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Evolution of Sri Lanka's Giant Danios (Teleostei: Cyprinidae: *Devario*): Teasing apart species in a recent diversification

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ABSTRACT

The small, colourful freshwater fishes of the cyprinid genus *Devario* are among the many vertebrate groups that appear to have diversified on Sri Lanka, a continental Indian Ocean island, which is part of the Western Ghats-Sri Lanka Biodiversity Hotspot. Despite Sri Lanka having been connected with India via a wide isthmus intermittently until the Plio-Pleistocene and almost continuously since then, during sea-level low-stands, the number of species of *Devario* on Sri Lanka is comparable with that on the Indian Peninsula, some 25 times its size. Here, from a sampling of 27 *Devario* populations across Sri Lanka's major river basins and climatic zones, we present and analyze a phylogeny based on mitochondrial and nuclear DNA data. We show that five species of *Devario* occur on the island, all but one of which are endemic: *Devario malabaricus* is widespread throughout the lowlands and parts of central hills of Sri Lanka and southern India. A new narrow-range endemic, here described as *D. memorialis* sp. nov., was discovered in a remnant rainforest habitat at Aranayake (Ma Oya basin) in this study. It is immediately distinguished from Sri Lankan congeners by having only 8 (vs 9–12) branched dorsal-fin rays and by uncorrected pairwise genetic distances of more than 4.0% and 7.8% for *cytochrome c oxidase subunit 1* and *cytochrome b*, respectively. Our results provide strong support for the monophyly of the entire Sri Lankan diversification of *Devario*. Divergence-timing analysis suggests the common ancestor dispersed to the island in the late Miocene, with the insular diversification in the island's south-western wet zone taking place during the Plio-pleistocene. There are signs of gene flow between the Indian and Sri Lankan populations of *D. malabaricus* until the late Pleistocene. Phylogenetic and haplotype-network analyses suggest basin-centric phylogeographic structure within the endemic species; *D. malabaricus,* however, shows little such structure in the island. Molecular and morphological analyses failed to identify *D. annnataliae* and *D. udenii* confidently as distinct species: they are considered synonyms of *D. micronema*. The morphological variation observed within *D. micronema* is likely attributable to polymorphism. The discordance between the mitochondrial and nuclear phylogenies for some samples of *Devario* in the present study suggest signs of mitochondrial introgression.

1. Introduction

Sri Lanka is a continental island separated from India by the ca 10 m deep, 30 km-wide Palk Strait. The island is, together with the Western Ghats of India, considered a Global Biodiversity Hotspot (Myers et al., 2000). Species richness and endemism across almost all taxonomic groups is markedly higher in the wet zone (rainfall > 2000 mm/y) of Sri Lanka's south-western quadrant, including the central hills, which rise to 2524 m above sea level (Gunatilleke et al., 2008). Although the present-day island experienced prolonged terrestrial connections with southern India during successive sea-level low-stands from the mid-Oligocene until ca 6 kya (Hansen et al., 2013; McLoughlin, 2001; Miller et al., 2013), it retains a strongly endemic biota (Bossuyt et al., 2004).

As part of a Global Biodiversity Hotspot (Myers et al., 2000), Sri Lanka is home to a remarkable wealth of endemic species (MOE, 2012). Bossuyt et al. (2004) showed, in a study of several vertebrate and invertebrate groups, that this endemism appears to be more a result of diversification within the aseasonal 'rain' forests of the island's south-western wet zone than insularity resulting from the island being separated from mainland India by the Palk Strait, a narrow, shallow-shelf marine channel. The diversifications within Sri Lanka of shrub frogs, horned agamid lizards, day geckoes and freshwater crabs (Agarwal

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et al., 2017; Beenaerts et al., 2010; Meegaskumbura et al., 2019; Schulte et al., 2002) have revealed a scenario that suggests that the remarkable diversifications that have occurred on the island began, in each case, with the immigration from India of one or a few founders subsequent to the Eocene-Oligocene boundary.

Freshwater fishes are interesting target organisms to study biogeographic linkages and patterns of evolution because they obligatorily depend on aquatic connectivity for dispersion. Molecular phylogenies based on finer geographic sampling have not, however, up to now been derived for any group of freshwater fishes in Sri Lanka. At the same time, new species of freshwater fishes continue to be discovered from the island's rainforests, highlighting the need for more intensive exploration amidst alarming rates of deforestation (Sudasinghe, 2017, 2018; Sudasinghe and Meegaskumbura, 2016).

Fishes of the cyprinid genus *Devario*, which comprises some 40 valid species, are ubiquitous in the freshwaters of South and Southeast Asia, spanning the region between the Indus and the Mekong basins (Fang Kullander, 2001; Kullander et al., 2017). *Devario* are among the most frequently encountered freshwater fishes also in Sri Lanka, their attractive blue-and-gold coloration resulting in their having figured prominently in the island's ornamental-fish export industry over the past half-century. Six species have been reported from the island, across all eco-climatic zones, from the rivers and ponds of the lowland floodplain to hill streams and torrents at altitudes of up to about 1400 m above sea level (asl) (Batuwita et al., 2017; Sudasinghe and Pethiyagoda, 2019): *D. annnataliae, D. malabaricus*, *D. micronema, D. monticola, D. pathirana* and *D. udenii.* Except for *D. malabaricus*, which occurs also in southern India, the remaining five nominal species are insular endemics restricted to streams traversing the island's south-western 'wet zone' quadrant. Remarkably, the diversity of the genus on the island is comparable to that reported from the whole of peninsular India, some 25 times its size.

The taxonomy of some of the Sri Lankan species, however, is marred by inconsistencies. Sudasinghe and Pethiyagoda (2019) cast doubt on the validity of *D. udenii* and *D. annnataliae* owing to confusion of morphological characters in their original descriptions by Batuwita et al. (2017). They also highlighted the difficulty of distinguishing between evidently closely-related species-pairs such as *D. malabaricus / D. monticola,* and *D. udenii / D. micronema*, based on the characters provided by Batuwita et al. (2017), which remains the most recent systematic review of these fishes. Given that all these nominal species are objects of conservation concern and investment, a clear understanding of the identity and distribution of valid species is a starting point for conservation planning, via the IUCN Red List (IUCN, 2019), for example. Objective means of delimiting the species and assessing molecular divergences reflected in morphological variation, are hence valuable.

The evolutionary history of Sri Lankan *Devario* too, remains to be elucidated. How many species does the diversification include? What are their phylogenetic relationships? What is the geographic and chronological context of their diversification?

Here, from a sampling of *Devario* populations representative of all the putative species across Sri Lanka's major river basins and climatic zones, we present and analyze a phylogeny based on a multi-gene dataset, also investigating the pattern of diversification. We apply divergence-timing analysis to show that the diversification of *Devario* in Sri Lanka is both recent and, in many instances, nuanced. We also explore the timing of historical dispersal or vicariance events between the island and India. Finally, we describe a remarkable new micro-endemic species of *Devario* discovered in this study.

2. Materials and methods

2.1. Ethics statement

Permission to carry out field work and sampling was obtained from the Department of Wildlife Conservation (permit no. WL/3/2/59/14) and the Forest Department (permit no. R&E/RES/NFSRCM/14-16-4) of Sri Lanka. At its 27th meeting on 4 August 2017, the ethics committee of the Postgraduate Institute of Science, University of Peradeniya, approved the methods of specimen collection, euthanisation (using MS-222 Tricaine methanesulfonate), tissue sampling, and fixation.

2.2. Material

Material referred to in the text is deposited in the collection of the Wildlife Heritage Trust of Sri Lanka (WHT), now at the National Museum of Sri Lanka (NH, NMSL, FF); the Evolutionary Ecology and Systematics Lab, Department of Molecular Biology and Biotechnology, University of Peradeniya (DZ); the Australian Museum, Sydney (AMS); and the Kerala University of Fisheries and Ocean Studies, Kochi, India (KUFOS) and presented in Table S1.

Except where stated otherwise, we follow the identities assigned to the species of Sri Lankan *Devario* by Batuwita et al. (2017), as clarified by Sudasinghe and Pethiyagoda (2019). In the molecular analysis, the names *D. annnataliae*, *D. micronema, D. monticola,* and *D. udenii* refer to the populations to which Batuwita et al. (2017) attributed those names.

2.3. Species concept

We adopt a general lineage concept of species (GLC), which recognizes species as independent evolutionary lineages diagnosed by multiple criteria (de Queiroz, 1998); and GLC is implemented in an integrative taxonomic framework (Dayrat, 2005; de Queiroz, 2007; Padial et al., 2010). This approach recognizes the multi-dimensional nature of species as ecological, morphological, behavioral, reproductive and historical entities. The multiple lines of evidence assessed in the present study are morphology, genetic data (based on mitochondrial and nuclear gene markers) and geographic distribution.

2.4. DNA protocols

The gene nomenclature is based on ZFIN Zebrafish Nomenclature Conventions (https://goo.gl/MdawKQ). DNA extraction, PCR amplification and PCR product purification for the mitochondrial *cytochrome c oxidase subunit 1* (*cox1*) and *cytochrome b* (*cytb*) genes and sequencing protocols follow Sudasinghe et al. (2018a). In addition, a ~1500 bp of the nuclear *recombination activating protein 1* (*rag1*) was amplified for a representative subset of Sri Lankan *Devario* by using the primer pair RAG1F1 (5′ CTG AGC TGC AGT CAG TAC CAT AAG ATG T 3′) and RAG1R1 (5′ CTG AGT CCT TGT GAG CTT CCA TRA AYT T 3′: López et al., 2004). The PCR of *rag1* was carried out in 25 μl reactions, using 2 μl of template DNA (10 to 100 ng), 12.5 μl of mastermix MangoMix™ (Bioline), 0.4 μl of each primer (10 μM) and 9.7 μl of deionized water. The PCR conditions for *rag1* followed an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 60 s, extension at 72 °C for 90 s and a final extension of 72 °C for 5 min. PCR product purification and sequencing protocols of *rag1* follow Sudasinghe et al. (2018a).

Sequenced data were checked and assembled in ChromasPro v1.34 (Technelysium Pty Ltd) and consensus sequences of the two strands were prepared using MEGA v. 7.0 (Kumar et al., 2016). Additional genetic data were obtained from GenBank (Table S2). The geographical origin of DNA samples for Sri Lankan *Devario* is shown in Fig. 1. 53 *cox1*, 52 *cytb*, and 12 *rag1* new marker sequences for Sri Lankan *Devario* were generated for the molecular analyses in the present study from sampling of 27 locations (Table 1). The *cytb*, *cox1* and *rag1* contig datasets were prepared and aligned separately using ClustalW in MEGA v. 7.0 (Kumar et al., 2016), verified manually, translated and checked for premature stop codons or frameshift mutations. Sequences of *Devario* from GenBank submissions were included only if their provenance had been declared (Table S2).

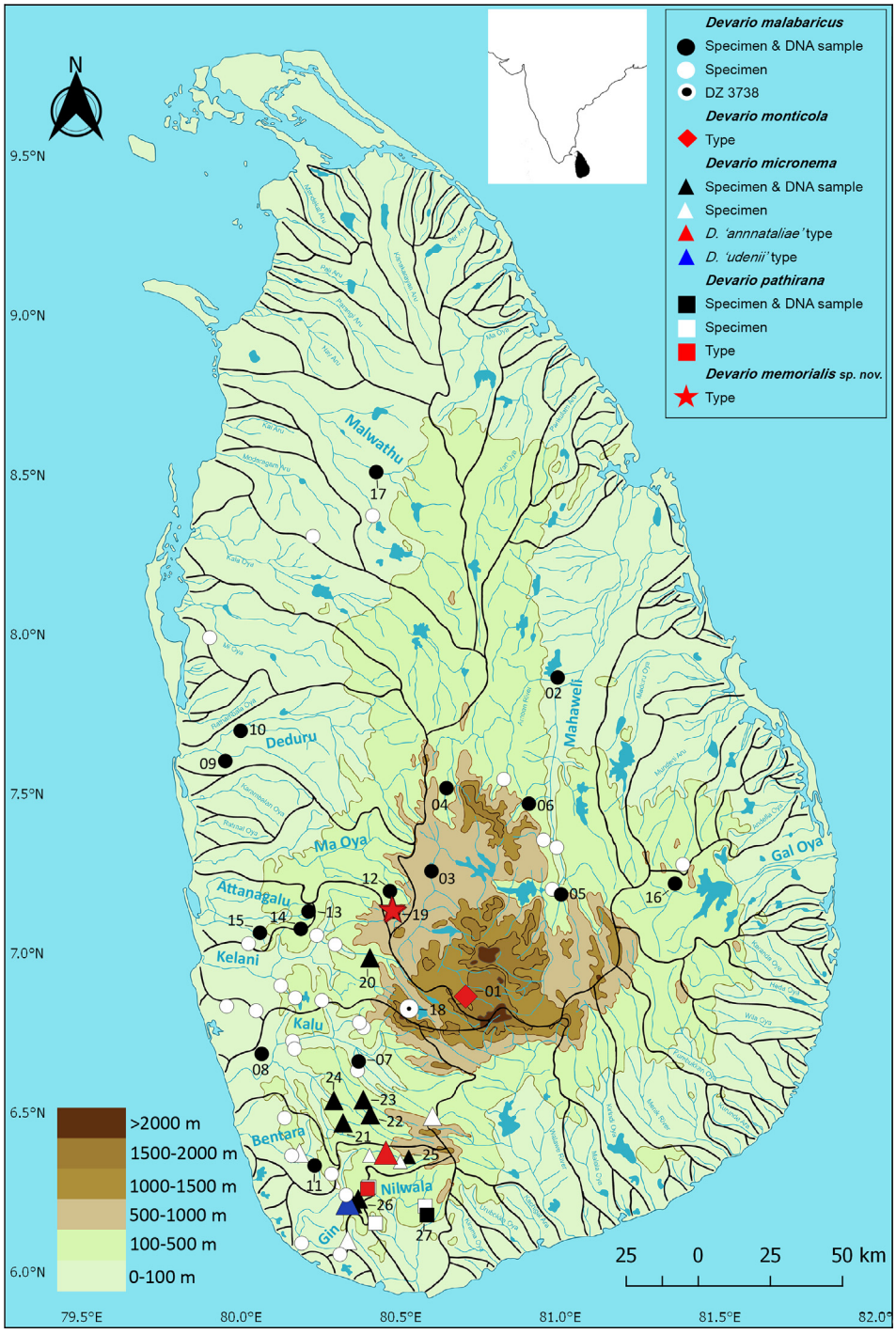

Fig. 1. Map of Sri Lanka showing geographical origin of samples of *Devario* used in this study. The 'wet zone' (rainfall $>$ 2 m·y^{-1}) broadly includes the central hills and the region southwest of them. The black lines represent drainage margins.

2.5. Phylogenetic analysis

For the phylogenetic inferences, the direct GenBank submissions were condensed into unique haplotypes by using ALTER (Glez-Pena et al., 2010). For each independent dataset, *cytb* (1092 bp), *cox1* (669 bp), *rag1* (1484 bp) and concatenated datasets of *cytb* + *cox1* (1761 bp) and *cytb* + *cox1* + *rag1* (3245 bp) prepared using SequenceMatrix (Vaidya et al., 2011), a Bayesian and a Maximum Likelihood analysis was carried out using MrBayes v3.2 (Ronquist et al., 2012) and IQ-TREE (Nguyen et al., 2015), respectively.

For the Bayesian phylogenetic inference (BI), PartitionFinder 2 (Lanfear et al., 2017) was used to determine the best nucleotide substitution model and the best partitioning scheme using the "greedy" algorithm (Lanfear et al., 2012) under the Bayesian information criterion (BIC) with each codon position of each gene given as the initial subset. The PhyML 3.0 (Guindon et al., 2010) was used as part of the PartitionFinder 2 package to evaluate specific nucleotide substitution model sets for MrBayes. The branch lengths of alternative partitions were given as "linked" in all the analyses. In the BI, four Metropolis coupled Markov-chain Monte Carlo (MCMCMC) chains were run for 10 million generations in two independent runs (sample frequency 1000) and the first 0.1% of the generations discarded as burn-in, which was

Table 1
Specimens of Sri Lankan species of *Devario* from which sequences were generated for the molecular analyses in the present study, with their localities, voucher references, and GenBank accession numbers.

Species	Voucher	Location	GPS coordinates	*cytb*	*cox1*	*rag1*
Devario monticola	DZ3197	Sri Lanka: Mahaweli basin, Agrapatana (1)	6.8643 N 80.7031 E	MN005753	MN005700	MN005809
Devario monticola	DZ3198	Sri Lanka: Mahaweli basin, Agrapatana (1)	6.8643 N 80.7031 E	MN005754	MN005701	–
Devario malabaricus	DZ3169	Sri Lanka: Mahaweli basin, Polonnaruwa (2)	7.8647 N 80.9913 E	MN005758	MN005705	MN005805
Devario malabaricus	DZ3174	Sri Lanka: Mahaweli basin, Polonnaruwa (2)	7.8647 N 80.9913 E	MN005759	MN005706	–
Devario malabaricus	DZ3190	Sri Lanka: Mahaweli basin, Polonnaruwa (2)	7.8647 N 80.9913 E	MN005760	MN005707	–
Devario malabaricus	DZ3273	Sri Lanka: Mahaweli basin, Peradeniya (3)	7.2575 N 80.5960 E	MN005761	MN005708	–
Devario malabaricus	DZ3275	Sri Lanka: Mahaweli basin, Peradeniya (3)	7.2575 N 80.5960 E	MN005762	MN005709	MN005807
Devario malabaricus	DZ3439	Sri Lanka: Mahaweli basin, Naula, Matale (4)	7.5178 N 80.6431 E	MN005763	MN005710	–
Devario malabaricus	DZ3504	Sri Lanka: Mahaweli basin, Kandeketiya (5)	7.1844 N 81.0023 E	MN005764	MN005711	–
Devario malabaricus	DZ3528	Sri Lanka: Mahaweli basin, Sulugune (6)	7.4689 N 80.9010 E	MN005765	MN005712	–
Devario malabaricus	DZ3529	Sri Lanka: Mahaweli basin, Sulugune (6)	7.4689 N 80.9010 E	MN005766	MN005713	–
Devario malabaricus	DZ3031	Sri Lanka: Kalu basin, Ratnapura, Elapatha (7)	6.6607 N 80.3678 E	MN005767	MN005714	–
Devario malabaricus	DZ3288	Sri Lanka: Kalu basin, Remuna, Horana (8)	6.6843 N 80.0641 E	MN005768	MN005715	–
Devario malabaricus	DZ3289	Sri Lanka: Kalu basin, Remuna, Horana (8)	6.6843 N 80.0641 E	MN005769	MN005716	–
Devario malabaricus	DZ3040	Sri Lanka: Deduru Oya basin, Kolamunuoya (9)	7.6029 N 79.9502 E	MN005770	MN005717	–
Devario malabaricus	DZ3043	Sri Lanka: Deduru Oya basin, Deduru Oya (10)	7.6976 N 79.9982 E	MN005771	MN005718	–
Devario malabaricus	DZ3307	Sri Lanka: Bentara basin, Pitigala (11)	6.3341 N 80.2299 E	MN005772	MN005719	–
Devario malabaricus	DZ3612	Sri Lanka: Ma Oya basin, Aranayake (12)	7.1419 N 80.4689 E	MN005773	MN005720	MN005806
Devario malabaricus	DZ3613	Sri Lanka: Ma Oya basin, Aranayake (12)	7.1419 N 80.4689 E	MN005774	MN005721	–
Devario malabaricus	DZ3614	Sri Lanka: Ma Oya basin, Aranayake (12)	7.1419 N 80.4689 E	MN005775	MN005722	–
Devario malabaricus	DZ3688	Sri Lanka: Kelani basin, Dunumana (13)	7.1480 N 80.2018 E	MN005776	MN005723	–
Devario malabaricus	DZ3689	Sri Lanka: Kelani basin, Dunumana (13)	7.1480 N 80.2018 E	MN005777	MN005724	–
Devario malabaricus	DZ3690	Sri Lanka: Attanagalu Oya basin, Malgahawita (14)	7.1308 N 80.2109 E	MN005778	MN005725	–
Devario malabaricus	DZ3691	Sri Lanka: Attanagalu Oya basin, Malgahawita (14)	7.1308 N 80.2109 E	MN005779	MN005726	–
Devario malabaricus	DZ3694	Sri Lanka: Attanagalu Oya basin, Wahareka (15)	7.0764 N 80.1867 E	MN005780	MN005727	–
Devario malabaricus	DZ3695	Sri Lanka: Attanagalu Oya basin, Wahareka (15)	7.0764 N 80.1867 E	MN005781	MN005728	–
Devario malabaricus	DZ3703	Sri Lanka: Gal Oya basin, Nilgala (16)	7.2183 N 81.3590 E	MN005782	MN005729	–
Devario malabaricus	DZ3704	Sri Lanka: Gal Oya basin, Nilgala (16)	7.2183 N 81.3590 E	MN005783	MN005730	–
Devario malabaricus	DZ3763	Sri Lanka: Malwathu Oya basin, Medawacchiya (17)	8.5091 N 80.4228 E	MN005784	MN005731	–
Devario malabaricus	DZ3764	Sri Lanka: Malwathu Oya basin, Medawacchiya (17)	8.5091 N 80.4228 E	MN005785	MN005732	–
Devario malabaricus	DZ3738	Sri Lanka: Kelani basin, Seethagangula (18)	6.8262 N 80.5256 E	MN005786	MN005733	MN005808
Devario memorialis sp. nov.	DZ3139	Sri Lanka: Ma Oya basin, Aranayake (19)	7.1299 N 80.4774 E	MN005787	MN005734	–
Devario memorialis sp. nov.	DZ3140	Sri Lanka: Ma Oya basin, Aranayake (19)	7.1299 N 80.4774 E	MN005788	MN005735	MN005816
Devario memorialis sp. nov.	DZ3147	Sri Lanka: Ma Oya basin, Aranayake (19)	7.1299 N 80.4774 E	MN005789	MN005736	–
Devario micronema	DZ3857	Sri Lanka: Kelani basin, Parawalathenna, Kitulgala (20)	6.9857 N 80.4033 E	MN005790	MN005737	MN005811
Devario micronema	DZ3858	Sri Lanka: Kelani basin, Parawalathenna, Kitulgala (20)	6.9857 N 80.4033 E	MN005791	MN005738	–
Devario micronema	DZ3859	Sri Lanka: Kelani basin, Parawalathenna, Kitulgala (20)	6.9857 N 80.4033 E	MN005792	MN005739	–
Devario micronema	DZ3615	Sri Lanka: Kalu basin, Runakanda (21)	6.4678 N 80.3190 E	MN005793	MN005740	MN005812
Devario micronema	DZ3616	Sri Lanka: Kalu basin, Runakanda (21)	6.4678 N 80.3190 E	MN005794	MN005741	–
Devario micronema	DZ3351	Sri Lanka: Kalu basin, Delgoda, Kalawana (22)	6.4935 N 80.4045 E	MN005795	MN005742	–
Devario micronema	DZ3352	Sri Lanka: Kalu basin, Delgoda, Kalawana (22)	6.4935 N 80.4045 E	MN005796	MN005743	–
Devario micronema	DZ3353	Sri Lanka: Kalu basin, Halwala, Kalawana (23)	6.5407 N 80.3823 E	MN005797	MN005744	–
Devario micronema	DZ3354	Sri Lanka: Kalu basin, Halwala, Kalawana (23)	6.5407 N 80.3823 E	MN005798	MN005745	–
Devario micronema	DZ3363	Sri Lanka: Kalu basin, Athwelthota (24)	6.5382 N 80.2901 E	MN005799	MN005746	MN005813
Devario micronema	DZ3364	Sri Lanka: Kalu basin, Athwelthota (24)	6.5382 N 80.2901 E	MN005800	MN005747	–
Devario 'annnataliae'	DZ3326	Sri Lanka: Gin basin, Deniyaya (25)	6.3615 N 80.5248 E	MN005755	MN005702	–
Devario 'annnataliae'	DZ3327	Sri Lanka: Gin basin, Deniyaya (25)	6.3615 N 80.5248 E	MN005756	MN005703	MN005814
Devario 'annnataliae'	DZ3328	Sri Lanka: Gin basin, Deniyaya (25)	6.3615 N 80.5248 E	MN005757	MN005704	–
Devario 'udenii'	DZ3862	Sri Lanka: Gin basin, Homadola, Udugama (26)	6.2137 N 80.3495 E	MN005801	MN005748	MN005810
Devario 'udenii'	DZ3863	Sri Lanka: Gin basin, Homadola, Udugama (26)	6.2137 N 80.3495 E	MN005802	MN005749	–
Devario 'udenii'	DZ3864	Sri Lanka: Gin basin, Homadola, Udugama (26)	6.2137 N 80.3495 E	–	MN005750	–
Devario pathirana	DZ3316	Sri Lanka: Nilwala basin, Mulatiyana (27)	6.1787 N 80.5820 E	MN005803	MN005751	MN005815
Devario pathirana	DZ3317	Sri Lanka: Nilwala basin, Mulatiyana (27)	6.1787 N 80.5820 E	MN005804	MN005752	–

determined using Tracer (Rambaut et al., 2014). The posterior probabilities (PP) of the clades (Huelsenbeck et al., 2001) were determined using the frequency of the remaining clades in trees that were sampled every 1000 generations.

Maximum Likelihood (ML) inference was carried out by IQ-TREE and node support was computed by ultrafast bootstrap support (BP) for 1000 iterations (Minh et al., 2013). The optimal partitioning models (Chernomor et al., 2016) for the datasets were determined through ModelFinder (Kalyaanamoorthy et al., 2017) in IQ-TREE under the minimum BIC score. Trees obtained from ML and BI were visualized using Figtree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Leptobarbus hoevenii, *Leptobarbus* sp. (NRM 51750), *Malayochela maassi*, *Neochela dadiburjori*, *Chela cachius*, *Danio rerio* and *Danio kerri* were used as the outgroups.

2.6. Molecular species delimitation

The terminology for species delimitation and specimen identification follows Collins and Cruickshank (2012). Molecular species delimitation methods were applied to achieve two goals. Firstly, to identify the direct GenBank submissions of specimens of *Devario*. For this purpose, we used the reference dataset of confidently identified species of *Devario* from Kullander et al. (2017) and sequences generated in the present study. In an integrative framework that combines geographical data and reference databases, this enables us to identify these sequences more reliably and hence to interpret our phylogenetic inferences more meaningfully.

Our second goal was to infer the boundaries between the putative Sri Lankan species of *Devario*. Because it is widely applied in DNA barcoding, we used the single locus of *cox1* (669 bp) for all these

analyses. The Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012) method was run via the ABGD website (http://wwwabi.snv.jussieu.fr/public/abgd/) using the whole *cox1* dataset for *Devario* and employing default settings under a JC69, K80 and uncorrected p-distances.

The *cox1* dataset was condensed into unique haplotypes and the optimal partitioning models and ML analysis was carried out in IQ-TREE. The ML tree obtained for this dataset was used as the gene tree to identify and delimit species based on Poisson Tree Processes (PTP), Bayesian Poisson Tree Processes (bPTP) (Zhang et al., 2013) and multi-rate Poisson Tree Processes (mPTP) methods (Kapli et al., 2017). The bPTP and PTP analyses were run via the bPTP web server (https://species.h-its.org/ptp/), while the mPTP analysis was run via the mPTP web server (https://mptp.h-its.org/#/tree). The same *cox1* dataset was used to construct an ultrametric bifurcating tree through BEAST v.1.10.0 (Suchard et al., 2018). PartitionFinder 2 was run with the model as "beast". The outgroups were removed from the analyses and the clock and tree models were set as strict clock and Speciation: Yule process, respectively. Two independent MCMC chains were run for 10 million generations, sampling every 1000 generations. Convergence of the two runs was determined by Tracer, and the first 0.1% of generations discarded as burn-in. The two runs were then combined and a maximum clade credibility (MCC) tree was constructed using the posterior sample of trees by using TREEANNOTATOR. This tree was used to delimit species boundaries by using the General Mixed Yule Coalescent model (GMYC) (Fujisawa and Barraclough, 2013) under single threshold, which was run via the Species delimitation server (https://species.h-its.org/).

In addition, the uncorrected pairwise genetic distances for the two genes *cox1* and *cytb* were calculated using MEGA for *Devario annnataliae, D. malabaricus, D. memorialis* sp. nov., *D. micronema, D. monticola*, *D. pathirana* and *D. udenii*.

2.7. Haplotype network

To explore the demographic histories of populations of *D. malabaricus* and *D. micronema*, which are sufficiently widespread to enable > 10 non-contiguous populations to be sampled, nucleotide diversity (π), haplotype diversity (Hd) and neutrality tests, Tajima's D (Tajima, 1989) and Fu and Li's F (Fu and Li, 1993) were conducted using DNAsp v.6 (Rozas et al., 2017). Reconstruction of the haplotype network for the *cytb*, *cox1* and *rag1* genes of Sri Lankan species of *Devario* was inferred by Median Joining Network (Bandelt et al., 1999) in PopArt (Leigh and Bryant, 2015). Direct GenBank submissions of *Devario* sequences from India which were identified as *D. malabaricus* in the present study were also incorporated in the analyses, to investigate the sharing of haplotypes of *D. malabaricus* between Sri Lanka and India.

2.8. Divergence-time estimation

Divergence timing was estimated using BEAST 2 (Bouckaert et al., 2014) using the combined dataset of *cytb* + *cox1* (1761 bp). The dataset was condensed into unique haplotypes and *Devario devario* was used as the outgroup. Two samples which are likely cases of mitochondrial introgression were excluded from the timing analysis (DZ 3197: *D. monticola* and DZ 3738: *D. malabaricus*). In accounting for over-parameterization, PartitionFinder 2 was run with the model as "beast" with a single partition per gene to determine the optimal substitution model. We carried out a log likelihood ratio test with and without enforcing the molecular clock in MEGA. The molecular clock test rejected the null hypothesis and hence a relaxed clock under log-normal distribution was used as the prior for the clock model. Substitution and clock models were unlinked for *cytb* and *cox1*, while the tree model was linked. A Bayesian coalescence method was used as the tree prior. We used the average cyprinid *cytb* substitution rate of 0.0082 substitutions per site per million years to calibrate the *cytb* clock rate (Rüber et al., 2007, 2004). This substitution rate for *cytb* had been obtained for European cyprinids in reference to two independent and reliably dated geological events (Zardoya and Doadrio, 1999). The clock rate for *cox1* was estimated relative to *cytb*. Two independent runs consisting of 20 million generations each were implemented, with a sampling frequency of the Markov Chain Monte Carlo (MCMC) chain set to every 1000 generations. Convergence of the two runs and ESS > 200 for the combined run was determined by Tracer, and the first 10% generations discarded as burn-in. The two runs were then combined and a maximum clade credibility (MCC) tree constructed using the posterior sample of trees by TREEANNOTATOR and visualized using FigTree v1.4.3.

2.9. Metrics and meristics

Measurements were taken on the left side of specimens, using digital calipers, to the nearest 0.1 mm. All measurements were taken point-to-point. Methods for taking counts and measurements follow Fang (1997) with the following exceptions: eye diameter is the horizontal distance between the medial anteriormost and posteriormost points of the exposed eye; caudal peduncle depth is taken as the least depth of the caudal peduncle. Dorsal-fin base length and anal-fin base length are the same as dorsal-fin length and anal-fin length, respectively, in Fang (1997). The following additional measurements were taken: postdorsal length, post-orbital head length, inter-orbital width, inter-narial width, barbel lengths as defined by Sudasinghe et al. (2018a). The transverse scale count was taken diagonally and expressed as the number of scale rows between the dorsal-fin origin and the lateral line, +1, plus the number of scale rows between the lateral line and the anal-fin origin. Counts of circumpeduncular, predorsal and prepelvic scales follow Sudasinghe et al. (2018a). Values in parentheses after a count represent the frequency of that count. Colour-pattern terminology is based on Fang (1998), as modified by Kullander (2015).

3. Results

3.1. Molecular phylogeny

The different nucleotide substitution models and the partitions used in the phylogenetic analyses are provided in Table S3. The phylogenetic analyses based on mitochondrial (*cox1*, *cytb*) and concatenated (*cytb* + *cox1*, *cytb* + *cox1* + *rag1*) genes and both phylogenetic methods performed (ML and BI) yielded highly congruent trees (Fig. 2, Figs. S1–S3) with differences observed mainly in branch lengths.

Three major clades of Sri Lankan *Devario* are identified. For convenient reference, we label these (i) the malabaricus clade, (ii) the micronema clade, and (iii) the memorialis clade (Fig. 2, Figs. S1–S3). Except for the phylogenetic position of the memorialis clade, the remaining two clades consistently recover the same relationships.

The malabaricus clade consists of the widespread species *D. malabaricus* from the lowland floodplains of both the dry and the wet zones of the island, as well as from parts of the central hills and the Indian peninsula (PP, BP ≥ 95), and *D. monticola*, apparently a micro-endemic restricted to a montane stream in the headwaters of Mahaweli basin around Agrapatana in Sri Lanka. Within the malabaricus clade, the populations of *D. malabaricus* from Sri Lanka and India are not clearly resolved. *Devario monticola* is recovered as the sister group to *D. malabaricus* in all the analyses except the BI analysis for *cox1*, in which it forms a polytomy with *D. malabaricus*. In the *cox1*, *cytb*, *cytb* + *cox1* and the *cytb* + *cox1* + *rag1* analyses, the sequences of *D. monticola* DZ 3197 are nested with *D. malabaricus*, while the sequences of *D. malabaricus* DZ 3738 are nested with *D. monticola* (Fig. 2, Figs. S1–S3).

The micronema clade, which includes *D. mirconema*, *D. pathirana*, *D. annnataliae* and *D. udenii*, is characterized morphologically by the presence of a process on the 1st infraorbital; it is broadly restricted to

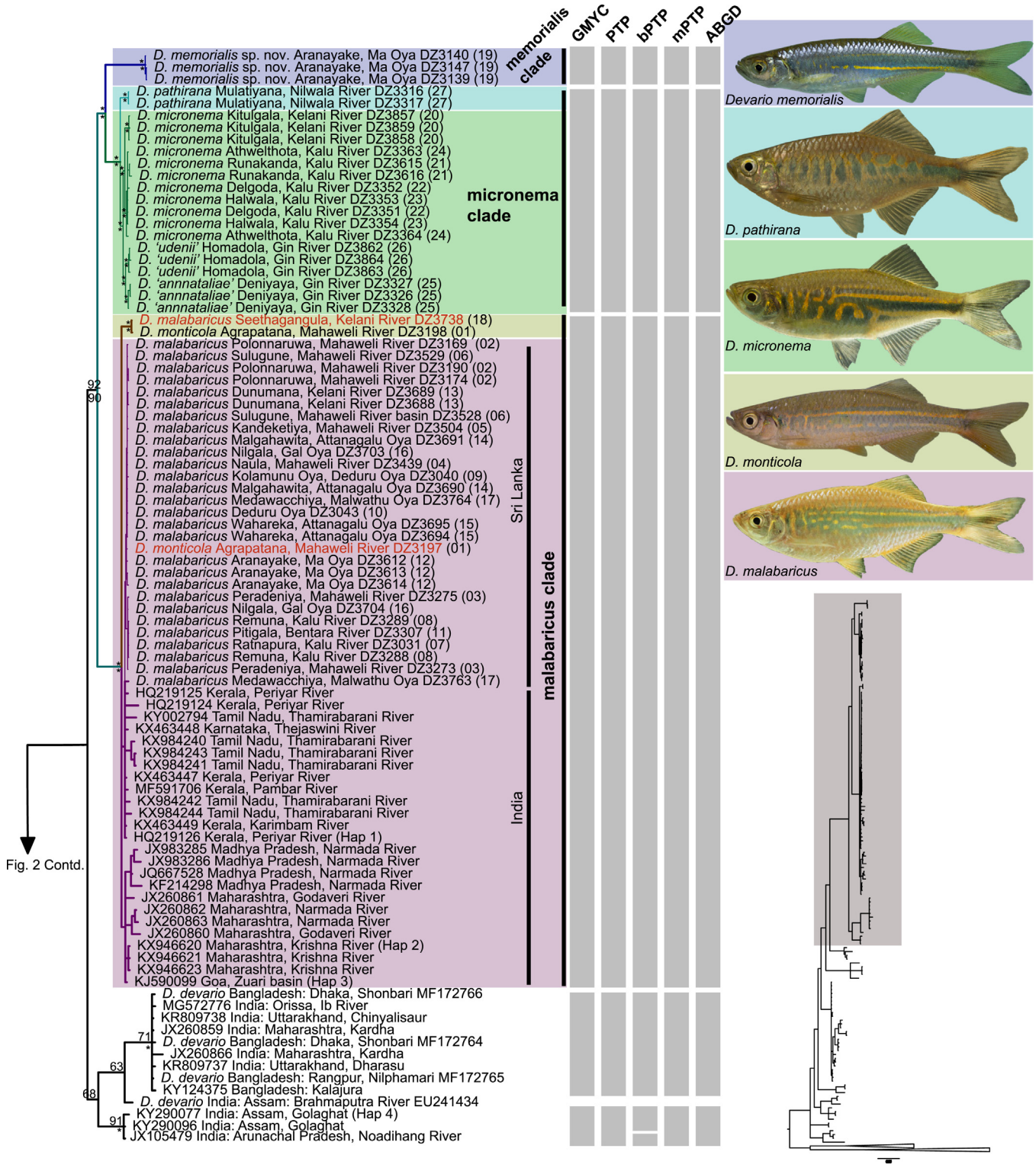

Fig. 2. Molecular phylogenetic relationships of South Asian *Devario*, based on Bayesian inference of the concatenated dataset of the *cytb* + *cox1* + *rag1* (3245 bp) genes, with emphasis on the Sri Lankan species. Asterisks (*) above and below nodes represent ≥ 95% Bayesian posterior probabilities and ML bootstrap values, respectively. Sequences generated in this study, and reference sequences from the published literature, begin with the species name, while identified sequences from GenBank begin with their accession numbers. Results of molecular species delimitation analyses (GMYC, PTP, bPTP, mPTP and ABGD) for *cox1* are shown as grey rectangles on the right. The results of the ABGD shown is based on the initial partition using the K80 model at p = ~0.01.

rainforest habitats in Sri Lanka's southwestern wet zone, drained principally by the Kelani, Kalu, Bentara, Gin and Nilwala rivers. Within the micronema clade, *D. pathirana* is the sister group of *D. micronema* + [*D. annnataliae* + *D. udenii*] (PP,BP ≥ 95). In the *cytb*, *cytb* + *cox1*, and *cytb* + *cox1* + *rag1* phylogenies, *D. udenii* + *D. annnataliae* (both from the Gin basin) form a sister group to *D. micronema* from the Kelani and Kalu basins (PP,BP ≥ 95) while *D. micronema*, *D. annnataliae* and *D. udenii* show a polytomy in the *cox1* analysis.

The memorialis clade, which consists of only the new species *D. memorialis* sp. nov., discovered in the present study, is recovered as the

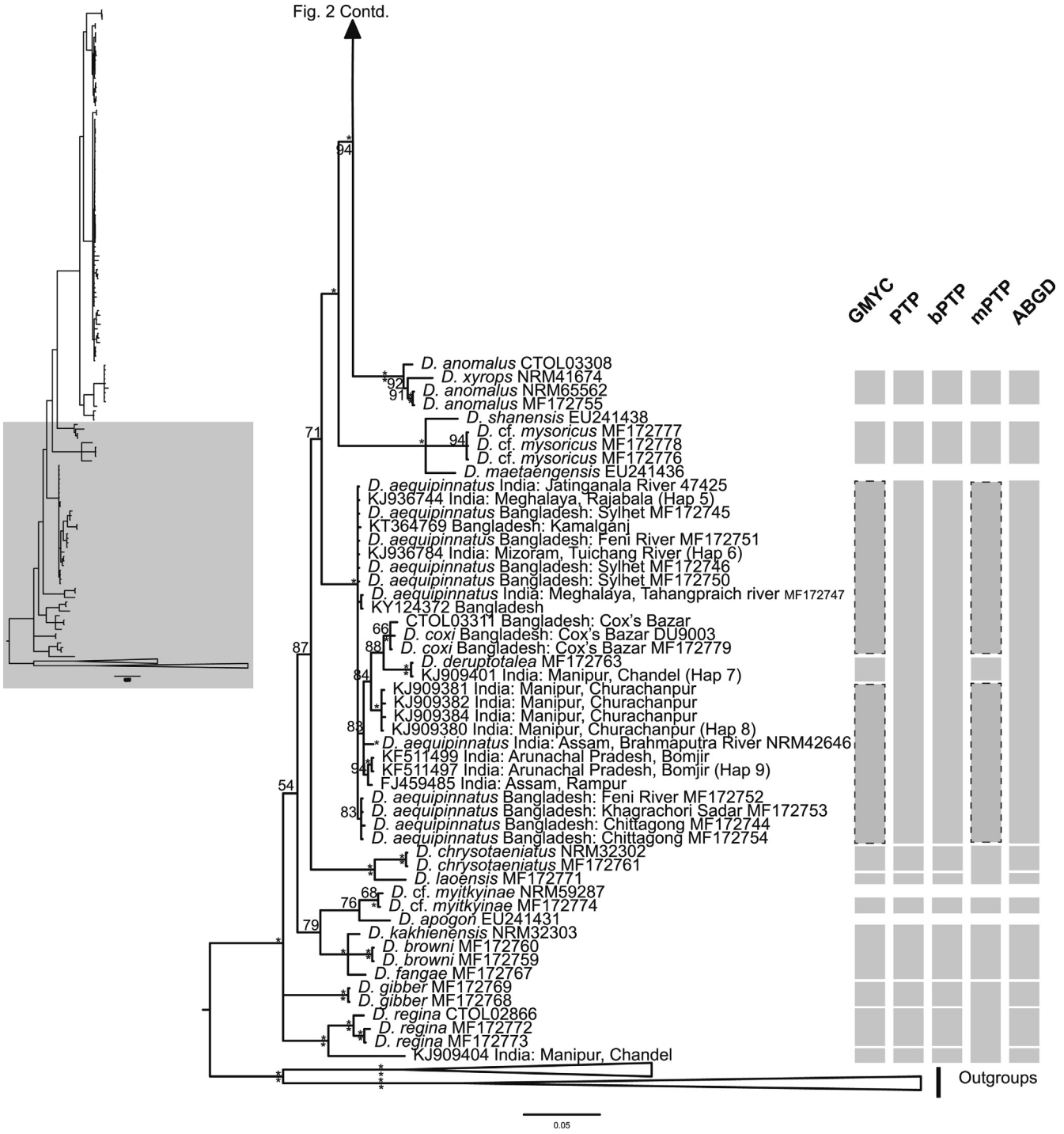

Fig. 2. (*continued*)

sister group to the micronema clade in the *cytb* (PP,BP ≥ 95), *cytb* + *cox1* (PP ≥ 95, BP = 89%) and *cytb* + *cox1* + *rag1* (PP,BP ≥ 95) analyses, while it is recovered as the sister group to the malabaricus clade in the *cox1* analysis, though with relatively weak support (PP = 66, BP = 86%, Fig. S2).

The Indian species *Devario devario* was recovered as the sister group to the Sri Lankan species of *Devario* in all the analyses.

In the *rag1* phylogeny (Fig. S4), however, the Sri Lankan species of *Devario* are not clearly resolved and form a polytomy. *Devario pathirana*, *D. micronema*, *D. annnataliae* and *D. udenii* (the micronema clade) nest together and form a polytomy (PP = 93, BP = 92%). *Devario monticola* (DZ 3197), which nested within *D. malabaricus* in the mitochondrial phylogeny (Figs. S1 and S2), does not nest with that species in the *rag1* phylogeny. Similarly, *D. malabaricus* (DZ 3738), which nested with *D. monticola* in the mitochondrial phylogeny (Figs. S1 and S2), nests with *D. malabaricus* in the *rag1* phylogeny.

3.2. Molecular species delimitation

Based on the reference dataset of confidently-identified samples of *Devario* from Kullander et al. (2017) and the sequences generated in the present study, the GenBank *cox1* sequences with known geographic localities were identified reliably (Table S2, Fig. S5). The various molecular species delimitation methods employed (ABGD, GMYC, PTP,

bPTP and mPTP) gave mostly congruent results. In the ABGD analysis, a definitive 'barcoding gap' was not observed for the dataset under all the distance matrices employed. Based on the initial partition using the K80 model at p= ~0.01, the ABGD analysis delimited only 3 of the 6 putative species in Sri Lanka. Similarly, GMYC, PTP, bPTP and mPTP delimited only 3 of the 6 putative species of *Devario* in Sri Lanka. [*D. pathirana* + *D. annnataliae* + *D. micronema* + *D. udenii*] and [*D. malabaricus* + *D. monticola*] were each delimited as a single species, while *D. memorialis* sp. nov., was recognized as a distinct species by all of the molecular species delimitation methods employed (Fig. S2).

The uncorrected pairwise genetic distances for the *cytb* and *cox1* genes are given in Table S4. *Devario memorialis* sp. nov., differs from the other Sri Lankan species by an uncorrected pairwise genetic distance of more than 4.0% for *cox1* and 7.8% for *cytb*. Sequences of Sri Lankan *D. malabaricus* differ from those of *D. monticola* (DZ 3198) minimally by 1.5% and 3.6%, from *D. micronema* by 3.3% and 7.7%, from *D. pathirana* by 4.2% and 8.2%, from the population identified as *D. annnataliae* by 3.7% and 8.1% and from that identified as *D. udenii* by 3.5% and 8.2% respectively, for the *cox1* and *cytb* genes. The Sri Lankan and Indian samples of *D. malabaricus* differ minimally, by 0.2%, for *cox1*. Within the micronema clade, the uncorrected pairwise genetic distances between *D. pathirana*, *D. micronema*, *D. annnataliae* and *D. udenii* are relatively low: *D. pathirana* differs from *D. micronema* minimally by 1.5% and 1.7%, from *D. annnataliae* by 1.9% and 2.1%, and from *D. udenii* by 1.7% and 2.3% for *cox1* and *cytb*, respectively. *Devario annnataliae* differs from *D. micronema* minimally by 0.4% and 1.3%, and from *D. udenii* by 0.2% and 1.2% for *cox1* and *cytb*, respectively. For the *cox1* and *cytb* genes, samples of *D. udenii* (Gin basin) differ from *D. micronema* from the Kelani basin minimally by 1.0% and 1.5%, and from *D. micronema* from the Kalu basin by 0.2% and 1.5%, respectively; while samples of *D. micronema* from the Kelani basin differ from those from the Kalu basin by 0.8% and 0.7%, respectively.

3.3. Haplotype network

3.3.1. The cox1 haplotype network

Devario memorialis sp. nov. (H1) and *D. pathirana* (H3) each possess a single unique haplotype. Populations of *D. micronema* from the Kelani, Kalu, and the Gin basins (*D. annnataliae* and *D. udenii*) contain one (H2), four (H4-H7), and three (H8-H10) haplotypes, respectively, with no sharing of haplotypes between the basins (Fig. 3A). Sri Lankan samples of *D. malabaricus* contain five haplotypes (H1-H5) while the Indian *D. malabaricus* form 15 haplotypes. No *D. malabaricus* haplotypes are shared between Sri Lanka and India (Fig. 4A). H4, which occurs in *D. malabaricus* from the lowland dry zone, wet zone and the central hills, appears to be a haplotype that signals an ancestral condition of *D. malabaricus* in Sri Lanka. The sequence of *D. monticola* (DZ 3197) forms a shared haplotype (H4) with *D. malabaricus*, while DZ 3198 of *D. monticola* forms a shared haplotype (H17) with *D. malabaricus* (DZ 3738). Populations of *D. micronema* (14 sequences) included 10 segregating sites and 8 parsimony-informative sites, while populations of Sri Lankan *D. malabaricus* (28 sequences) included 9 segregating sites and 4 parsimony-informative sites. π and Hd were greater in *D. micronema* than in *D. malabaricus* (0.00454, 0.879 vs. 0.00182, 0.751), respectively. Tajima's D test was negative but insignificant ($p > 0.05$) for both *D. micronema* (-0.31502) and *D. malabaricus* (-1.49242). Fu and Li's F test statistic was positive for *D. micronema* (0.39137) and negative for *D. malabaricus* (-1.73597), but insignificant ($p > 0.02$) for both.

3.3.2. The cytb haplotype network

Two unique haplotypes (H1-H2) were detected in *D. memorialis* sp. nov. *Devario pathirana* possesses a unique haplotype (H8). Populations of *D. micronema* from the Kelani, Kalu and the Gin basins (*D. annnataliae* and *D. udenii*) contain one (H3), five (H9-H13) and four (H4-H7) haplotypes each, respectively, with no sharing of haplotypes between the three basins (Fig. 3B). The largest haplotype groups were identified in the malabaricus clade (H1-H7, H9-H12). The H9 haplotype of *D. malabaricus,* which represents samples from the lowland dry zone, the wet zone and the central hills, may signal an ancestral condition in this species in Sri Lanka (Fig. 4B). The sequence of *D. monticola* (DZ 3197) forms a unique haplotype (H8), while DZ 3198 of *D. monticola* forms a shared haplotype (H13) with *D. malabaricus* (DZ 3738). Populations of *D. micronema* (n = 13) included 27 segregating sites and 20 parsimony-informative sites, while those of *D. malabaricus* (n = 28) included 14 segregating sites and 8 parsimony-informative sites. π and Hd were greater in *D. micronema* than in *D. malabaricus* (0.00777, 0.885 vs. 0.00333, 0.772), respectively. Tajima's D test was negative but insignificant ($p > 0.05$) for both *D. micronema* (-0.31266) and *D. malabaricus* (-1.28627), while Fu and Li's F test statistic was positive for *D. micronema* (0.24742) and negative for *D. malabaricus* (-2.31170), but insignificant ($p > 0.02$) for both species.

3.3.3. The rag1 haplotype network

Devario monticola (DZ 3197), which nested within the *cox1* and *cytb* haplotype network of *D. malabaricus*, shares a haplotype (H2) with *D. memorialis* sp. nov. (Fig. S4). Similarly, *D. malabaricus* DZ 3738, which nested with *D. monticola* in the mitochondrial haplotype network, shares a haplotype (H3) with *D. malabaricus* in the *rag1* haplotype network. *Devario micronema* from the Kelani, Kalu and Gin (*D. annnataliae* and *D. udenii*) basins shares a single haplotype (H1) with *D. pathirana*.

3.4. Divergence timing

The phylogenetic relationships and the topology obtained for the species of *Devario* in the BEAST analysis of the *cytb* + *cox1* concatenated dataset were largely congruent with those resulting from the BI and ML analyses of the same dataset. Based on the time-calibrated tree obtained for the *cytb* + *cox1* dataset (Fig. 5), the diversification of *Devario* in Sri Lanka is recent, during the Plio-pleistocene. The BEAST analysis estimated that the most recent common ancestor of malabaricus, micronema and memorialis clades diverged from *D. devario* ca 6.2 (95% highest posterior density (HPD): 4.6–7.6) Mya in the late Miocene. The basal split between the malabaricus clade and [micronema + memorialis] clades was estimated 5.6 (95% HPD: 4.1–7.1) Mya, also in the late Miocene or early Pliocene. The divergence between the micronema and memorialis clades was dated at 4.2 Mya (95% HPD: 2.9–5.4), in the early Pliocene, while the split between *D. malabaricus* and *D. monticola* was dated at 2.1 Mya (95% HPD: 1.4–2.8), and that between *D. pathirana* and *D. micronema* around 1.4 Mya (0.9–1.9), in the Pleistocene. The divergence between *D. micronema* from the Gin basin (*D. annnataliae* + *D. udenii*) from *D. micronema* from the Kelani and Kalu river basins was dated at 0.9 (95% HPD: 0.6–1.2) Mya. The populations of *D. micronema* from the Kelani and Kalu river basins have been isolated from each other for ca 0.5 (95% HPD: 0.3–0.8) Mya. The isolation between Sri Lankan and Indian populations of *D. malabaricus* was dated at 0.5 (95% HPD: 0.3–0.7) Mya and continued until ca 0.1 (95% HPD: 0.02–0.3) Mya.

3.5. Taxonomy

Devario memorialis, new species.

3.5.1. Holotype

2020.03.06.NH, 54.9 mm SL, Sri Lanka, Ma Oya basin, Aranayake, 7°08′14″N 80°28′18″E, 238 m asl, H. Sudasinghe, May 2015.

3.5.2. Paratypes

2020.03.07.NH–10.NH, 4, 46.6–52.6 mm SL, same data as holotype; DZ3147, 1, 55.1 mm SL, Sri Lanka, Ma Oya basin, Aranayake, 7°07′57″N 80°28′28″E, 251 m asl, H. Sudasinghe, Jan 2017; DZ3619, 4, 40.6–55.8 mm SL, Sri Lanka, Ma Oya basin, Aranayake, 7°07′47″N

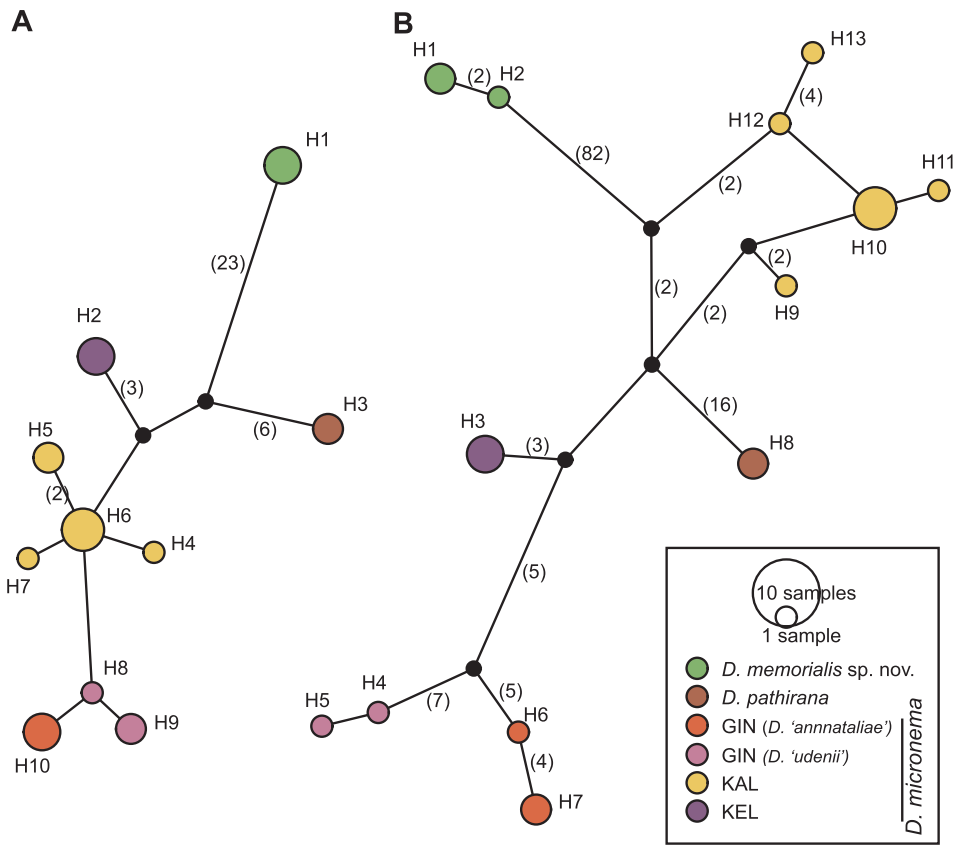

Fig. 3. Median-joining haplotype network for the micronema and memorialis clades of Sri Lankan *Devario*, based on the analysis of (A) a 669 bp fragment of the *cox1* gene; (B) a 1092 bp fragment of the *cytb* gene. The areas of the circles are proportional to the number of individuals sharing a given haplotype. The number of mutational steps > 1 is indicated in parentheses. The black circles are hypothetical nodes. Legend colors correspond to the river basin (*Devario micronema*: Kelani (KEL); Kalu (KAL); Gin ('*D. annnataliae*'); Gin ('*D.udenii*'); or species (*D. pathirana* and *D. memorialis* sp. nov.).

80°28′38″E, 266 m asl, H. Sudasinghe, Jan 2017.

3.5.3. Diagnosis

Devario memorialis sp. nov. (Fig. 6) is distinguished from Sri Lankan and the peninsular-Indian congeners by the combination of the following characters and character states: 8 branched dorsal-fin rays; anal-fin origin on vertical through dorsal-fin origin; dorsal-fin base length (13.1–16.5% SL); P stripe 2 scales wide anteriorly, uninterrupted P stripe commencing between verticals through pectoral- and pelvic-fin origins; anterior bars on body indistinct or absent; interstripes I and I + 1 narrow, about ¼-⅓ times width of P stripe; a mediodorsal band of 2–3 rows of tubercles on lower jaw; rostral barbel 4.1–8.9% HL; body depth 20.4–23.5% SL; 1st infraorbital smooth, with no process; 38–46 lateral-line scales; 15–16 circumpeduncular scales.

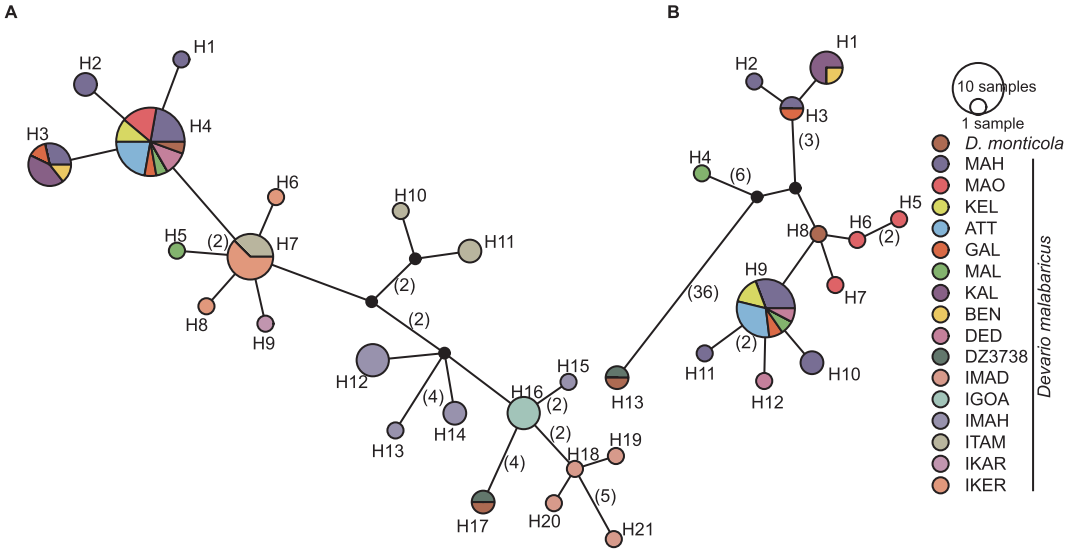

Fig. 4. Median-joining haplotype network for the malabaricus clade, based on the analysis of (A) a 669 bp fragment of the *cox1* gene; (B) a 1092 bp fragment of the *cytb* gene. The areas of the circles are proportional to the number of individuals sharing a given haplotype. The number of mutational steps > 1 is indicated in parentheses. The black circles are hypothetical nodes. Legend colors correspond to the river basin (Sri Lankan *Devario malabaricus*: Mahaweli (MAH); Ma Oya (MAO); Kelani (KEL); Attanagalu Oya (ATT); Gal Oya (GAL); Malwathu Oya (MAL); Kalu (KAL); Bentara (BEN); Deduru Oya (DED), species (*D. monticola*) or region (Indian *D. malabaricus*: Madhya Pradesh (IMAD); Goa (IGOA); Maharashtra (IMAH); Tamil Nadu (ITAM); Karnataka (IKAR); Kerala (IKER)).

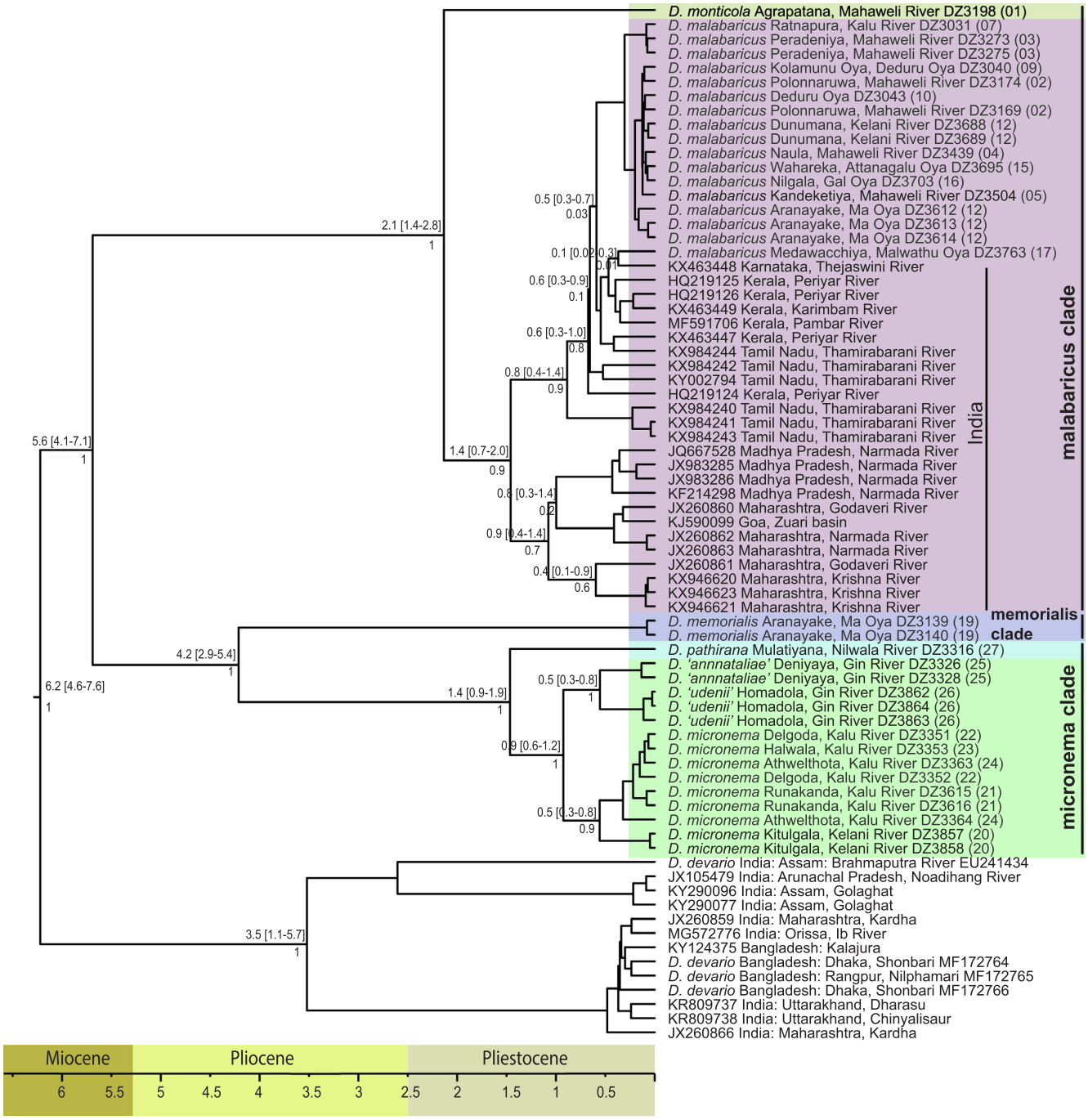

Fig. 5. Bayesian time-calibrated tree of *Devario* based on the concatenated *cytb* + *cox1* (1761 bp) dataset. Values above and below major nodes represent median, 95% HPD and Bayesian posterior probabilities, respectively.

3.5.4. Description

For general appearance, see Fig. 6; morphometric data are provided in Table S5. Head and body laterally compressed, elongate. Body depth greatest at pelvic-fin origin. Snout length subequal to eye diameter. Medial margins of dentaries straight, parallel, with an indentation (danionine notch) anteriorly. Well-developed dermal grooves along supraorbital shelves. Symphysial knob present, small, rounded, fitting into shallow groove on inner margin of upper jaw. Lower jaw slightly longer than upper. 1st infraorbital smooth, lacking a process. Maxilla just reaching vertical through anterior margin of orbit. Tubercles on lower jaw minute, rounded, in a mediodorsal band of 2–3 rows, tapering to a single row posteriorly and towards symphysis; tuberculation uncorrelated with sex. Single row of small, rounded tubercles on upper margin of upper jaw. Pectoral-fin tubercles absent (all 4 specimens dissected to determine sex were females). Both maxillary and rostral barbels present. Rostral barbel not reaching anterior margin of orbit, slightly longer than or subequal to maxillary barbel; maxillary barbel just reaching anterior margin of orbit.

Lateral line complete, declining steeply for first 7 scales, then proceeding in a curve parallel to ventral body outline, low on caudal peduncle, ending on caudal-fin base, with 38 (4), 39 (1), 40 (1), 41 (1), 42 (1), 43 (1) or 46 (1) scales on body, plus 1–2 on caudal-fin base. Median predorsal scales 17 (2), 18 (4) or 19 (4). Lateral scale rows between dorsal and pelvic fins ½7 + 1 + 1 (9) or ½7 + 1 + 1½ (1). Circumpeduncular scales 15 (4) or 16 (6). Anal-fin scales 12(1), 13 (1), 14 (1) or 15 (6).

Fig. 6. Coloration of *Devario memorialis* sp. nov., (A) in aquarium (~50 mm SL); (B) in preservative, holotype, 2020.03.06.NH, 54.9 mm SL. All from Aranayake, Ma Oya basin, Sri Lanka.

Dorsal fin with 2–3 unbranched rays, 8 branched rays, origin on vertical through anal-fin origin, distal margin straight. Anal fin with 3 unbranched rays, 14 (5) or 15 (5) branched rays, distal margin slightly concave. Pectoral-fin with 1 unbranched and 9 (7), 10(2) or 11 (1) branched rays, adpressed fin just reaching or surpassing pelvic-fin origin; axial lobe well developed. Pelvic fin with 1 unbranched and 7 (10) branched rays, origin closer to anal-fin origin than to pectoral-fin origin; tip of adpressed pelvic fin not reaching anal-fin origin. Pelvic 'axillary' scale present. Caudal fin with 8 + 8 (1), 8 + 9 (2) or 9 + 8 (7) branched rays, forked, lobes subequal, rounded distally.

3.5.5. Color pattern

For condition in life, see Fig. 6A; in preservative, see Fig. 6B. In preservative, mid-dorsal stripe narrow, extending from occiput to caudal peduncle. Cleithral spot vertically elongate, covering part of first lateral-line scale and scale above it. P stripe broad, two scale-widths wide anteriorly, 1 scale-width wide posteriorly, continuing to end of medial caudal-fin rays; anteriorly uninterrupted above pectoral and pelvic fins, bordered dorsally and ventrally by narrow I + 1 and I, respectively. Interstripe I narrow, of uniform width, about ¼-⅓ width of P stripe, subequal to P-1 (when present), slightly wider than I + 1. Interstripe I + 1 narrow, of uniform width, about ¼-⅓ width of P stripe, fragmented into spots anterior to pelvic-fin origin. P + 1 originating above middle of pectoral fin, narrower than P, wider than I + 1, tapering to caudal peduncle, not reaching caudal-fin base. Interstripe I + 2 absent or only faintly visible. P + 2 absent. P-1 narrower than P, faint, with scattered melanophores, tapering to caudal peduncle, not reaching caudal-fin base; I-1, P-2 absent. Anterior bars indistinct or absent. Dorsal and anal fins hyaline, with light scattering of small melanophores along interradial membranes. Caudal-fin rays pigmented anteriorly; both rays and interradial membranes pigmented posteriorly. Pectoral and pelvic fins hyaline.

3.5.6. Etymology

The species is named in memory of those who perished in the disastrous landslide at Aranayake, the type locality, in May 2016, while our fieldwork was in progress.

3.5.7. Natural history

Devario memorialis sp. nov., has been recorded only from a 3-km stretch of a headwater stream of the Ma Oya (river), in an elevation range of 238–266 m asl, in the vicinity of Aranayake. It was the most abundant fish in this area, usually observed in fast-flowing parts of the stream in shoals of > 50 individuals. While *D. malabaricus* too, were observed in syntopy with *D. memorialis* sp. nov., the latter was the dominant species. Other cyprinids recorded in the vicinity were *Dawkinsia singhala*, *Garra ceylonensis*, *Puntius bimaculatus*, *P. dorsalis*, *Rasbora microcephalus* and *Tor khudree*.

3.5.8. Comparative remarks

Devario memorialis sp. nov., is immediately distinguished from all other Sri Lankan species of *Devario* by possessing only 8 (vs. 9–12) branched dorsal-fin rays; and having the anal-fin origin vertically beneath dorsal-fin origin (vs. posterior to, beneath 3rd branched dorsal-fin ray). It is additionally distinguished from species of the micronema clade (*D. micronema*: sensu Batuwita et al., 2017, *D. pathirana*, *D. annataliae* and *D. udenii*) by the absence (vs. presence) of a process on the 1st infraorbital.

4. Discussion

We evaluated the genetics, morphology and geographic distribution of Sri Lankan *Devario* in the context of GLC and show the existence of a distinct new species, which we describe as *Devario memorialis* sp. nov. However, several species evaluated did not meet one or two of the criteria evaluated. Here we place our results within the broader context of the taxonomy and the evolutionary history of these fishes.

4.1. Species delimitation

Our Bayesian inference phylogeny based on the *cox1* gene (Fig. S2), recovers *Devario udenii* and *D. annnataliae* nested within a clade that includes *D. micronema*, with an uncorrected p-distance of only 0.2–0.4% between them. The genetic divergences between the topotypes of *D. udenii* and *D. annnataliae* (Gin basin), and *D. micronema* from the Kalu basin, are 0.2–0.6% and 0.4–0.6%, respectively, while those between these two species and *D. micronema* from the Kelani basin are 1.0–1.2% and 1.2%, respectively (Table S4). As shown by Sudasinghe and Pethiyagoda (2019), the diagnoses of *D. udenii* and *D. annnataliae* provided by Batuwita et al. (2017) are deficient. Our morphological

comparison of 31 examples of *D. udenii* against a series of 100 examples of *D. micronema* from the Kelani, Kalu, Bentara and (elsewhere in the) Gin basin uncovered no autapomorphies which, whether singly or in combination, could serve to reliably distinguish this species from *D. miconema* sensu Batuwita et al. (2017), of which we consider it a synonym.

The population to which Batuwita et al. (2017) gave the name *D. annnataliae* (11 ex.), however, can be distinguished from *D. micronema* (31 ex., including '*D. udenii*') by a shorter rostral barbel (6.3–9.6% HL vs. 10.1–16.2 in *D. micronema*); a lesser body depth (18.5–27.3% SL vs. 27.0–35.4); and usually, a smaller, knob-like (vs. rounded or square) infraorbital process. These characters do not however, by themselves suggest a specific distinction between *D. annnataliae* and *D. micronema.* As shown in Fig. S6, body depth is highly variable in *D. micronema* even within individual river basins. The shallower body and shorter barbels in the population referred to *D. annnataliae* may be a consequence of phenotypic plasticity, possibly driven by factors such as stream gradient and predator abundance (e.g., see de Barros et al., 2019; Langerhans and DeWitt, 2004; Malato et al., 2017). Further, the p-distance of 0.2–0.4% between the population assigned to *D. annnataliae* and *D. micronema* elsewhere in the same (Gin River) basin is substantially lower than the ~1.5% usually observed for closely related, morphologically diagnosable species of *Devario* (Kullander et al., 2017; present study); for instance, for *cox1*, *D. pathirana* and *D. micronema* differ by a minimum p-distance of 1.5% (present study) and *D. aequipinnatus* and *D coxi*, and *D. fangae* and *D. kakhienensis* by 1.8% (Kullander et al., 2017). Given the modest morphological disparity between *D. annnataliae* and *D. micronema*, taken together with the low divergence time (Fig. 5), the former cannot be considered an example of a cryptic species (Struck et al., 2018). We therefore follow a conservative taxonomic approach and consider *D. annnataliae* a synonym of *D. micronema* sensu Batuwita et al. (2017).

We suspect that the morphological variation observed within *D. micronema* (including '*D. annnataliae*') may be a result of polymorphism and changes to plastic characters resulting from adaptation to local environments. As observed by Parsons and Robinson (2006), populations of fishes exposed to novel environments could diverge rapidly as a result of the genetic assimilation of such variation. Notwithstanding this polymorphism, *D. micronema* can be reliably distinguished from its Sri Lankan congeners by a suite of characters: a prominent process on the 1st infraorbital, supraorbital usually transgressing the orbital rim, and 4–6 wide bars anteriorly on the side of the body, reaching the pelvic-fin origin or beyond. Our phylogenetic analyses and haplotype networks revealed that there is phylogeographic structure in *D. micronema,* with restricted gene flow within populations in Kelani, Kalu and Gin basins despite recent isolation and geographic proximity. Speciation, however, is not an inevitable consequence of such recently diverged lineages, which can also coalesce over time (Sukumaran and Knowles, 2017).

Comparison of genetic divergences among the closely related taxa that are likely to share a recent common ancestry is more suitable than using barcode gaps, as it aids the understanding of both cryptic diversity and the speciation process (Singhal et al., 2018). Kullander et al. (2017) showed that the p-distance among 15 morphologically distinguishable species of striped *Devario* sampled from across a wide range from southern China and Laos to the Western Ghats of India ranged from 1.8 to 12.8. These data are broadly consistent with the minimum inter-species p-distances among the five morphologically distinguishable species of Sri Lankan *Devario* (*D. malabaricus, D. micronema, D. monticola, D. pathirana* and *D. memorialis* sp. nov.) in our dataset, of 1.5–4.2%.

The exploratory molecular species delimitation algorithms (ABGD, GMYC, PTP, bPTP and mPTP) employed in our study did not discriminate between *D. pathirana* and *D. micronema*, and between *D. malabaricus* and *D. monticola* (Fig. S2), both of which we however, consider as valid species based on multiple lines of evidence assessed in the present study.

The series of 5 examples of *Devario pathirana* examined is reliably distinguished from *D. micronema* by their striking barred colour pattern and by the P-stripe originating posterior to (vs. anterior to) the anal-fin origin. Further, *D. pathirana* occurs only in rainforest streams in the Nilwala basin, whereas *D. micronema* is restricted to rainforest streams in the Kelani, Kalu, Bentara and Gin basins.

Devario monticola is distinguished from *D. malabaricus* by the presence (vs. absence) of occipital tubercles in males, the pectoral fin tip usually falling short of (vs. reaching or surpassing) the pelvic-fin origin, having the tubercles on the lower jaw large (vs. minute), rounded (vs. conical), mostly in 2, rarely 3 rows (vs. 3–6 rows) and by having a shorter rostral barbel (9.1–12.8% HL vs. 10.3–21.7) not reaching (vs. reaching) the anterior margin of the orbit. Although the two species are separated by a p-distance of only 1.5–1.7%, comparably low *cox1* divergences have been recorded also between other species-pairs of *Devario* (Kullander et al., 2017), as well as other freshwater fish groups (Valdez-Moreno et al., 2009).

The underestimation of the true species diversity here could be a coupling effect of small sample size, low genetic distances and low divergence timings between the lineages (Fujisawa and Barraclough, 2013; Luo et al., 2018; Puillandre et al., 2012). Our species delimitations were based only on a single mitochondrial marker, *cox1,* for which only two sequences were available for *D. pathirana* and a single sequence for *D. monticola* (see below on mitochondrial introgression). The *cox1* p-distances between *D. pathirana* and *D. micronema*, and between *D. malabaricus* and *D. monticola,* are only 1.5–1.9% and 1.5–1.7%, respectively. Further, our data show the divergence between *D. pathirana* and *D. micronema*, and between *D. malabaricus* and *D. monticola*, to have occurred as recently as 1.4 Mya and 2.1 Mya, respectively. Future work using larger datasets of rapidly evolving nuclear loci will likely provide robust statistical power to test the species boundaries between these closely related species (Fujita et al., 2012; Leaché et al., 2014; Pedraza-Marrón et al., 2019; Wagner et al., 2013).

4.2. Diversification and biogeography

The phylogeny inferred from our concatenated *cytb* + *cox1* + *rag1* dataset shows the five species of *Devario* recorded from Sri Lanka to belong to a monophyletic group dating to ~5.6 Mya. Sea levels dropped to their lowest since the Oligocene at about this time (De Boer et al., 2010; Hansen et al., 2013) and remained below present levels until the Holocene (Bintanja and van de Wal, 2008). The ~30 km-wide Palk Strait, which now separates Sri Lanka from India, is < 10 m deep and was an isthmus several tens of kilometers wide during the successive sea-level low-stands from the mid-Oligocene until ca 6 kya, facilitating the exchange of biotas between Sri Lanka and southern India (Hansen et al., 2013; McLoughlin, 2001; Miller et al., 2013). Despite this connectivity, however, the island retained a high level of endemism (Bossuyt et al., 2004).

Devario malabaricus is the only member of the genus that is shared between Sri Lanka and India. This species is abundant and widespread throughout the lowlands of southern peninsular India and the lowland 'floodplain' that surrounds Sri Lanka's central hills (Fig. 1). Our phylogeny suggests there has been gene flow until ~0.1 Mya between the Sri Lankan and South Indian populations of *D. malabaricus*, suggestive of the species also having inhabited the erstwhile Palk Isthmus, with the potential to disperse in both directions. The time-calibrated molecular phylogeny of Beenaerts et al. (2010) too, showed that a lineage of parathelphusid (freshwater) crabs adapted to open lowland habitats migrated between Sri Lanka and India ca 5.5 Mya, which is consonant with our timing.

The diversification of the remaining four species of Sri Lankan *Devario* was the result of autochthonous insular speciation in the mountains and the moist south western quarter of the island. The Pleistocene was an epoch that witnessed the rapid diversification in

these rainforests and montane habitats also of shrub frogs (Meegaskumbura et al., 2019) and freshwater crabs (Beenaerts et al., 2010).

The split between the micronema clade and *D. memorialis* sp. nov., the oldest speciation event in the genus in Sri Lanka, occurred in the early Pliocene, dated at ~4.2 Mya. This appears to have been coincident with Late Miocene and early Pliocene climatic changes: global cooling, drying and changing phytography (Beenaerts et al., 2010; Cerling et al., 1997; Milá et al., 2017). Meanwhile, Himalayan-Tibetan orogeny resulted in the intensification of the South Asian monsoons (Chatterjee et al., 2013; Clift et al., 2008; Prell and Kutzbach, 1992; Zhisheng et al., 2001) facilitating the formation and expansion of the rainforests and providing niches for the diversifying lineages (Meegaskumbura et al., 2019).

Devario micronema is the most widely distributed of the endemic species of *Devario* in Sri Lanka. It is restricted largely to shaded streams traversing 'rain' forests in the foothills, rather than the more open habitats of the coastal floodplain across which there appear to be no barriers to dispersion. The absence of shared haplotypes between populations of *D. micronema* in the major river basins (Kelani, Kalu, Gin) it inhabits, all of which debouch into the Indian Ocean across a common lowland floodplain (Fig. 1), suggest restricted gene-flow even between adjacent basins of this evidently 'forest-adapted' species.

There is, by contrast, no such phylogeographic structure observed in *D. malabaricus*, which is widely distributed in the lowlands, including the south-western floodplain (Fig. 1). It appears to have enjoyed unrestricted gene flow throughout the lowlands of the island's dry and wet zones. The star-like pattern in the ancestral haplotypes (H4 in *cox1*, H9 in *cytb*) and negative values for Fu and Li's F tests (though not significant) are suggestive of a recent population expansion, possibly a consequence of more 'open' (unshaded) habitats becoming available as a result of Holocene anthropogenic modifications.

The splits between *D. monticola* and *D. malabaricus*, and between *D. pathirana* and *D. micronema*, were estimated at 2.1 Mya and 1.4 Mya, respectively. *Devario pathirana* and *D. micronema* are restricted to per-humid 'rain' forests in the island's south-western quadrant, while *D. monticola* is restricted to a montane sub-basin at an altitude of ~1400 m in the central hills.

Sri Lanka's montane freshwater-fish fauna is depauperate. Unlike in the Western Ghats of southern India, there is a striking absence of rheophilic fishes adapted to life in torrential waters. Although Senanayake (1980) explained this by invoking the isolating action of waterfalls that inhibit the upward dispersion of fishes, the case of the Western Ghats, in which dozens of torrent-adapted species flourish (Jayaram, 2010) suggests this cannot by itself explain the absence of torrent fishes in Sri Lanka. The only three native fish genera that now occur in the island's mountains (*Devario, Garra* and *Schistura*) have representatives also in the lowlands. This may suggest a recent colonization of mountain streams following a general extinction, perhaps caused by prolonged desiccation.

4.3. Mitochondrial introgression in Sri Lankan Devario

Hybridization between *Devario* species in nature was first mentioned by Kullander et al. (2017), where two specimens morphologically identified as *D. anamolus* nested within the *D. aequippinatus* clade for the mitochondrial *cox1* gene while the nuclear *rag1* phylogeny nested them within *D. anamolus*. Kullander et al. (2017) mentioned this as a putative example of introgression between *D. aequippinatus* and *D. anamolus*. Similarly, in our analysis too, a specimen morphologically identified as *D. monticola* (DZ 3197) nested with *D. malabaricus* in the mitochondrial phylogenies, but separately in the nuclear phylogeny (Fig. S4). Conversely, DZ 3738, a juvenile morphologically identified as *D. malabaricus* from the central hills nested with *D. monticola* in the mitochondrial phylogeny, while nesting with the remaining *D. malabaricus* in the nuclear phylogeny.

These results are suggestive of hybridization and mitochondrial introgression in *Devario* in Sri Lanka. Given the recent diversification of this group in the island, it is possible that there has been insufficient time for reproductive barriers to evolve between some of the species. Hybridization and introgression are hence plausible at their zones of contact, or as a result of undocumented translocations (e.g. by fisheries agencies) or river diversions for irrigation or hydroelectricity projects (Sudasinghe et al., 2018b; Wikramanayake, 1990). We note, nevertheless, that *D. malabaricus* (e.g., DZ3612-14) occurs together with *D. memorialis* sp. nov., in the headwaters of Ma Oya. Interestingly, this is the only one of the 64 sites from which specimens have been examined in this study, at which two species of *Devario* occur in syntopy. Though based on limited sampling, our molecular analysis exhibits no evidence of mitochondrial introgression between these two species. Indeed, we hypothesize that hybridization and mitochondrial introgression may be common in species pairs like *D. micronema/D. malabaricus* and *D. monticola/D. malabaricus* at their zones of contact.

5. Conclusions

Devario is one of the few groups of freshwater fishes that show remarkable diversification on Sri Lanka. Our findings, based on mitochondrial and nuclear data, support the monophyly of the five species we report from the island. Our molecular dating analysis estimates the diversification of *Devario* on Sri Lanka to have occurred during the Pliopleistocene. Phylogenetic and haplotype network analyses suggest strong basin-centric phylogeographic structure within the endemic species of *Devario*, which are largely confined to forest habitats in the island's south-western wet zone. In contrast, the widely distributed *D. malabaricus,* the only non-endemic species, shows little phylogeographic structure in Sri Lanka, suggesting geneflow unconstrained by basin boundaries. Molecular and morphological analyses failed confidently to identify *D. annnataliae* and *D. udenii* as distinct species. Both are considered synonyms of *D. micronema*. The most widespread of the endemic species, *D. micronema,* shows morphological variation within different populations, likely attributable to polymorphism. The discordance between the mitochondrial and nuclear phylogenies for some samples of *Devario* suggest signs of mitochondrial introgression. A new species of *Devario* from a remnant rainforest habitat in Aranayake, discovered in the present study, highlights the need for further biodiversity exploration in Sri Lanka.

CRediT authorship contribution statement

Hiranya Sudasinghe: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Rohan Pethiyagoda:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition. **Madhava Meegaskumbura:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

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Appendix A. Supplementary material

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