontiformes) – *Vertebrate Zoology*, **63**: 139–154.
- CROTTINI, A., MADSEN, O., POUX, C., STRAUSS, A., VIEITES, D.R. & VENCES, M. (2012): Vertebrate time-tree elucidates the biogeographic pattern of a major biotic change around the K–T

← **Fig. 1.** Phylogenetic tree obtained by Bayesian Inference analysis of two mitochondrial and three nuclear gene segments (16S, COX1, RAG1, SREB2, GLYT). Numbers are posterior probabilities in percent. Asterisks mark taxa assigned to different subfamilies in the Poeciliidae. Analysis (tree with black lines) carried out without inclusion of sequences of *Pantanodon stuhlmanni*; red dotted lines mark the position of this taxon after its inclusion in a separate analysis, and red numbers are posterior probabilities that changed in this second analysis. The inset tree on upper right shows the same tree topology, branches colored by continental distribution of the taxa.

- boundary in Madagascar. – Proceedings of the National Academy of Sciences of the USA, **109**: 5358–5363.
- DOADRIO, I. & DOMÍNGUEZ, O. (2004): Phylogenetic relationships within the fish family Goodeidae based on cytochrome *b* sequence data. – Molecular Phylogenetics and Evolution, **31**: 416–430.
- FRIEDMAN, M., KECK, B.P., DORNBURG, A., EYTAN, R.I., MARTIN, C.H., HULSEY, C.D., WAINWRIGHT, P.C. & NEAR, T.J. (2013): Molecular and fossil evidence place the origin of cichlid fishes long after Gondwanan rifting. – Proceedings of the Royal Society B, **280**: 20131733.
- FROESE, R. & PAULY, D. (editors) (2014): FishBase. World Wide Web electronic publication. www.fishbase.org, version (04/2014).
- HERTWIG, S.T. (2008): Phylogeny of the Cyprinodontiformes (Teleostei, Atherinomorpha): the contribution of cranial soft tissue characters. – Zoologica Scripta, **37**: 141–174.
- HRBEK, T. & MEYER, A. (2003): Closing of the Tethys Sea and the phylogeny of Eurasian killifishes (Cyprinodontiformes: Cyprinodontidae). – Journal of Evolutionary Biology, **16**: 17–36.
- HRBEK, T., SECKINGER, J. & MEYER, A. (2007): Molecular phylogeny of the Poeciliidae (Teleostei, Cyprinodontiformes): Biogeographic and taxonomic implications. – Molecular Phylogenetics and Evolution, **43**: 986–998.
- HRBEK, T., TAPHORN, D.C. & THOMERSON, J.E. (2005): Molecular phylogeny of *Austrofundulus* Myers (Cyprinodontiformes: Rivulidae), with revision of the genus and the description of four new species. – Zootaxa, **825**: 1–39.
- HUBER, J.H. (2006): Killi-Data Catalogue. Killi-Data Editions, Paris: 1028 pp.
- JONES, J.C., FAN, S., FRANCHINI, P., SCHARTL, M. & MEYER, A. (2013): The evolutionary history of *Xiphophorus* fish and their sexually selected sword: a genome-wide approach using restriction site-associated DNA sequencing. – Molecular Ecology, **22**: 2986–3001.
- LANFEAR, R., CALCOTT, B., HO, S.Y.W. & GUINDON, S. (2012): PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. – Molecular Biology and Evolution, **29**: 1695–1701.
- LOISELLE, P.V. (2006): A review of the Malagasy *Pachypanchax* (Teleostei: Cyprinodontiformes, Aplocheilidae), with description of four new species. – Zootaxa, **1366**: 1–44.
- MURPHY, W.J. & COLLIER, G.E. (1997): A molecular phylogeny for aplocheiloid fishes (Atherinomorpha, Cyprinodontiformes): the role of vicariance and the origins of annualism. – Molecular Biology and Evolution, **14**: 790–799.
- MURPHY, W.J., NGUYEN, T.N.P., TAYLOR, E.B. & COLLIER, G.E. (1998): Mitochondrial DNA phylogeny of West African aplocheiloid killifishes (Cyprinodontiformes, Aplocheilidae). – Molecular Phylogenetics and Evolution, **11**: 343–350.
- MURPHY, W.J., THOMERSON, J.E. & COLLIER, G.E. (1999): Phylogeny of the Neotropical killifish family Rivulidae (Cyprinodontiformes, Aplocheiloidei) inferred from mitochondrial DNA sequences. – Molecular Phylogenetics and Evolution, **13**: 289–301.
- NEAR, T.J., DORNBURG, A., EYTAN, R.I., KECK, B.P., SMITH, W.L., KUHN, K.L., MOORE, J.A., PRICE, S.A., BURBRINK, F.T., FRIEDMAN, M. & WAINWRIGHT, P.C. (2013): Phylogeny and tempo of diversification in the superradiation of spiny-rayed fishes. – Proceedings of the National Academy of Sciences of the U.S.A., **110**: 12738–12743.
- NEAR, T.J., EYTAN, R.I., DORNBURG, A., KUHN, K.L., MOORE, J.A., DAVIS, M.P., WAINWRIGHT, P.C., FRIEDMAN, M. & SMITH, W.L. (2012): Resolution of ray-finned fish phylogeny and timing of diversification. – Proceedings of the National Academy of Sciences of the U.S.A., **109**: 13698–136703.
- PALUMBI, S., MARTIN, A., ROMANO, S., McMILLAN, W.O., STICE, L. & GRABOWSKI, G. (1991): The Simple Fool's guide to PCR. Honolulu: Department of Zoology.
- PARENTI, L.R. (1981): A phylogenetic and biogeographic analysis of cyprinodontiform fishes (Teleostei, Atherinomorpha). – Bulletin of the American Museum of Natural History, **168**: 335–557.
- RAMBAUT, A. & DRUMMOND, A.J. (2007): Tracer: MCMC Trace Analysis Tool. Institute of Evolutionary Biology, University of Edinburgh, Edinburgh.
- REZNICK, D.N., GHALAMBOR, C.K. & CROOKS, K. (2008): Experimental studies of evolution in guppies: a model for understanding the evolutionary consequences of predator removal in natural communities. – Molecular Ecology, **17**: 97–107.
- RONQUIST, F., TESLENKO, M., VAN DER MARK, P., AYRES, D.L., DARLING, A., HÖHNA, S., LARGET, B., LIU, L., SUCHARD, M.A. & HUELSENBECK, J.P. (2012): MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. – Systematic Biology, **61**: 539–542.
- SAMONDS, K.A., GODFREY, L.R., ALI, J.R., GOODMAN, S.M., VENCES, M., SUTHERLAND, M.R., IRWIN, M.T. & KRAUSE, D.W. (2012): Spatial and temporal arrival patterns of Madagascar's vertebrate fauna explained by distance, ocean currents, and ancestor type. – Proceedings of the National Academy of Sciences of the U.S.A., **109**: 5352–5357.
- SANDERSON, M.J. & SHAFFER, H.B. (2002): Troubleshooting molecular phylogenetic analyses. – Annual Review of Ecology and Systematics, **33**: 49–72.
- SCHARTL, M. (2014): Beyond the zebrafish: diverse fish species for modeling human disease. – Disease Models and Mechanisms, **7**: 181–192.
- SEDLÁČEK, O., BACIAKOVÁ, B., KRATOCHVÍL, L. (2014) Evolution of body colouration in killifishes (Cyprinodontiformes: Aplocheilidae, Nothobranchiidae, Rivulidae): Is male ornamentation constrained by intersexual genetic correlation? – Zoologischer Anzeiger, **253**: 207–215.
- SETIAMARGA, D.H., MIYA, M., YAMANOUÉ, Y., MABUCHI, K., SATOH, T.P., INOUE, J.G. & NISHIDA, M. (2008): Interrelationships of Atherinomorpha (medakas, flyingfishes, killifishes, silversides, and their relatives): The first evidence based on whole mitogenome sequences. – Molecular Phylogenetics and Evolution, **49**: 598–605.
- SPARKS, J.S. (2003): *Pantanodon*. p. 871-872. In: S.M. GOODMAN & J.P. BENSTEAD (eds.): The natural history of Madagascar. The University of Chicago Press, Chicago and London. 1709 p.
- SPARKS, J. & SMITH, W. (2005): Freshwater fishes, dispersal ability, and nonevidence: “gondwana life rafts” to the rescue. – Systematic Biology, **54**: 158–165.
- TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. (2011): MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. – Molecular Biology and Evolution, **28**: 2731–2739.
- YODER, A.D. & NOWAK, M.D. (2006): Has vicariance or dispersal been the predominant biogeographic force in Madagascar?

- Only time will tell. – Annual Review of Ecology, Evolution and Systematics, **37**: 405–431.
- VENCES, M., FREYHOF, J., SONNENBERG, R., KOSUCH, J. & VEITH, M. (2001): Reconciling fossils and molecules: Cenozoic divergence of cichlid fishes and the biogeography of Madagascar. – Journal of Biogeography, **28**: 1091–1099.
- WEBB, S.A., GRAVES, J.A., MACIAS-GARCIA, C., MAGURRAN, A.E., FOIGHIL, D.O. & RITCHIE, M.G. (2004): Molecular phylogeny of the livebearing Goodeidae (Cyprinodontiformes). – Molecular Phylogenetics and Evolution, **30**: 527–544.

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Supplementary Table S1. Substitution models and partition scheme.

Supplementary Table S2. Detailed collection localities for selected species.

Supplementary Figure S1 – S5. Single-gene trees based on DNA sequences of each of the five genes used for analysis.

Appendix

Table 1. Sample provenance and Genbank accession numbers for the killifish species used in the multigene phylogenetic analysis. See Supplementary Materials Table S2 for detailed collection data of selected species. Asterisks mark sequences taken from GenBank; all other sequences were newly determined for this study. NA: Not available.

Species	Collecting locality	16S	Cox1	RAG1	SREB2	GLYT
Aplocheilidae						
<i>Aplocheilus blockii</i>	India: Alappuzha	KJ844775	KJ844712	KJ844666	KJ844833	KJ844618
<i>Aplocheilus dayi wernerii</i>	Sri Lanka: Kottawa Forrest	KJ844776	KJ844713	KJ844667	KJ844834	KJ844619
<i>Aplocheilus lineatus</i>	NA	KJ844777	KJ844714	NA	NA	KJ844620
<i>Aplocheilus panchax</i>	NA	KJ844778	KJ844715	NA	KJ844835	KJ844621
<i>Aplocheilus</i> sp.	NA	KJ844779	KJ844716	KJ844668	NA	KJ844622
<i>Pachypanchax playfairii</i>	Seychelles	KJ844807	KJ844743	KJ844691	KJ844854	KJ844644
<i>Pachypanchax sakaramyi</i>	Madagascar: Montagne d’Ambre	KJ844808	KJ844744	KJ844692	NA	KJ844645
<i>Pachypanchax sakaramyi</i>	Madagascar: Montagne d’Ambre	KJ844809	KJ844745	NA	KJ844855	NA
<i>Pachypanchax sakaramyi</i>	Madagascar: Montagne d’Ambre	KJ844810	KJ844746	NA	NA	NA
<i>Pachypanchax sakaramyi</i>	Madagascar: Montagne d’Ambre	KJ844811	KJ844747	NA	KJ844856	KJ844646
<i>Pachypanchax sakaramyi</i>	Madagascar: Montagne d’Ambre	KJ844812	KJ844748	NA	KJ844857	NA
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844813	KJ844749	KJ844693	NA	NA
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844814	KJ844750	KJ844694	NA	NA
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844815	KJ844751	KJ844695	NA	KJ844647
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844816	KJ844752	KJ844696	KJ844858	KJ844648
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844817	KJ844753	NA	KJ844859	KJ844649
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844818	KJ844754	NA	KJ844860	KJ844650
<i>Pachypanchax arnoulti</i>	Madagascar: Tsingy de Bemaraha	KJ844819	KJ844755	KJ844697	KJ844861	KJ844651
Cyprinodontidae						
<i>Aphanius saourensis</i>	Algeria	KJ844770	KJ844707	KJ844662	NA	KJ844613
<i>Aphanius baeticus</i>	Spain	KJ844771	KJ844708	NA	NA	KJ844614
<i>Cubanichthys pengelleyi</i>	Jamaica	KJ844786	KJ844722	NA	NA	NA
<i>Cyprinodon dearborni</i>	NA	KJ844788	KJ844724	KJ844674	KJ844841	NA
<i>Jordanella floridiae</i>	USA	KJ844796	KJ844733	KJ844683		KJ844635
Fundulidae						
<i>Fundulus heteroclitus</i>	USA	NA	KJ844731	KJ844681	EF033030*	NA
<i>Lucania goodei</i>	USA	KJ844800	KJ844736	NA	NA	KJ844638
Nothobranchiidae						
<i>Aphyosemion herzogi</i>	NA	KJ844772	KJ844709	KJ844663	KJ844831	KJ844615
<i>Aphyosemion plagitaenium</i>	Republic of the Congo	KJ844773	KJ844710	KJ844664	KJ844832	KJ844616
<i>Aphyosemion</i> sp.	NA	KJ844774	KJ844711	KJ844665	NA	KJ844617
<i>Chromaphyosemion loennbergii</i>	Cameroon: Likouk	KJ844785	NA	NA	KJ844839	KJ844626
<i>Diapteron cyanostictum</i>	Gabon	KJ844789	NA	NA	KJ844842	KJ844627

Table 1 continued.

Species	Collecting locality	16S	Cox1	RAG1	SREB2	GLYT
Nothobranchiidae						
<i>Epiplatys dageti</i>	Liberia	NA	KJ844725	KJ844675	KJ844843	KJ844628
<i>Epiplatys sexfasciatus</i>	Gabon	KJ844790	KJ844726	KJ844676	KJ844844	KJ844629
<i>Epiplatys spilargyreus</i>	Sudan: Kosti	KJ844791	KJ844727	KJ844677	KJ844845	KJ844630
<i>Fenerbahce devosi</i>	Democratic Republic of the Congo	KJ844792	KJ844728	KJ844678	KJ844846	KJ844631
<i>Fundulopanchax scheeli</i>	Nigeria	KJ844793	KJ844729	KJ844679	KJ844847	KJ844632
<i>Fundulopanchax</i> sp.	NA	KJ844783	KJ844720	KJ844672	KJ844838	KJ844624
<i>Fundulosoma thierryi</i>	Ghana: Ada	KJ844794	KJ844730	KJ844680	NA	KJ844633
<i>Nimbapanchax viridis</i>	NA	KJ844803	KJ844739	KJ844687	KJ844852	KJ844640
<i>Nothobranchius korthausae</i>	Tanzania: Mafia Island	KJ844804	KJ844740	KJ844688	NA	KJ844641
<i>Nothobranchius furzeri</i>	NA	NC_011814 *	NC_011814 *	GAIB01120750 *	GAIB01005857 *	GAIB01101555 *
<i>Pseudepiplatys annulatus</i>	Liberia: Monrovia	KJ844825	KJ844762	KJ844701	KJ844864	KJ844656
<i>Scriptaphysemon roloffi</i>	NA	NA	KJ844766	KJ844704	KJ844867	KJ844659
Poeciliidae, Aplocheilichthyinae						
<i>Micropanchax kingii</i>	Sudan: Kosti	KJ844801	KJ844737	KJ844685	KJ844850	NA
<i>Poropanchax luxophthalmus</i>	NA	KJ844823	KJ844759	KJ844700	NA	NA
<i>Poropanchax normani</i>	NA	NA	KJ844760	NA	NA	NA
Poeciliidae, Poeciliinae						
<i>Poecilia reticulata</i>	NA	KJ844822	KJ844758	NA	NA	KJ844654
<i>Xiphophorus helleri</i>	NA	KJ844830	KJ844768	KJ844705	NA	KJ844660
Poeciliidae, Procatopodinae						
<i>Lamprichthys tanganicanus</i>	NA	KJ844799	KJ844735	NA	NA	NA
<i>Plataplochilus miltotaenia</i>	NA	KJ844821	KJ844757	KJ844699	KJ844862	KJ844653
<i>Procatopus nototaenia</i>	NA	KJ844824	KJ844761	NA	KJ844863	KJ844655
Rivulidae						
<i>Anablepsoides igneus</i>	NA	KJ844769	KJ844706	KJ844661	NA	NA
<i>Anablepsoides rubrolineatus</i>	NA	KJ844828	KJ844765	NA	NA	KJ844658
<i>Austrofundulus lehoignei</i>	Venezuela	KJ844780	KJ844717	KJ844669	KJ844836	NA
<i>Austrolebias nigripinnis</i>	NA	KJ844781	KJ844718	KJ844670	NA	NA
<i>Austrolebias wolterstorffi</i>	NA	KJ844782	KJ844719	KJ844671	KJ844837	KJ844623
<i>Campellolebias brucei</i>	Brazil	KJ844784	KJ844721	NA	NA	KJ844625
<i>Cynodonichthys tenuis</i>	NA	KJ844787	KJ844723	KJ844673	KJ844840	NA
<i>Hypsolebias hellneri</i>	Brazil	KJ844795	KJ844732	KJ844682	KJ844848	KJ844634
<i>Kryptolebias marmoratus</i>	NA	KJ844797	NA	KJ844684	KJ844849	KJ844636
<i>Laimosemion geayi</i>	NA	KJ844798	KJ844734	NA	NA	KJ844637
<i>Nematolebias whitei</i>	Brazil	KJ844802	KJ844738	KJ844686	KJ844851	KJ844639
<i>Ophthalmolebias constanciae</i>	Brazil	KJ844805	KJ844741	KJ844689	KJ844853	KJ844642
<i>Rachovia brevis</i>	NA	KJ844826	KJ844763	KJ844702	KJ844865	
<i>Rivulus cylindraceus</i>	Cuba	KJ844827	KJ844764	KJ844703	KJ844866	KJ844657
Valenciidae						
<i>Valencia letourneuxi</i>	Greece: Corfu: Sidari	KJ844829	KJ844767	NA	KJ844868	NA
Incertae sedis						
<i>Pantodon stuhlmanni</i>	Kenya: Koreni	KJ844820	KJ844756	KJ844698	NA	KJ844652
Outgroups						
<i>Arrhamphus sclerolepis</i> (Beloniformes; Hemiramphidae)	NA	AY693481*	NA	JX190870*	JX189951*	NA
<i>Labrisomus multiporosus</i> (Perciformes; Labrisomidae)	NA	AY539069*	NA	JX189914*	JX190051*	JX188812*
<i>Oryzias woworae</i> (Beloniformes; Adrianichthyidae)	Indonesia	KJ844806	KJ844742	KJ844690	NA	KJ844643
<i>Paratilapia polleni</i> (Perciformes; Cichlidae)	NA	AY662719*	AY263886*	JX189869*	AFN28951*	JX188772*
<i>Perccottus glenii</i> (Gobiiformes; Odontobutidae)	NA	KF415440*	AY722171*	JX189917*	JX190055*	JX188816*
<i>Pseudomugil gertrudae</i> (Atheriniformes; Pseudomugilidae)	NA	AY266083*	AY290817*	KF141336*	KF140258*	KF139857*
<i>Rheocles wrightae</i> (Atheriniformes; Bedotiidae)	NA	AY266069*	AY290803*	JX189788*	JX189945*	JX188685*
<i>Sphaeramia orbicularis</i> (Perciformes; Apogonidae)	NA	NA	FJ346823*	KF141362*	KF140274*	KF139886*
<i>Xenentodon cancila</i> (Beloniformes; Belontiidae)	NA	AF243967*	FJ459541*	JX189791*	JX189948*	JX188688*

Multigene phylogeny of cyprinodontiform fishes suggests continental radiations and a rogue taxon position of *Pantanodon*

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Supplementary Materials

Supplementary Table S1. Substitution models and partition scheme.

Supplementary Table S2. Detailed collection localities for selected species.

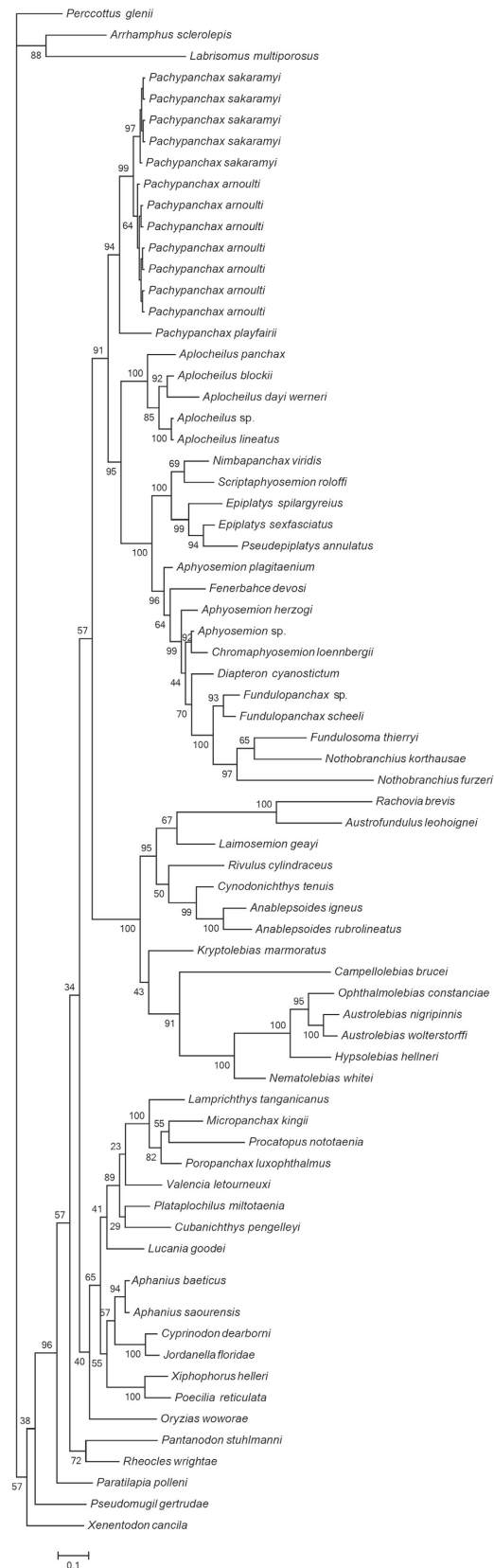
Supplementary Figure S1 – S5. Single-gene trees based on DNA sequences of each of the five genes used for analysis.

Supplementary Table S1. Substitution models and partitions applied for multi-locus phylogenetic reconstructions, as identified by PartitionFinder 1.1.1 software under the AICc criterion for concatenated analysis.

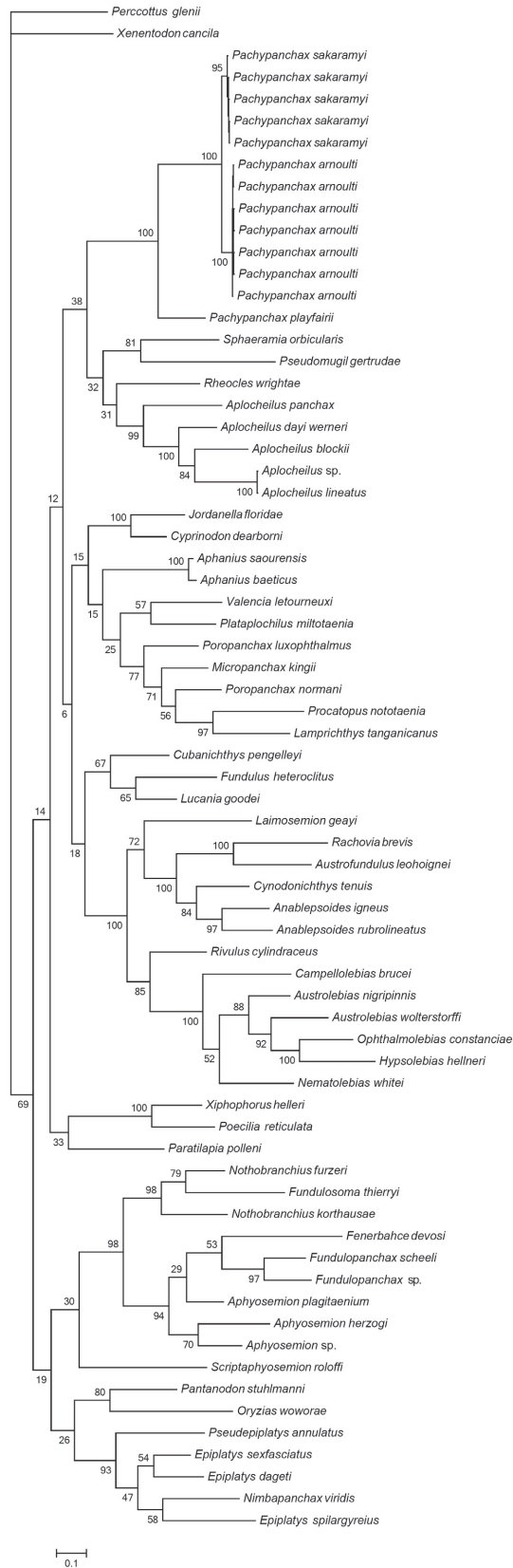
Subset	Best Model	Subset Partitions
1	GTR+I+G	16s
2	F81+I	1stPos_COI
3	GTR+I+G	2ndPos_COI
4	SYM+I+G	3rdPos_COI
5	K80+G	1stPos_RAG1
6	HKY+I+G	2ndPos_RAG1
7	GTR+I+G	3rdPos_GLYT, 3rdPos_RAG1
8	SYM+I+G	1stPos_SREB
9	F81+I	2ndPos_SREB
10	GTR+G	3rdPos_SREB
11	HKY+I+G	2ndPos_GLYT
12	GTR+G	1stPos_GLYT

Supplementary Table S2. Detailed collection localities for samples of selected species. Taxonomic note: Species assignment of the Malagasy *Pachypanchax* is based on their locality only and therefore in need of confirmation. The specimens herein named *P. sakaramyi* are part of the "*homalonotus* cluster" based on mtDNA.

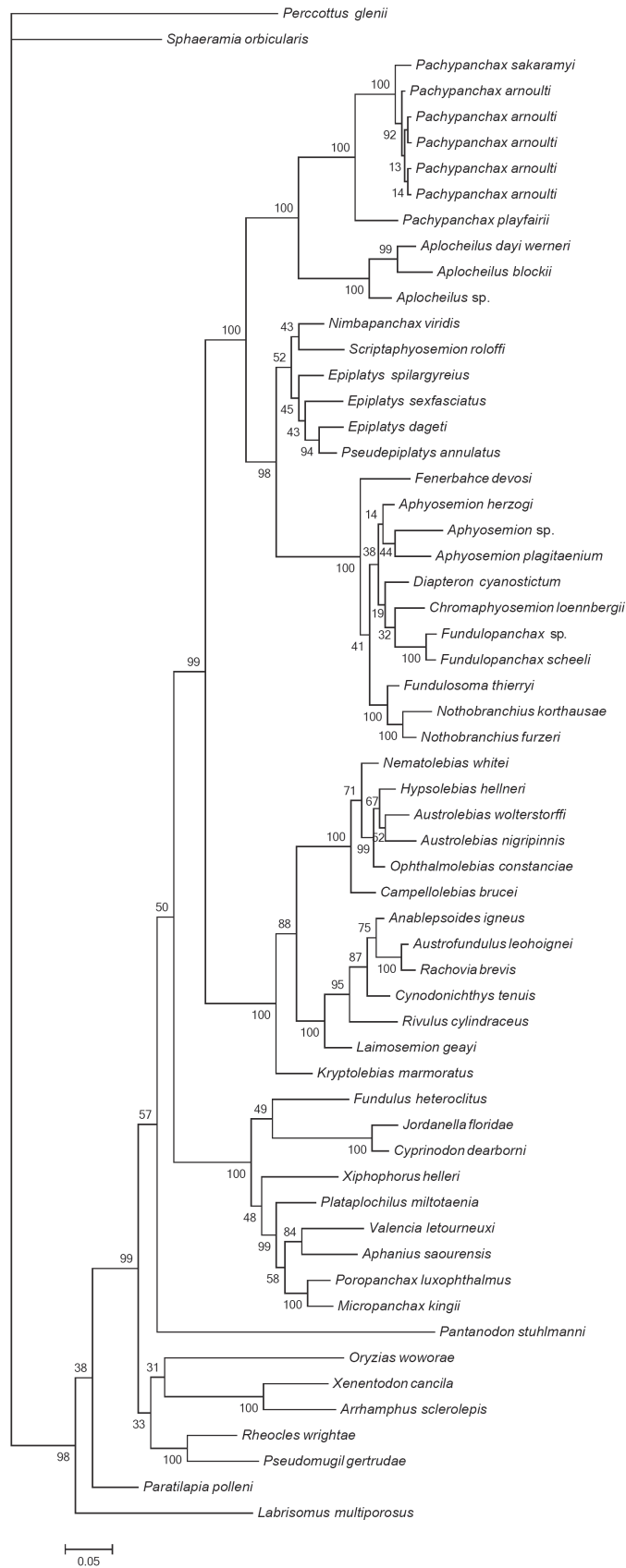
Species	Description of locality	Geographical coordinates	Date of collection
<i>Pantanodon stuhlmanni</i>	Brackish water pond, connected to a small temporary river, emptying into the Indian Ocean just west of the Lamu Island, Kenya.	02°12'24"S, 40°42'16"E, 9m asl	11. January 2010
<i>Fundulosoma thierryi</i>	roadside pool northwest of the town Ada, Ghana	05°53'19"N, 00°27'45"E	November 2006
<i>Micropanchax kingii</i>	flood plain of the White Nile. Adjacent shallow backwater, heavily vegetated, inundated and connected to the river	13°10'15"N, 32°40'22"E	11. November 2010
<i>Epiplatys spilargyreus</i>	flood plain of the White Nile. Adjacent shallow backwater, heavily vegetated, inundated and connected to the river	13°10'15"N, 32°40'22"E	11. November 2010
<i>Pachypanchax arnouliti</i>	various specimens, all collected at Antranopasasy, Tsingy de Bemaraha, Madagascar	18° 42' 31" S, 44° 43' 08" E, 146 m asl	23.03.2006
<i>Pachypanchax sakaramyi</i>	various specimens, all collected at Forêt d'Ambre, near Montagne d'Ambre National Park	12°28'00" S, 49°13'37" E, 470 m asl	12.03.2007



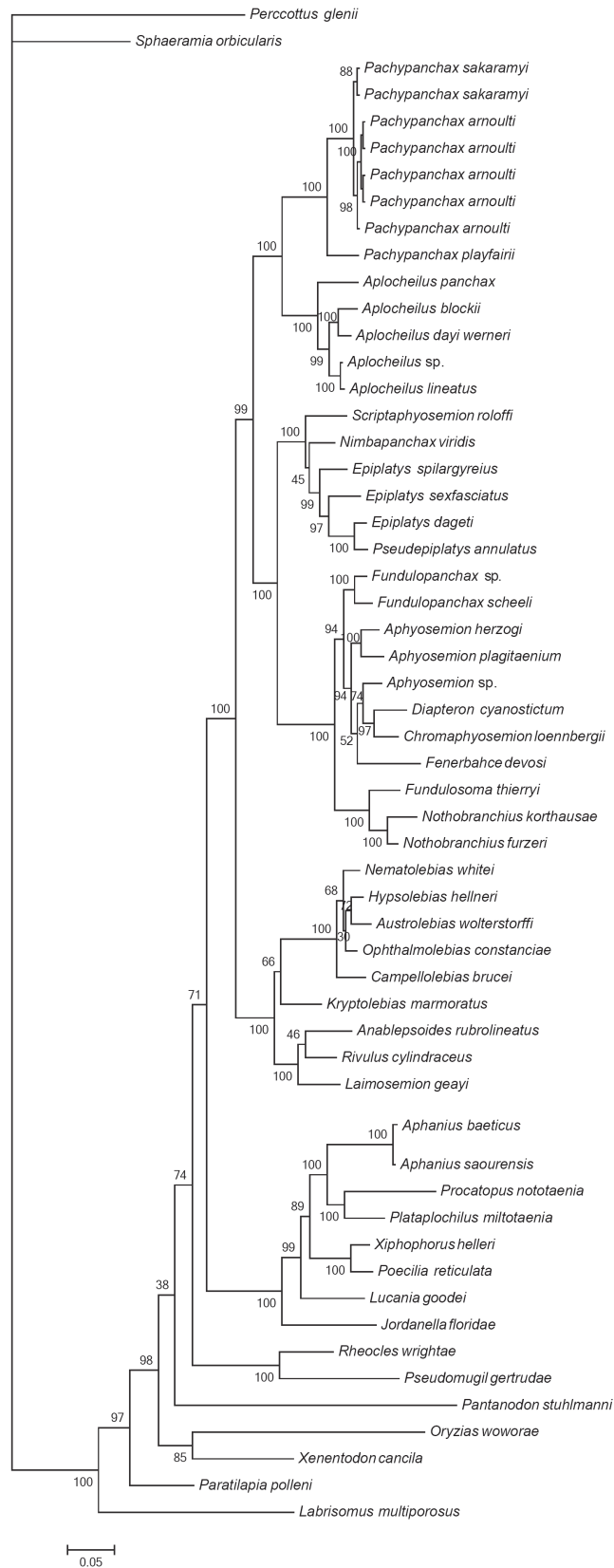
Supplementary Figure S1. Single-gene phylogenetic tree obtained by Bayesian inference (unpartitioned) from the analysis of DNA sequences of the mitochondrial 16S rRNA gene (alignment length 603 bp). Numbers are posterior probabilities in percent.



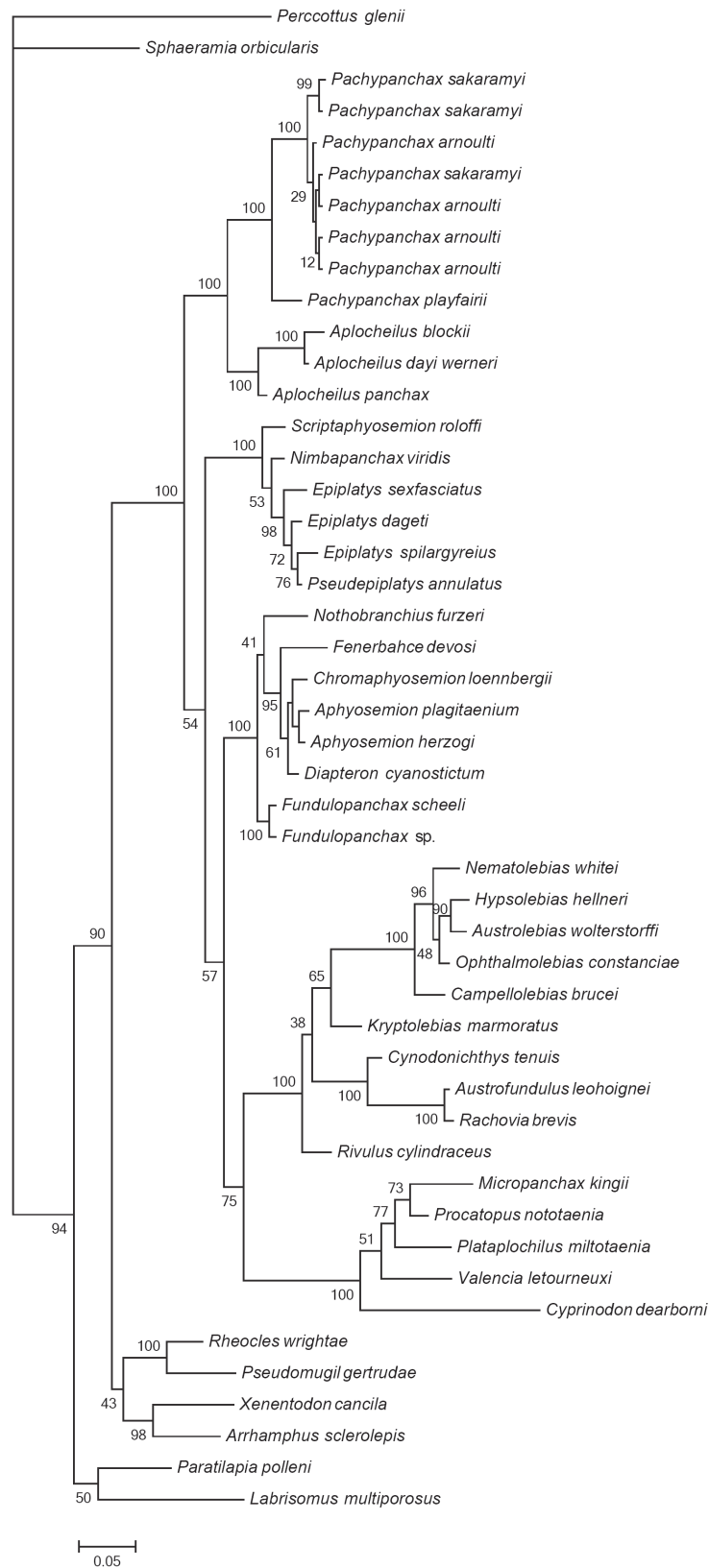
Supplementary Figure S2. Single-gene phylogenetic tree obtained by Bayesian inference (unpartitioned) from the analysis of DNA sequences of the mitochondrial cytochrome oxidase subunit 1 (COX1) gene (alignment length 571 bp). Numbers are posterior probabilities in percent.



Supplementary Figure S3. Single-gene phylogenetic tree obtained by Bayesian inference (unpartitioned) from the analysis of DNA sequences of the recombination-activating gene 1 (RAG1) (alignment length 433 bp). Numbers are posterior probabilities in percent.



Supplementary Figure S4. Single-gene phylogenetic tree obtained by Bayesian inference (unpartitioned) from the analysis of DNA sequences of the glycosyltransferase (GLYT) gene (alignment length 597 bp). Numbers are posterior probabilities in percent.



Supplementary Figure S5. Single-gene phylogenetic tree obtained by Bayesian inference (unpartitioned) from the analysis of DNA sequences of the brain super conserved receptor (SREB2) gene (alignment length 599 bp). Numbers are posterior probabilities in percent.