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A near-complete species-level phylogeny of uropeltid snakes harnessing historical museum collections as a DNA source

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ABSTRACT

Uropeltidae is a clade of small fossorial snakes (ca. 64 extant species) endemic to peninsular India and Sri Lanka. Uropeltid taxonomy has been confusing, and the status of some species has not been revised for over a century. Attempts to revise uropeltid systematics and undertake evolutionary studies have been hampered by incompletely sampled and incompletely resolved phylogenies. To address this issue, we take advantage of historical museum collections, including type specimens, and apply genome-wide shotgun (GWS) sequencing, along with recent field sampling (using Sanger sequencing) to establish a near-complete multilocus species-level phylogeny (ca. 87% complete at species level). This results in a phylogeny that supports the monophyly of all genera (if *Brachyophidium* is considered a junior synonym of *Teretrurus*), and provides a firm platform for future taxonomic revision. Sri Lankan uropeltids are probably monophyletic, indicating a single colonisation event of this island from Indian ancestors. However, the position of *Rhinophis goweri* (endemic to Eastern Ghats, southern India) is unclear and warrants further investigation, and evidence that it may nest within the Sri Lankan radiation indicates a possible recolonisation event. DNA sequence data and morphology suggest that currently recognised uropeltid species diversity is substantially underestimated. Our study highlights the benefits of integrating museum collections in molecular genetic analyses and their role in understanding the systematics and evolutionary history of understudied organismal groups.

1. Introduction

Uropeltidae Müller, 1832 is a family of small (generally < 40 cm total length) fossorial snakes endemic to peninsular India and Sri Lanka (e.g., Pyron et al., 2016), and mostly to the Western Ghats and Sri Lanka global biodiversity hotspot (Bossuyt et al., 2004; Myers et al., 2000). Comprising eight genera and ca. 64 currently recognised species (Uetz and Hošek, 2022), this little-studied squamate clade is poorly understood, in part because of the secretive habits of many of these snakes. Barriers (especially financial and administrative e.g. Pethiyagoda et al., 2007; Prathapan et al., 2009) to the flow of information and material among biologists and museum collections in South Asia and the West have also hindered progress, resulting in a confusing taxonomy that, for the most part, was established in the late 1800s and early 1900s with

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little subsequent revision. In the last decade there has been a resurgence in taxonomic activity, with several new species being described — often based solely on external morphological characters — and other species resurrected from synonymy and/or reallocated to different genera (Aengals and Ganesh, 2013; Cyriac et al., 2020; Ganesh et al., 2021; Ganesh and Achyuthan, 2020; Gower et al., 2008; Gower, 2020; Gower and Wickramasinghe, 2016; Jins et al., 2018; Pyron et al., 2016; Sampaio et al., 2020; Wickramasinghe et al., 2009, 2020).

Attempts to quantitatively analyse evolutionary relationships among uropeltids were based initially on a limited number of species and immunological (Cadle et al., 1990) or cranial data (Rieppel and Zaher, 2002), but more recent studies have used additional species and generated phylogenies for the family based on mitochondrial and nuclear sequence data (Bossuyt et al., 2004; Cyriac and Kodandaramaiah, 2021, 2017; Jins et al., 2018; Pyron et al., 2016, 2013; Sampaio et al., 2020). Despite this recent progress, which has resulted in an improved genus-level classification, uropeltid phylogenetics requires further examination, to increase taxonomic sampling and to resolve incongruences in the relationships among genera inferred by some of the latest analyses (Cyriac and Kodandaramaiah, 2017; Jins et al., 2018). In addition, although all genera were sampled in the latest molecular phylogenetic studies, the monophyly of some genera (*Platyplectrurus* and *Plectrurus*) remains unaddressed because only a single species of these genera were included in the analyses. Sampling of uropeltids in molecular phylogenetic studies has been challenging for two main reasons: (1) fossorial squamates are not easily encountered without dedicated effort, such that some species have rarely been collected and/or are known only from very few museum records, and (2) identification of sequenced vouchers to species level has proven challenging because of an incompletely resolved taxonomy, lack of working identification keys, and lack of precise and/or accurate locality data for the types of many species.

Given the challenges of sampling and identifying some uropeltids, they are good candidate taxa to apply recently developed ancient DNA molecular approaches to generate DNA sequence data from historical natural history collections. Most uropeltid specimens in European and North American natural history collections (where most types reside) are old, and of unknown preservation history. From the early 1900s, fixing specimens in formalin prior to long-term storage in alcohol became common practise for vertebrate wet specimen preservation (Simmons, 2014). There are several challenges in sequencing ancient DNA or DNA from specimens from historical collections (reviewed in Dabney et al., 2013): i) DNA degraded into short fragments (Pääbo, 1989); ii) high occurrence of nucleotide substitutions from C to T at the 5′ and G to A at the 3′, with nucleotide misincorporations caused by cytosine deamination occurring more frequently in single stranded overhangs at the 5′ of ancient DNA strands (Briggs et al., 2007; Brotherton et al., 2007); iii) cross-linkage between DNA strands or between DNA and other molecules, which does not allow the strands to be separated and thus preventing DNA polymerases moving along a strand (Pääbo, 1989). Additionally, samples fixed in formalin are also known to have DNA misincorporations in the form of C to T and G to A (Williams et al., 1999; Wong et al., 2014), and cross-linkage between DNA and proteins (Gilbert et al., 2007 and references therein). Due to these issues with DNA fragmentation and quality, traditional PCR-based approaches aiming to amplify target fragments of DNA are typically inappropriate to obtain sequence data from historical fluid-preserved specimens, especially for those fixed in formalin (Gilbert et al., 2007 and references therein; Williams et al., 1999; Wong et al., 2014).

Advances in high-throughput sequencing (HTS) technologies, including genome-wide shotgun (GWS) sequencing and sequence capture, have made it possible to obtain large amounts of DNA sequence data and even whole genomes from degraded DNA. State-of-the-art bioinformatic tools have been an important associated development to assemble reads de novo or through alignment to a reference genome. Recent studies using these HTS methods on herpetological fluid-preserved and in some cases formalin-fixed specimens have had varying levels of success (Hykin et al., 2015; McGuire et al., 2018; O'Connell et al., 2022; Pyron et al., 2022; Ruane and Austin, 2017). Molecular data from type and historical museum specimens would be useful to resolve outstanding issues in uropeltid taxonomy, especially through matching of extant populations of known locality data with historical and type specimens lacking precise collection data. Additionally, generating DNA sequence data from historical museum specimens would greatly help maximise taxon sampling to build near-complete species-level phylogenies that could be used for downstream analyses of uropeltid evolution.

Here, we report a near-complete species-level phylogeny for Uropeltidae, combining Sanger sequencing data for recent samples with GWS sequencing data from fluid-preserved historical museum specimens, including type specimens. We use our results to ask 1) Are currently recognised uropeltid genera monophyletic?; 2) Are Sri Lankan uropeltids (a subset of endemic species of the genus *Rhinophis*) monophyletic, consistent with there being a single colonisation event from Indian ancestors?; 3) Does current uropeltid taxonomy accurately catalogue the group's extant species diversity?; and 4) Can sufficient amounts of DNA sequence data be generated from fluid-preserved museum specimens of uropeltids using ancient DNA methods?

2. Materials and methods

2.1. Sanger sequencing

2.1.1. Taxon sampling and identification

A total of 230 uropeltid samples were used to infer phylogenetic relationships. The samples employed in the Sanger sequencing part of this study were obtained through relatively recent (mostly 21st Century) fieldwork in the Western Ghats and Sri Lanka. This included representatives of all genera and included specimens referable to the majority of currently recognised species (ca. 43/64 species). Additional sequence data were retrieved from GenBank. Details on sample codes, species, locality, and GenBank accession numbers for all samples included in the analyses are listed in Table S1 (Appendix A). Specimens were identified based on external morphology, using the most recent understanding of uropeltid taxonomy and diagnostic characters (Cyriac et al., 2020; Ganesh et al., 2021; Ganesh and Achyuthan, 2020; Ganesh and Murthy, 2022; Gower, 2020; Gower and Wickramasinghe, 2016; Jins et al., 2018; Pyron et al., 2016; Sampaio et al., 2020; Wickramasinghe et al., 2020 and references cited in these works). Samples in Table S1 bearing names including "sp." or "cf." were not able to be identified as described species with confidence. Some samples were identified as being most similar to species currently regarded as junior subjective synonyms (e.g. Pyron et al., 2016; Uetz and Hošek, 2022). For specimens that were not types, these identifications were informed by morphology and sometimes by locality data. More detailed reasoning and justification for our identification and labelling of samples in trees and tables is presented in Appendix A.

2.1.2. DNA extraction, amplification and sequencing of recent tissue samples

Genomic DNA was extracted from absolute ethanol-preserved muscle or liver using Qiagen's DNeasy Blood and Tissue kits (Qiagen, Valencia, CA). DNA was amplified using PCR for four mitochondrial (mtDNA) markers: 12S rRNA (*12s*), 16 s rRNA (*16s*), NADH dehydrogenase subunit 4 (*nd4*) and cytochrome *b* (*cytb*), and two nuclear (nuDNA) loci: oocyte maturation factor (*cmos*) and prolactin receptor (*prlr*). The mtDNA markers and *cmos* have been used successfully in previous phylogenetic studies of uropeltids (Bossuyt et al., 2004; Cyriac and Kodandaramaiah, 2017; Jins et al., 2018; Pyron et al., 2016) and thus also provide additional data. *Cmos* is a relatively conserved marker that is typically useful to infer deeper phylogenetic divergences among extant reptiles (Saint et al., 1998). Because this marker is not particularly informative at and below the species level, the additional nuDNA

marker *prlr* was also selected because it has been shown to be relatively rapidly evolving in other squamates (Townsend et al., 2008). Detailed information on PCR protocols and DNA sequencing can be found in Supplementary methods and Table S3 (Appendix A). Sequence chromatograms were checked manually and edited using Geneious v.8.1.9 (Kearse et al., 2012) (Biomatters). Heterozygous positions in nuclear sequences were scored with IUPAC ambiguity codes.

2.2. Museum samples

2.2.1. Taxon sampling

A total of 44 uropeltid historical museum specimens were selected (Table S4), including type specimens, with the aim that resulting sequence data would fill sampling gaps and/or help resolve taxonomic issues impeding accurate and precise identification of vouchers sampled from fresh tissue. Historical specimens of different accession ages (1801 to 1977) were included. Where multiple specimens were available, those with existing incisions and/or in seemingly better overall condition were selected. Sampling included some species for which recent or fresh tissue was also available (compare Tables S1 and S4) to provide an opportunity to verify DNA sequences from historical specimens. Although it is highly likely that the museum specimens preserved after 1970 were fixed in formalin and those preserved prior to the early 1900s were highly likely not (see Introduction), data on the preservation history of each specimen were not available.

2.2.2. DNA extraction and library build

Genomic DNA was extracted from historical specimens to generate low-level whole-genome coverage to assemble mitochondrial and nuclear markers to match the Sanger dataset. See Table S4 for information on samples used, and supplementary methods (Appendix A) for detailed historical-specimen DNA extraction and library-build protocols. DNA concentration was quantified using Qubit dsDNA HS kit, and fragment lengths were analysed with TapeStation (prior to pooling and after pooling). A total of 48 double-indexed libraries (44 samples and four negative controls) were pooled together at an equimolar concentration. Multiplexed samples were subjected to shotgun sequencing using a mid-output kit for 75 base pairs (bp) paired-end reads on an Illumina NextSeq500 platform (Natural History Museum (NHM) Sequencing Facility). Raw reads for samples successfully sequenced have been deposited in Sequence Read Archive (BioProject PRJNA879208; accession numbers can be found in Table S5).

2.2.3. Bioinformatics

Reads were trimmed for Illumina adapters with AdapterRemoval v.2.2.4 (Schubert et al., 2016). Given that DNA damage is typically more likely to occur at the ends of fragments, three base pairs were removed from the 3′ and 5′ ends of each read. As an additional quality check, read ends were trimmed for both Ns and low-quality bases. Minimum read length was set to 25 bp and overlapping paired reads (of at least 11 nucleotides by default) were merged with a mismatch rate of three. Quality of trimmed reads was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) (results not shown).

In the absence of complete mitochondrial or nuclear genomes of conspecific or very closely related taxa to be used as references in a mapping approach, a two-step approach was employed. First, a de novo assembly (Zerbino and Birney, 2008) was carried out to obtain new draft reference partial mitochondrial genomes for each sample. Second, the trimmed reads were mapped to the newly generated draft references (Figure S4).

Multiple kmer sizes (17, 27, 37, 47, 57) were used in a de novo assembly using Velvet v.1.2.10 (Zerbino and Birney, 2008). Kmer length is an important choice for a successful assembly, though kmer size selection is not straightforward, and analysing a set of different kmer lengths can aid deeper genome coverage (Bi et al., 2012). This approach has been employed successfully in previous studies using ancient or museum specimen DNA for de novo genome assembly (Hykin et al., 2015; Seitz and Nieselt, 2017). The coverage cut-off value was initially set at 5, and if that was deemed too high (i.e., by having only a few contigs mapping against a reference), cut-offs of 3 and then of 1 were applied for selected samples.

For each sample, contigs from all Velvet runs were imported into Geneious and using Map to Reference (in the Tools > Align/Assemble menu) contigs were mapped against a partial uropeltid mitochondrial genome (GenBank accession number: GC200594 – *Rhinophis* cf. *philippinus*, which lacks the duplicated control regions). The Geneious mapper option was employed with a Medium-Low Sensitivity setting (except for sample FS85, for which Medium Sensitivity was applied, to increase the number of contigs mapping to the reference sequence), with up to five iterations. Some assembled contigs showed mismatches in some positions (particularly toward the ends of contigs) due to incorporation of possibly deaminated bases. Consensus sequences (25% threshold) were extracted and used as new draft references for each respective sample in a second step, mapping trimmed reads in Burrows Wheeler Aligner (BWA) aln (Li and Durbin, 2009). Default options were used except for no seeding (-l1024), a setting typically applied when working with degraded DNA to avoid deamination driven nucleotide mismatches occurring in the seed region, forcing a global alignment of the reads to the reference sequence (Rasmussen et al., 2015; Schubert et al., 2012). Samtools v.1.9 (Li et al., 2009) was used to remove unmapped reads, and to remove reads with mapping quality Phred scores of<30. Picard MarkDuplicates v.2.18.16 (https://broadinstitute.github.io/picard/) was used to remove PCR duplicates obtained during library builds. Mapping statistics for total reads, initial mapped reads, quality filtered reads (MAPQ30), and quality filtered reads with duplicates removed were calculated with Samtools (flagstat). DNA damage patterns were assessed by plotting nucleotide misincorporation in mapDamage v.2.0 (Jónsson et al., 2013). Mean read length and partial mtDNA genome coverage were calculated with QualiMap v.2.2.1 (Okonechnikov et al., 2015). Resulting BAM files were imported into Geneious, where consensus sequences for partial mitochondrial genomes for each sample were generated, and sequences (homologous with those generated by Sanger sequencing for fresh tissues) for up to four mitochondrial markers (*12s*, *16s*, *nd4* and *cytb*) were extracted. These data were then added to the data matrix of Sanger sequences for downstream phylogenetic analyses.

Attempts were made to also retrieve nuDNA sequences of greatest relevance to this phylogenetic study (i.e. *cmos* and *prlr*). Trimmed reads were imported into Geneious and mapped to reference sequences (generated from Sanger sequencing and from GenBank), using the same settings and process as described above. These attempts were unsuccessful (see below).

2.3. Sequence alignments, data partitions and model selection

Sequence data were aligned using ClustalW (Thompson et al., 1994) implemented in Geneious with default settings (gap open cost = 15; gap extended cost = 6.66). Ambiguously aligned positions in *12s* and *16s* alignments were removed using Gblocks v.0.91b (Castresana, 2000) via an online server (https://phylogeny.lirmm.fr/, Dereeper et al., 2008) selecting the 'less stringent' option. The tRNA regions were removed from *nd4* sequences. Protein-coding gene (*cytb*, *nd4*, *cmos* and *prlr*) alignments were checked for unexpected stop codons and reading-frame shifts.

Alignments were concatenated and assembled into multiple datasets: 1) Sanger sequencing data for uropeltids (mtDNA and nuclear data); 2) combined Sanger and HTS data (mtDNA and nuclear data); 3) mtDNA data for all samples (for input in single locus species delimitation methods); and 4) *cmos*-, and 5) *prlr*-only datasets (Table S6). These concatenated datasets are available in nexus and fasta format via the NHM Data Portal (data.nhm.ac.uk/dataset/Sampaio_uropeltidae; https://doi.org/10.5519/97dm3yy8) and in Appendix B. The five

datasets were analysed with PartitionFinder v.2.1.1 (Guindon et al., 2010; Lanfear et al., 2016, 2012), to determine the best-fit partition schemes and models of nucleotide substitution, implementing a greedy search algorithm and applying the Bayesian information criterion (BIC) for model selection. Data were partitioned by gene for *12s* and *16s*, and by gene and codon position for the protein-coding genes, giving a total of 14 possible partitions (PartitionFinder results are presented in Appendix A Table S6).

2.4. Phylogenetic analyses

2.4.1. Concatenated mt- and nuDNA phylogeny

Phylogenetic relationships were inferred using Bayesian Inference (BI) and Maximum Likelihood (ML), implementing data partitions and substitution models based on PartitionFinder results (Table S6). BI analyses were performed in MrBayes v.3.2 (Ronquist et al., 2012), conducting two independent runs for $5x10^7$ generations, sampling every 5,000 generations, resulting in 10,000 trees. Runs were checked for convergence by visually examining trace plots and ensuring that effective sample sizes (ESS) were greater than 200 using Tracer v.1.7.1 (Rambaut et al., 2018). The first 25 % trees were discarded as burnin and the remaining trees were used to determine Bayesian posterior probability values (PP) for branch support. Additional BI analyses for individual nuclear markers were generated in MrBayes by conducting two independent runs for $1x10^6$ generations, sampling every 100 generations, resulting in 10,000 trees for each dataset.

Analyses were carried out using the online CIPRES Science Gateway v3.1 server (Miller et al., 2010). Trees were visualised and edited in R v.3.6.0 (R Development Core Team, 2019) with packages ape v.5.3 (Paradis and Schliep, 2019) and phytools v.0.6-99 (Revell, 2012). Trees were rooted with a monophyletic *Melanophidium* (in R using the root function implemented in ape v.5.3 (Paradis and Schliep, 2019)) based on evidence from previous studies that this genus is sister to all other uropeltids (Cyriac and Kodandaramaiah, 2017; Jins et al., 2018).

ML analyses were conducted using IQ-TREE v.2.2.0 (Nguyen et al., 2015), applying ModelFinder (Kalyaanamoorthy et al., 2017) to find the best partition models. Branch support was assessed using an ultra-fast bootstrap (BP) approximation (Minh et al., 2013) with 1,000 replicates.

2.4.2. Concatenated mtDNA phylogeny

A fully resolved tree (lacking polytomies) for the concatenated mtDNA matrix (dataset 3), for use in lineage (potential species) delimitation analyses, was generated using MrBayes v.3.2 conducting two independent runs for 10^7 generations, sampling every 1,000 generations, resulting in 10,000 trees. All other settings were implemented as described in Section 2.4.1.

An ultrametric tree (an input requirement for the species delimitation method bGMYC (Reid and Carstens, 2012)), was built for the same dataset (3) using BEAST2 v.2.4.8 (Bouckaert et al., 2014). A strict clock model was selected (ucld.stdev was $\ll 1$), using a Yule model as the tree prior. Three independent analyses were run for $1x10^8$ million generations sampling every 10,000 generations. Convergence of runs was detected as reported above. Runs were merged in LogCombiner (Drummond et al., 2012), and a maximum clade credibility (MCC) tree was obtained using TreeAnnotator (Drummond et al., 2012), discarding 10% of trees as burnin. Additionally, the three runs were merged in LogCombiner discarding 10% as burnin, and 100 trees were resampled from the posterior probability distribution. MrBayes and Beast2 runs were conducted in the CIPRES Science Gateway v3.1 server (Miller et al., 2010).

2.5. Species delimitation

To attempt to obtain greater confidence in identifying candidate species (e.g. Amador et al., 2018), we employed four different tree-based species delimitation methods – Bayesian general mixed Yule-coalescent (bGYMC: Reid and Carstens, 2012), Poisson tree processes (PTP: Zhang et al., 2013), multi-rate PTP (mPTP: Kapli et al., 2017) and Bayesian Phylogenetics and Phylogeography (BPP: Yang, 2015). These methods were applied to the following four major clades, A) *Melanophidium*; B) *Teretrurus* + *Platyplectrurus*; C) *Uropeltis*; D) *Pseudoplectrurus* + *Plectrurus* + *Rhinophis*, which were extracted from the Maximum clade credibility (MCC) tree (using the extract.clades function implemented in ape). This was necessary because when these ingroup taxa were analysed together using bGMYC, log ratio values (coalescent rate/Yule rate) were below zero. This indicated that the model was not a good fit to the data, likely due to substantial divergences among the main clades, and/or high rate heterogeneity (Talavera et al., 2013). In addition, there is a limit to the number of species in the guide tree for BPP analyses, with greater than 30 species causing issues with the program's memory.

2.5.1. Single-locus species delimitation

Three species-delimitation methods were applied to the mtDNA sequence data for four main clades (labelled A–D: see Results). Each clade was analysed using bGMYC for $1x10^6$ generations, with a thinning of 100, and with the first 10% of samples discarded, ensuring log ratio values were above zero. Multiple probability thresholds of delimitation ranging from 0.1 to 0.9 in intervals of 0.1 were implemented (0.1 being the most conservative and 0.9 being more liberal in terms of estimated number of species). Additionally, PTP and mPTP were also implemented. Unlike bGMYC, these methods do not require an ultrametric tree, and the concatenated mtDNA tree from MrBayes was used. bPTP (the bayesian implementation of the PTP model) was run for $1x10^6$ MCMC iterations, sampling every 100, with a burnin of 10 %, and mPTP was run using both ML and MCMC searches, the latter analyses were run for $1x10^8$ iterations, sampling every 10,000 steps, with a burnin of 10%.

2.5.2. Multilocus species delimitation

Results from single-locus species delimitation analyses were compared and used to designate the putative species to be included in the multilocus delimitation software BPP v.4 (Flouri et al., 2018). Analysis A10 compares species delimitation models based on a user specified fixed tree that guides the Markov chain (Rannala and Yang, 2013; Yang and Rannala, 2010). The guide trees employed for each clade were based on the BI tree inferred from the concatenated mt + nuDNA dataset, in which all polytomies were resolved using ape's multi2di function in R. BPP analyses were conducted for concatenated mitochondrial and nuclear data, as well as for mitochondrial and nuclear data separately (dataset 6 — Table S1, Appendix A). Multiple BPP analyses were conducted, implementing different prior settings for ancestral population sizes (θ) and divergence times (τ), based on those previously applied in studies of squamate reptiles and amphibians (Bellati et al., 2015; Gehara et al., 2017; Leaché and Fujita, 2010). Prior values were converted from a gamma to an inverse gamma distribution IG(α,β), because the latter is implemented in the most recent version of BPP, by preserving the priors mean ($\beta/(\alpha-1)$) and variance ($b^2/((\alpha-1)^{2*}(\alpha-2))$) the same. The three different prior settings assumed: 1) large ancestral population sizes and deep divergences among species ($\theta \sim$ IG (3, 0.2) and $\tau \sim$ IG(3, 0.2); θ and τ with mean = 0.1 and variance = 0.01); 2) small ancestral population sizes and recent divergences ($\theta \sim$ IG (4, 0.003) and $\tau \sim$ IG(4, 0.003); θ and τ with mean = 0.001 and variance = $5x10^{-7}$); and 3) large ancestral population sizes and recent divergence ($\theta \sim$ IG(3, 0.2); $\tau \sim$ IG(4, 0.003)). To account for rate variation among loci, a symmetric Dirichlet prior was set with $\alpha = 5$. After a burnin of 10^6 samples, each analysis ran for $4x10^6$ steps with a sampling frequency of 10 (i.e., 400,000 samples were logged). All prior combinations were run four times in total, twice for each of the two rjMCMC algorithms (0 and 1) available in BPP to assess reliability of results. The results of the multiple runs were compared to identify potential convergence problems.

3. Results

3.1. Shotgun sequencing and historical-DNA recovery

Of the 44 samples included in the shotgun sequencing, 22 yielded contigs from Velvet that successfully mapped to the reference uropeltid partial mitochondrial genome (GenBank accession: GC200594) (Appendix A Table S7). For the 22 samples that yielded mtDNA sequences, BWA mapping results after quality trimming and duplicate removal revealed mean coverage depths between 2.3x and 78.2x, with < 1% of the original reads being mapped for all samples. Read lengths were relatively short, averaging between 36.8 and 52 bp. Summary statistics for mapped reads are reported in Table S7. Although the age of the sampled specimens is not always clear from their accession numbers, at least eight of the 22 successfully sequenced samples were from the late 1800s (Table S4). MapDamage analyses revealed patterns of deamination at the ends of the reads, typical signatures of post-mortem DNA damage (Jónsson et al., 2013; Schubert et al., 2012), with a relative increase of frequency of C to T misincorporations at the 5′ ends and an increase of G to A transitions at the 3′ ends of the reads. Example nucleotide-misincorporation pattern plots for two samples mapped in BWA after quality trimming and duplicates removal are presented in Figures S5 and S6. Sequences of up to four mitochondrial markers (*12s*, *16s*, *nd4* and *cytb*) were extracted for the 22 samples and used in subsequent phylogenetic analyses (Appendix A, Table S1). No nuclear sequence data for *cmos* or *prlr* were retrieved from the shotgun data.

3.2. Phylogenetic relationships

For each of the two multilocus datasets (datasets 1 and 2), ML and BI analyses yielded similar topologies that were congruent for all well-supported clades. The only exceptions were the relationships among some Indian *Rhinophis* (discussed below). The inferred trees include several polytomies, almost all of which are confined to low-level (largely intraspecific) relationships. Given the general agreement for well-supported relationships across different analyses, we summarise here the results for the more taxonomically complete dataset 2 (Sanger and shotgun data). The ML tree for dataset 2 is available in Appendix A Figure S1, and the BI tree based on dataset 1 (Sanger only dataset) is provided in the supplementary materials data files (Appendix B).

Overall, the monophyly of all genera was strongly supported. Within Uropeltidae four well-supported main clades were found: Clade A (*Melanophidium*), Clade B (*Platyplectrurus* and *Teretrurus*), Clade C (*Uropeltis*) and Clade D (*Pseudoplectrurus* + *Plectrurus* and *Rhinophis*). Clade B is resolved as the sister group to clades C + D.

Within *Melanophidium* (Clade A; Fig. 1), all four currently recognised species were included in the analyses, and all are resolved as monophyletic with the exception of *M. wynaudense*, which is paraphyletic with respect to *M. khairei*. Terminal branches within *Melanophidium* are generally long, indicating high levels of genetic diversity within currently recognised species, particularly for *M. punctatum* and *M. wynaudense*. Clade B (Fig. 2) comprises the two currently recognised *Platyplectrurus* species together resolved as sister, with maximum support, to a clade comprising *Brachyophidium* and at least four well-supported lineages of *Teretrurus*. Clade C includes approximately 21 out of the 25 currently recognised *Uropeltis* species, and an additional ten or so lineages that likely represent named (i.e., known synonyms) or as yet undescribed species. The two clades (C1 and *C*2; Fig. 3) separated by the basal split within *Uropeltis* comprise species with notably different external tail morphologies – taxa in clade C1 have Pyron et al.'s (2016) tail types III and IV (equivalent to types I and III of Smith (1943)), and those in clade *C*2 have tail type V (equivalent to Smith's (1943) type II). Within clade D (Fig. 4), the monotypic *Pseudoplectrurus* is sister to a maximally supported group comprising all three currently recognised *Plectrurus* species. Together, *Pseudoplectrurus* + *Plectrurus* is the sister group to all (Indian and Sri Lankan) *Rhinophis,* with the monophyly of the latter genus also receiving maximum support.

Some disagreement between the results of the ML and BI analyses was identified regarding the relationships among some of the Indian *Rhinophis*. All analyses resolved three main Indian lineages (Fig. 4): *R. goweri*, *R. travancoricus*, and a clade comprising all other Indian *Rhinophis.* However, the relationships among these three Indian lineages and the Sri Lankan *Rhinophis* are not consistent except in that *R. goweri* is more closely related to Sri Lankan *Rhinophis* than to other Indian *Rhinophis*. In some analyses, the Sri Lankan *Rhinophis* are paraphyletic with respect to *R. goweri,* though support is not compelling.

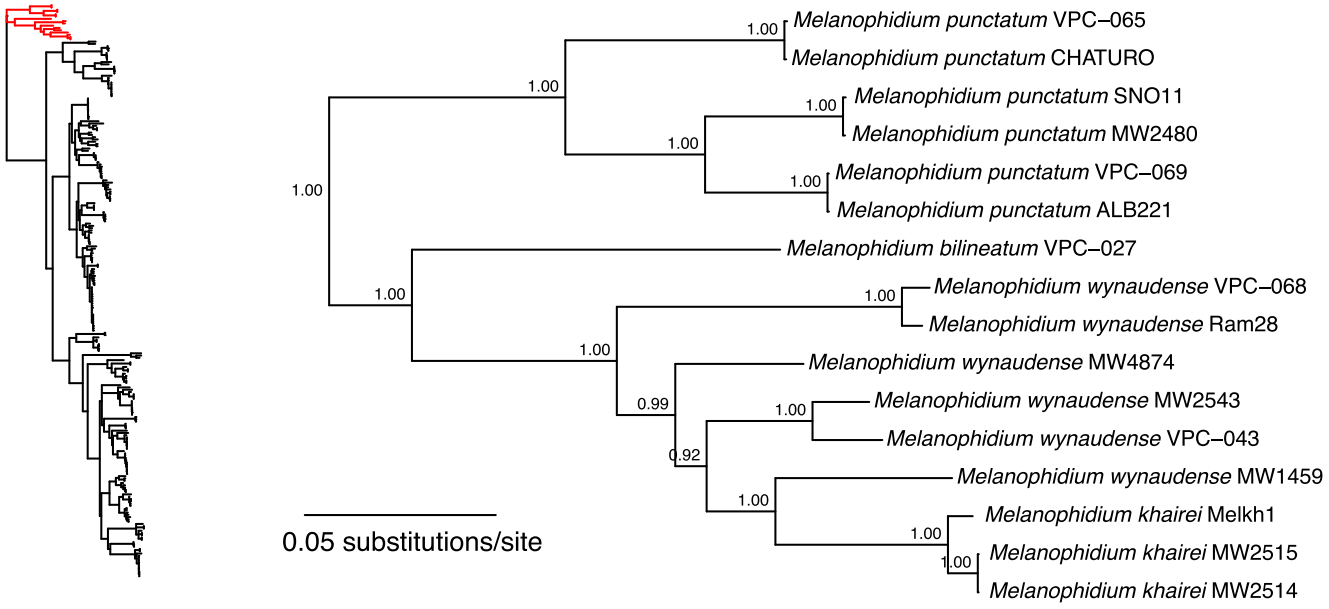

Fig. 1. A subset of the Bayesian phylogeny, representing clade A (*Melanophidium*). The entire tree is plotted on the left, with the position of clade A highlighted in red. Tree based on mt- (*12s*, *16s*, *nd4* and *cytb*) and nuDNA markers (*cmos* and *prlr*) generated through Sanger and whole-genome shotgun sequencing. Numbers at internal branches are Bayesian posterior probabilities (above, given to two decimal places). Scale bar indicates substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

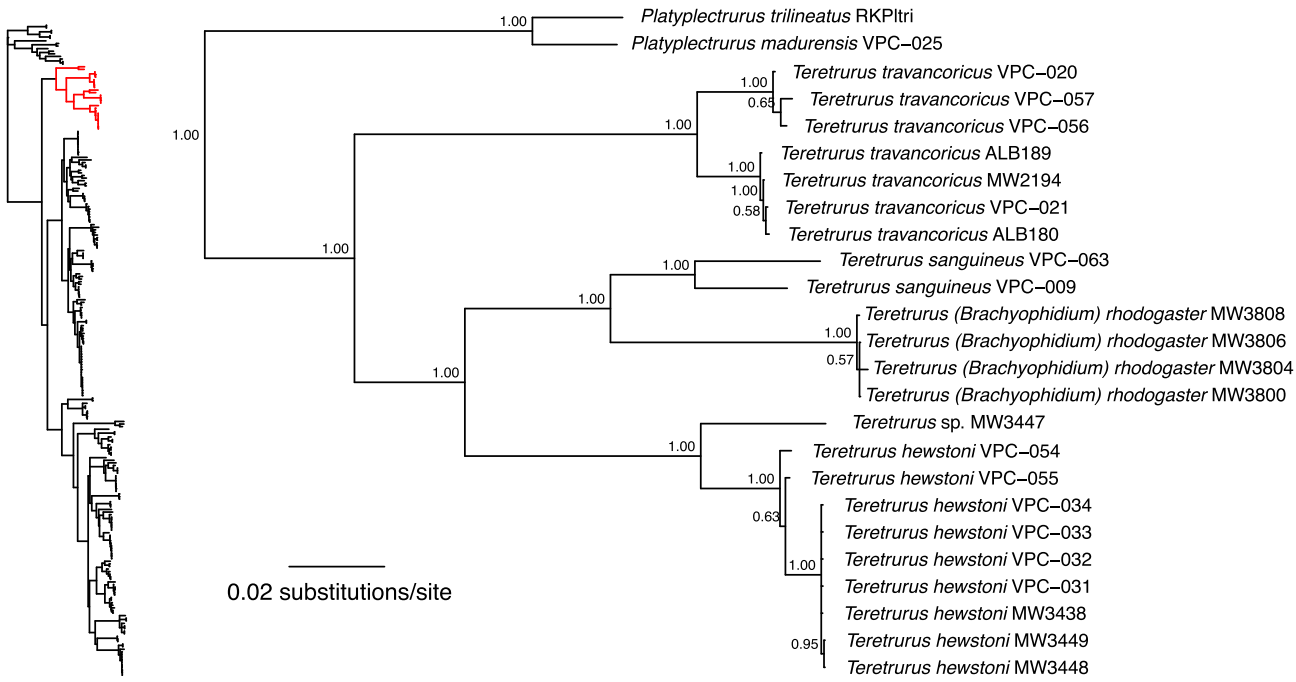

Fig. 2. A subset of the Bayesian phylogeny, representing clade B (*Platyplectrurus* + *Teretrurus*). The entire tree is plotted on the left, with the position of clade B highlighted in red. Tree based on mt- (*12s*, *16s*, *nd4* and *cytb*) and nuDNA markers (*cmos* and *prlr*) generated through Sanger and whole-genome shotgun sequencing. Numbers at internal branches are Bayesian posterior probabilities (above, given to two decimal places). Scale bar indicates substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In general, there was seemingly accurate placement of historical samples, where this could be assessed through closeness of relationships to conspecific specimens sampled from fresh tissue (Figs. 1–4). Thus, the historical samples (including types) of specimens of *Uropeltis ellioti* (though see Section 4.2), *U. liura*, *U. rubrolineata, U. petersi, U. arcticeps, U. woodmasoni, Rhinophis saffragamus*, *R. oxyrhynchus* and *Pseudoplectrurus canaricus* all clustered most closely with fresh samples of vouchers identified morphologically as the same (or most similar) respective species. P-distances between these conspecific historical and recent samples are generally small (Appendix B).

3.3. Species delimitation

The numbers of operational taxonomic units (OTUs), which may represent putative or candidate species recovered by each single-locus analysis are summarised in Table 1 (detailed results in Table S8). Results of bGMYC analyses employing different threshold values were consistent for all genera except *Uropeltis* and *Teretrurus,* for which estimates of OTUs were 21–48 and 9–12, respectively. bPTP results were similar to those for bGMYC that employed 0.6 or 0.7 threshold values, while mPTP consistently detected substantially fewer OTUs than the other single-locus methods (Table 1). The results from mPTP were more in line with the number of currently recognised species, although some samples that we are confident represent different species were clustered together (e.g., samples identified as *P. madurensis* and *P. trilineatus*; and *U. macrorhyncha*, *U. petersi*, and other closely related *Uropeltis* species). Our sampling includes a number of paraphyletic 'species' (based on voucher identifications) and obvious singletons, which mPTP tends to clump together with other nested or sister lineages, respectively (Kapli et al., 2017). Consequently, results from mPTP were not considered further for comparison with the other delimitation methods employed in this study.

There are no precise guidelines for which bGMYC threshold is likely to yield the most accurate results, and most workers have seemingly arbitrarily selected those generated using a midpoint (0.5) value. Here, we further examine bGMYC results from application of a threshold of 0.6 and results from bPTP (because they were broadly in agreement; Table 1) to select the putative species-level units to be included in the guide tree in the BPP analyses. Beyond simple counts (one more *Uropeltis* OTU detected by bPTP than by bGMYC 0.6), there were also some discordances between the bGMYC 0.6 and bPTP results in the composition of OTUs, which were treated for the BPP analyses on a case-by-case basis, as follows. In both analyses, the *U. broughami* sample BMNH1946.1.16.29 was grouped in the same unit as its sister lineage comprising all *U. woodmasoni* samples. These two species are morphologically similar (e.g., they have the derived condition of 19 midbody dorsal scale rows) but specimens attributed to each species are very distinct in, for example, the number of ventral scales (> 200 in *broughami* versus < 180 in *woodmasoni*), and therefore were retained as distinct species for input in BPP. The two samples of *Uropeltis* cf. *dupeni* VPC-038 and VPC-039 were grouped as a single OTU by bGMYC but as different OTUs by bPTP. The two specimens are from nearby localities and lack obvious morphological differences (pers. obs.) and so were grouped into a single species for BPP and divergence analyses input. *Uropeltis ellioti* VPC-004 was recovered as a singleton (the sole sampled member of a species as determined by molecular species delimitation) in bGMYC analysis and clustered with a monophyletic lineage of other *U. ellioti* samples in bPTP. Because this sample is a singleton, it was used as single OTU in BPP. *Uropeltis phipsonii* U19 3765 grouped with other *U. phipsonii* samples in the bGMYC results but was a separate OTU in the bPTP results – only *12s* data are available for this sample, so it was not considered as distinct from other *U. phipsonii* samples for input in BPP and divergence analyses.

Results from BPP analysis with the concatenated mtDNA and nuDNA dataset varied substantially depending on the starting parameters (Table 2). Overall, for the four main clades, settings based on assuming small ancestral-population sizes and recent divergences (prior settings 2) tended to split samples into more putative species (output support values close to 1), though analysis under these settings still erroneously clustered together all *Plectrurus* samples as a single OTU, and did the same for the *Uropeltis woodmasoni* and *U. broughami* samples. In the concatenated mt- and nuDNA dataset, runs for clade C (*Uropeltis*) and D

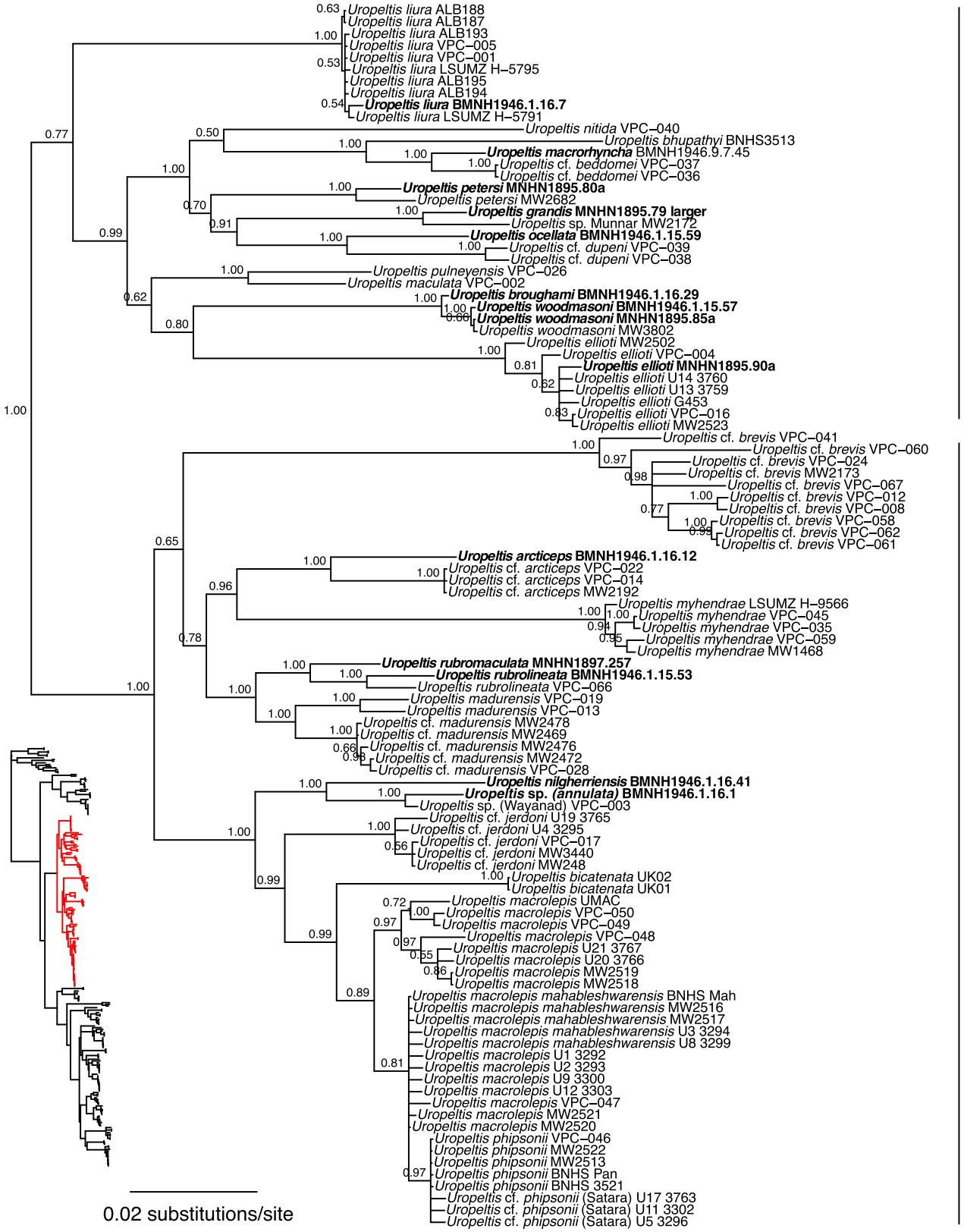

Fig. 3. A subset of the Bayesian phylogeny, representing C (*Uropeltis*). The entire tree is plotted on the left, with the position of clade C highlighted in red. Tree based on mt- (*12s*, *16s*, *nd4* and *cytb*) and nuDNA markers (*cmos* and *prlr*) generated through Sanger and whole-genome shotgun sequencing. Tips in bold indicate historical museum samples obtained through shotgun sequencing. Numbers at internal branches are Bayesian posterior probabilities (above, given to two decimal places). Scale bar indicates substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

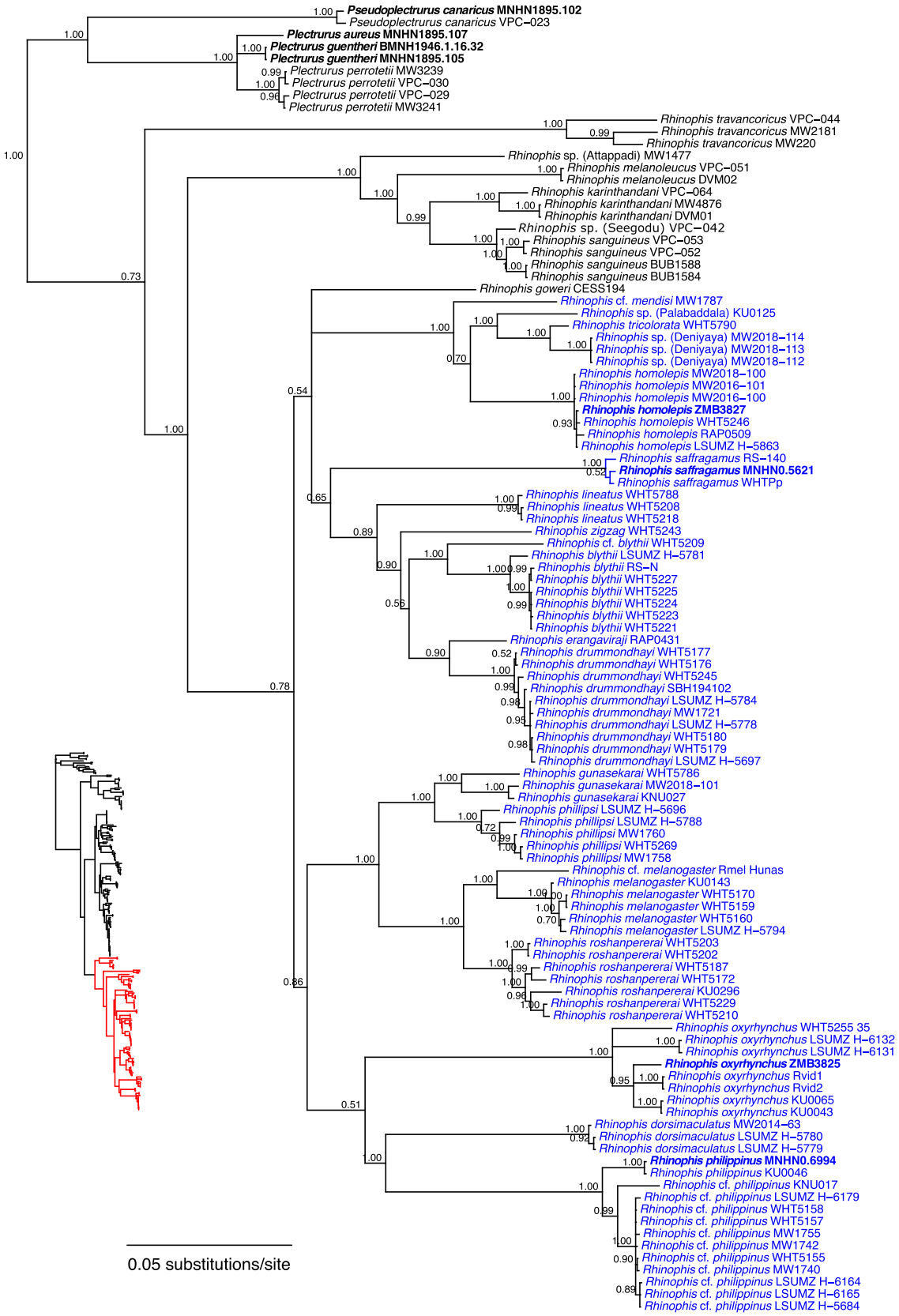

Fig. 4. A subset Bayesian phylogeny, representing clade D (*Pseudoplectrurus*, *Plectrurus* + *Rhinophis*). The entire tree is plotted on the left, with the position of clade D highlighted in red. Tree based on mt- (*12s*, *16s*, *nd4* and *cytb*) and nuDNA markers (*cmos* and *prlr*) generated through Sanger and whole-genome shotgun sequencing. Tips in bold indicate historical museum samples obtained through shotgun sequencing. Numbers at internal branches are Bayesian posterior probabilities (above, given to two decimal places). Scale bar indicates substitutions per site. Indian sample tips coloured in black and Sri Lankan in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Number of putative lineages identified by alternative species-delimitation analyses. Results from single-locus delimitation analyses used to inform species input into the multilocus method BPP are highlighted in bold. Number of currently recognised species in each major clade are presented based on Pyron et al. (2016) with additions of species described by Jins et al. (2018), Ganesh & Achyuthan (2020), Cyriac et al. (2020), Wickramasinghe et al. (2020); Gower (2020), Sampaio et al. (2020). Values in parentheses are the number of currently recognised species initially thought to have been sampled.

	Clade A	Clade B	Clade C	Clade D
	Melanophidium	*Teretrurus*, *Brachyophidium*, *Platyplectrurus*	*Uropeltis*	*Plectrurus*, *Pseudoplectrurus*, *Rhinophis*
Method	4 (4)	4 (4)	25 (22)	27 (24)
bGMYC 0.1	11	9	21	48
bGMYC 0.2	12	9	26	48
bGMYC 0.3	12	10	29	49
bGMYC 0.4	12	10	34	49
bGMYC 0.5	12	10	39	49
bGMYC 0.6	**12**	**11**	**43**	**49**
bGMYC 0.7	12	11	45	50
bGMYC 0.8	12	11	46	50
bGMYC 0.9	12	12	48	51
bPTP	**12**	**11**	**44**	**49**
mPTP multi	5	7	17	36

(*Pseudoplectrurus*, *Plectrurus* and *Rhinophis*) were inconsistent, having failed to converge. The other two prior settings, based on assumptions of large ancestral-population sizes and either deep or recent divergences among species (prior settings 1 and 3, respectively), resulted in similar delimitations and tended to cluster together samples into fewer putative species, including combining into single units samples that we identified as currently recognised distinct species.

4. Discussion

Recent molecular phylogenetics studies of Uropeltidae have made some progress in clarifying the relationships among these understudied fossorial squamates (Cyriac and Kodandaramaiah, 2017; Jins et al., 2018; Pyron et al., 2016, 2013; Sampaio et al., 2020). The historically confusing taxonomy of uropeltids and availability of relatively few recent tissue samples have limited further progress. Here, we substantially increased sampling of individuals and species by generating new Sanger multilocus data and combining this with high-throughput derived mtDNA sequence data from historical museum specimens. This has allowed us to infer a near-complete species-level molecular phylogeny that offers new insights into species diversity, taxonomy and evolution of the group.

4.1. Molecular phylogenetics of Uropeltidae and taxonomic implications

Only two of the few previous molecular phylogenetic studies of Uropeltidae had sampled all genera of the family (Cyriac and Kodandaramaiah, 2017; Jins et al., 2018) and none had sampled more than 60% of the 64 species currently recognised. The present study represents the most extensive molecular and taxonomic sampling of uropeltids across both India and Sri Lanka, sampling all currently recognised genera and more than 50 named species. This greatly expanded taxonomic sampling has provided the basis for greater confidence in the inferred relationships and generic classification of many taxa.

Our phylogenetic results, like those of all molecular studies thus far (Bossuyt et al., 2004; Cadle et al., 1990; Cyriac and Kodandaramaiah, 2017; Jins et al., 2018; Pyron et al., 2016, 2013; Sampaio et al., 2020), support Pyron et al.'s (2016) recent reclassification of the species recognised between 1943 and 2015 as *Uropeltis phillipsi, U. melanogaster* and *Pseudotyphlops philippinus* under the binomials *Rhinophis phillipsi*, *R. melanogaster* and *R. saffragamus*, respectively. The inclusion of additional taxa in our analyses also provides evidence for the first molecular test of (and support for) the monophyly of *Platyplectrurus* and of *Plectrurus*.

Relationships among genera are congruent with those inferred by Cyriac and Kodandaramaiah (2017) including that *Rhinophis* is more closely related to *Uropeltis* than to *Brachyophidium* (=*Teretrurus*), whereas other studies with smaller sample sizes instead found *Rhinophis* to be more closely related to *Brachyophidium* (Bossuyt et al., 2004; Jins et al., 2018; Pyron et al., 2016). As found by Cyriac and Kodandaramaiah (2017), *B. rhodogaster* is nested within a paraphyletic *Teretrurus*, supporting the conclusion that *Brachyophidium* and *Teretrurus* can be considered congeneric, as has been proposed previously (e.g. Cadle et al., 1990; Ganesh and Murthy, 2022; Rieppel and Zaher, 2002; Smith, 1943). On this matter we are less cautious than Cyriac and Kodandaramaiah (2017) and agree with Ganesh & Murthy (2022) that *Brachyophidium* Wall, 1921 can be emphatically relegated to the subjective junior synonymy of *Teretrurus* Beddome, 1886, with *B. rhodogaster* reclassified as *T. rhodogaster*. In terms of major external features, the two genera differ only in that *Brachyophidium* lacks separate supraocular scales, and in this respect resembles all known species of *Melanophidium*, *Pseudoplectrurus*, *Rhinophis*, and *Uropeltis* (e.g. Pyron et al., 2016; Smith, 1943), with separate supraoculars likely being lost convergently on multiple occasions. Our results and those of Cyriac and Kodandaramaiah (2017) are consistent with Ganesh and Murthy's (2022) removal of *T. hewstoni* and *T. travancoricus* from the synonymy of *T. sanguineus*. Some of the relationships we infer among species of *Uropeltis* differ from the results of Cyriac and Kodandaramaiah (2017), which is likely explained by the overlapping but larger taxon sampling in the present study, and associated differences in alignments, partitions and models.

Some of the phylogenetic results are consistent with morphological taxonomic work, including the suggestion (Jins et al., 2018) that the

Table 2
BPP results summary. For each prior, three different values of branch support (0.75, 0.85, 0.95) were considered to delimit species. Where there is a range rather than a single value that is because there was variation among the four runs in each set.

		Prior settings 1			Prior settings 2			Prior settings 3		
Clade	# tips	0.75	0.85	0.95	0.75	0.85	0.95	0.75	0.85	0.95
Melanophidium	12	6	5	3	11–12	11	9	6	5	4
Teretrurus (+*Brachyophidium*)	9	3	3	3	9	9	8–9	3	3	3
Platyplectrurus	2	1	1	1	2	2	1–2	1	1	1
Pseudoplectrurus + *Plectrurus*	4	2	2	2	2–4	2–4	2–4	2	2	2
Uropeltis	44	19	19	18	30	30	23–26	19	19	18
Rhinophis	45	21–22	20–21	20	31–45	31–45	31–45	20–21	19–20	19–20
Totals			**47–53**			**74–102**			**47–52**	

previously unsampled *Uropeltis macrorhyncha* is closely related to *U. bhupathyi*, and Wickramasinghe et al.'s (2020) interpretation that *Rhinophis gunasekarai* is closely related to *R. phillipsi.* Similarly, it is unsurprising that *U. broughami* is sister to one of only two congeners (*U. woodmasoni*) that also has the derived condition of 19 dorsal scale rows at midbody. The relationships of historical specimen DNA for the types of *Silybura melanogaster* (BMNH 1946.1.15.57) and *Silybura nigra* (MNHN 1895.85a), clustered with a fresh sample of *U. woodmasoni* (MW 3802) (Fig. 3), provide support for the morphology based hypotheses that these two taxa are junior synonyms of the latter (e.g. Gans, 1966; McDiarmid et al., 1999; Pyron et al., 2016; Smith, 1943). Except for *R. gunasekarai*, these examples come from specimens sampled from historical samples, reinforcing our confidence in the HTS data.

4.2. Uropeltid species-level diversity

In understudied organismal groups for which assigning samples to species is sometimes challenging, single-locus species discovery analyses and multilocus coalescent validation methods can assist taxonomic revision by assigning samples to candidate species or operational taxonomic units (OTUs) (Carstens et al., 2013 and references therein). Consistency between different methods can provide greater confidence in identifying candidate species (e.g. Amador et al., 2018). Results for BPP, the validation method employed here, were however unclear and varied widely among different prior settings, possibly due to the lack of dense sampling for the two nuclear markers and lack of additional nuclear data available for analyses. When employing these methods there are limitations associated with the characteristics of the data analysed, such as sampling coverage, number of samples per species, effective population size, or species divergence times (Ahrens et al., 2016; Luo et al., 2018; Talavera et al., 2013; Zhang et al., 2011). Additionally, there are other caveats associated with the methods, such as the inability to distinguish between population and species-level structure, which might lead to inaccurate delimitation, overestimating species numbers (Sukumaran and Knowles, 2017). Due to these issues, these methods may serve as a first approach to inspect possibly unrecognised species diversity (Zhang et al., 2011), and identify candidate species for more integrative taxonomic assessments (Carstens et al., 2013; Dayrat, 2005; Padial et al., 2010).

The molecular species-delimitation results fall into two main groups, those for the single locus (mtDNA) analyses plus the BPP mt + nuDNA analyses applying prior settings 2 (assuming smaller ancestral populations; recent divergences) versus those for the BPP mt + nuDNA analyses applying prior settings 1 and 3 (larger ancestral populations). The former identifies many more lineages (74–116) than there are currently recognised species (ca. 60), whereas the latter are much more conservative (47–53). Based on a priori morphological identification and current understanding of diagnostic features, our sampling comprises approximately 68 species-level taxa. This already indicates that uropeltid species diversity is greater than currently recognised and that new species require description and/or junior synonyms await resurrection. Other recent molecular studies of Western Ghats-Sri Lanka herpetofauna have also found a higher number of putative than currently recognised species (e.g. Agarwal and Karanth, 2015; Lajmi and Karanth, 2019; Vijayakumar et al., 2014).

Our morphological identifications indicate that the BPP analyses under prior settings 1 and 3 are too conservative, but it is unclear as to whether the much higher number of OTUs recovered by single locus and BPP under prior settings 2 realistically capture species-level diversity or (to some extent) merely intraspecific lineages. Interpretation of our BPP analyses is complicated by our dataset having patchy coverage and only a small number of loci. The wide range of BPP results also indicates the sensitivity of the method to the priors applied. The issue is exemplified by *Melanophidium*, recently reviewed by Gower et al. (2016), for which all four currently described species were sampled. BPP analyses with prior settings 2 largely agreed with bGMYC (0.6 threshold) and PTP analyses in returning up to 12 OTUs, identifying multiple OTUs within groups of vouchers identified as *M. punctatum*, *M. wynaudense* and as *M. kharei*, suggesting possibly cryptic species diversity. All *M. khairei* samples are from the same locality, and we have no reason to suspect that they represent more than a single species. The *M. wynaudense* samples are clustered partly geographically, though the three Wayanad samples are not each other's closest relatives. Among *M. punctatum* samples, species delimitation analyses largely separate lineages according to geography, with samples representing a lowland southern lineage (VPC-065, CHATURO), a highland southern lineage (VPC-069, ALB221), and a highland northern lineage (MW2480 and SN011). The possible over-splitting of *M. punctatum* by DNA species-delimitation methods might be explained by geographical isolation rather than these lineages being specifically distinct. Phenotypic and/or additional nuclear data are required to assess this in more detail.

Although molecular species delimitation methodologies help generate hypotheses of candidate species, these ought to be further tested in an integrative taxonomy framework (Dayrat, 2005; Padial et al., 2010) with morphological, spatial, and ecological data. We view the results of the molecular species-delimitation analyses as an interesting 'first pass' but probably less useful at this stage in uropeltid taxonomic history than combined considerations of morphology and the raw molecular data and trees. The dataset available for analysis was not specifically designed a priori with species delimitation in mind. Future species-delimitation analyses with an expanded dataset (more dense sampling, more nuclear markers) may well be worthwhile for some uropeltid groups. Given that we did not sample several currently recognised species but nonetheless sampled approximately 68 species-level taxa, and because there is no compelling or widespread evidence that current taxonomy overestimates true diversity, we would not be surprised if extant uropeltid diversity comprised a much higher number of species. Although the species-delimitation results are not conclusive, the molecular data are undoubtedly very useful in highlighting where further morphological and molecular research would be best applied. Obvious cases include reassessment of *Melanophidium punctatum* and *M. wynaudense*, the likely resurrection of junior synonyms within *Uropeltis ceylanica,* careful examination of possibly overlooked species-level taxa within *Rhinophis travancoricus* and *U. madurensis,* and untangling of the *U. macrolepis* + *U. phipsoni* 'complex'.

4.3. Museum specimen preservation and HTS success

Generation of mtDNA sequences from the regions of interest for 22 out of the 40 historical samples included in the shotgun sequencing not only increased taxon sampling but also allowed several extant populations (of sometimes uncertain identity) to be linked with type specimens. This is exceptionally useful in a group where species-level taxonomy is still confusing and in places incompletely resolved (e.g. Jins et al., 2018; Pyron et al., 2016). The only historical sample from a type specimen that did not cluster with freshly sampled conspecifics was MNHN 1895.90a. This specimen has been reported as a possible type of *U. beddomii* (Pyron et al., 2016 but questioned by Jins et al., 2018: appendix 1), but based on DNA sequence data it clustered within freshly sampled specimens identified as *U. ellioti*. *Uropeltis beddomii* and *U. ellioti* are undoubtedly distinct species, the former having many more ventral scales and a longer rostral shield. Subsequent re-examination of this specimen supports the result from our DNA data analyses — it has fewer than 170 ventral scales and is thus not *U. beddomii*. The very close relationships of historical samples of type specimens and fresh samples of conspecifics (and small p-distances between them) provides additional confidence in the quality of the historical DNA data.

Regarding the HTS of historical DNA, short read lengths, read-end deamination, and low coverage are consistent with poor endogenous DNA preservation, post mortem degradation and fragmentation, and DNA damage (Dabney et al., 2013; Jónsson et al., 2013; Schubert et al., 2012). Shotgun sequencing of mtDNA overall produced good results,

with 22 samples of 44 samples yielding data of sufficient quality to be used in phylogenetic analysis, but nuDNA sequences for target markers (present in much lower copy numbers than the mt locus) could not be retrieved. Lack of success for some samples with seemingly sufficient DNA concentrations (e.g., MCZ18038) is perhaps explained by highly degraded DNA and/or presence of contaminants for those samples.

The preservation history of the historical museum specimens used in this study is not recorded, but accession dates provide a rough guide to likely fixation and storage fluids, based on historical information presented by Simmons (2014). Alcohol was used for specimen preservation as early as 1662, with specimens often fixed and stored in spirits such as brandy or rum. Due to high costs and/or low availability of ethyl alcohol, specimens were often preserved in diluted concentrations and/or used alcohol with additives. Industrial methylated spirits (IMS), which went into production in 1855, is cheaper than ethyl alcohol, and is still used to store specimens in some major collections (e.g., NHM, London). Formaldehyde was not commonly used to fix specimens before 1900. Experimental data are scant, but it seems that for fluid-preserved specimens the following generalisations apply: i) DNA from IMS preserved specimens is lower quality than from those stored in ethyl alcohol; ii) dilution of ethyl ethanol in water degrades DNA; iii) formaldehyde degrades DNA considerably, though it might still be possible to extract and amplify and/or sequence DNA (e.g. Carter, 2003; McGuire et al., 2018; Turvey et al., 2019).

The oldest historical museum specimen for which DNA sequence data were successfully generated in this study was accessioned in 1801 (probably one of the oldest fluid-preserved vertebrate specimens successfully sequenced thus far) and the most recent one in 1897. Note that we did not generate viable DNA sequence data for all the specimens accessioned between those dates, so other factors might have inhibited successful extraction and sequencing of DNA from museum specimens. All five samples from historical specimens with precise accession dates more recent than 1897 failed, so the laboratory methods employed in this study might not be suitable for some material, including formalin-fixed specimens, to which recently developed protocols might be applied in the future (e.g. O'Connell et al., 2022). Further experimentation is required (i) to test the hypothesis that the protocol used here is not effective for extracting, sequencing and assembling DNA from formalin-fixed material, and (ii) to modify the protocol to increase effectiveness. Previous studies employing HTS methods for obtaining DNA data from herpetological fluid-preserved specimens between 145 and 30 years old have had varying degrees of success (Hykin et al., 2015; McGuire et al., 2018; Ruane and Austin, 2017). Given that preservation history records are typically lacking, it seems unlikely that a one-size-fits-all DNA extraction and amplification method will suffice in these circumstances.

4.4. Dispersal between India and Sri Lanka

The first evidence for Indian uropeltids being paraphyletic with respect to a clade of Sri Lankan uropeltids was based on allozyme and albumin immunology data (Cadle et al. 1990). Cadle et al. inferred that *R. travancoricus*, a low-elevation species endemic to the southern Western Ghats of India (Rajendran, 1985), was sister to the Sri Lankan uropeltids. These authors thus suggested an Indian origin for crown-group uropeltids, and proposed a single colonisation event from lowland India to Sri Lanka, with further lineage diversification occurring within the island accompanied by species adapting from lowland to montane habitats. Support for Cadle et al.'s hypothesis has come from previous DNA sequence phylogenies (Bossuyt et al., 2004; Cadle et al., 1990; Cyriac and Kodandaramaiah, 2017; Jins et al., 2018; Pyron et al., 2016; Sampaio et al., 2020), which all found the same pattern of paraphyletic Indian *Rhinophis* lineages as successive sister taxa to the monophyletic Sri Lankan uropeltids. Although unobserved extinctions and range expansions and contractions could have generated misleading patterns, the phylogeny and present-day distributions more strongly support this scenario (Indian origin) that an origin of crown-group uropelids in Sri Lanka, or outside the Indian subcontinent with separate dispersals into India and into Sri Lanka.

There are six Indian *Rhinophis* species currently recognised – *R. travancoricus, R. sanguineus, R. fergusonianus, R. goweri*, *R. karinthandani*, and *R. melanoleucus*. The present study is the first to sample *R. goweri* and has incorporated molecular phylogenetic data for all Indian congeners except *R. fergusonianus*, which is known from only one specimen (BMNH 1946.1.16.77) (McDiarmid et al., 1999) for which the HTS DNA library was not successful. All but one of the sampled Indian *Rhinophis* are strongly supported as lying outside the least inclusive clade containing Sri Lankan species, but we were unable to provide a compelling resolution of the phylogenetic relationships of *R. goweri*. Despite the inconsistent and poorly supported position of *R. goweri*, there seems to be no compelling support for the placement of that species within the Sri Lankan radiation, and thus no firm basis for rejecting Cadle et al.'s (1990) hypothesis of a single dispersal of lowland, dry adapted taxa from India to Sri Lanka. If *R. goweri* is in fact nested within the Sri Lankan clade, this would be indicative of multiple dispersals from India to Sri Lanka or a 'back-dispersal' of a single lineage back to southern India, such as has been hypothesised for some freshwater fishes, frogs and freshwater crabs (Bossuyt et al., 2004; Meegaskumbura et al., 2019).

The lack of clarity on the monophyly of Sri Lankan *Rhinophis* due to the inconclusively resolved relationships of *R. goweri* is likely a result of failing to obtain sequence data for more than *12s* and *16s* for a single specimen of this taxon. Future studies including more genetic data for this species might be able to clarify its phylogenetic position more precisely and allow firmer conclusions to be drawn regarding the biogeographic history of the genus in South India and Sri Lanka.

5. Conclusions

This study was successful in generating HTS data from fluid-preserved historical specimens, by utilising ancient DNA techniques and methodology. The protocols employed here proved useful for samples accessioned in collections before 1897 but failed for specimens accessioned more recently that are more likely to have been exposed to formalin. This study provides the most complete understanding of uropeltid phylogenetic relationships to date by including all but seven or eight of the ca. 64 currently recognised species (species missing: *R. fergusonianus*, *R. porrectus*, *R. punctatus*, *U. ceylanica*, *U. dindigalensis*, *U. shortii, U. rajendrani*, and possibly *U. jerdoni* but see Table S2 in Appendix A), plus potential new species and synonyms that likely require resurrection and potential new species. The monophyly of all genera has been corroborated, providing a firm platform for taxonomic revisionary work. Species delimitation analyses based on our DNA sequence dataset are not especially conclusive or convincing, but combined consideration of morphology and DNA sequences indicates that there are likely at least 30% more extant uropeltid species than currently recognised.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

GenBank and SRA accession numbers are provided in Appendix A tables S1 and S5. Alignments are available in Appendix B.

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Appendices A and B. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107651.

References

Aengals, R., Ganesh, S.R., 2013. *Rhinophis goweri* - a new species of Shield-tailed snake from the Southern Eastern Ghats. India. Russ. J. Herpetol. 20, 61–65.

Agarwal, I., Karanth, K.P., 2015. A phylogeny of the only ground-dwelling radiation of Cyrtodactylus (Squamata, Gekkonidae): Diversification of Geckoella across peninsular India and Sri Lanka. Mol. Phylogenet. Evol. 82, 193–199. https://doi.org/10.1016/j.ympev.2014.09.016.

Ahrens, D., Fujisawa, T., Krammer, H.-J., Eberle, J., Fabrizi, S., Vogler, A.P., 2016. Rarity and Incomplete Sampling in DNA-Based Species Delimitation. Syst. Biol. 65, 478–494. https://doi.org/10.1093/sysbio/syw002.

Amador, L., Parada, A., D'Elía, G., Guayasamin, J.M., 2018. Uncovering hidden specific diversity of Andean glassfrogs of the Centrolene buckleyi species complex (Anura: Centrolenidae). PeerJ 6, e5856.

Bellati, A., Carranza, S., Garcia-Porta, J., Fasola, M., Sindaco, R., 2015. Cryptic diversity within the *Anatololacerta* species complex (Squamata: Lacertidae) in the Anatolian Peninsula: Evidence from a multi-locus approach. Mol. Phylogenet. Evol. 82, 219–233. https://doi.org/10.1016/J.YMPEV.2014.10.003.

Bi, K., Vanderpool, D., Singhal, S., Linderoth, T., Moritz, C., Good, J.M., 2012. Transcriptome-based exon capture enables highly cost-effective comparative genomic data collection at moderate evolutionary scales. BMC Genomics 13, 403. https://doi.org/10.1186/1471-2164-13-403.

Bossuyt, F., Meegaskumbura, M., Beenaerts, N., Gower, D.J., Pethiyagoda, R., Roelants, K., Mannaert, A., Wilkinson, M., Bahir, M.M., Manamendra-Arachchi, K., Ng, P.K.L., Schneider, C.J., Oommen, O.V., Milinkovitch, M.C., 2004. Local Endemism Within the Western Ghats-Sri Lanka Biodiversity Hotspot. Science 306, 479–481. https://doi.org/10.1126/science.1100167.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A., Drummond, A.J., 2014. BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Comput. Biol. 10, e1003537.

Briggs, A.W., Stenzel, U., Johnson, P.L.F., Green, R.E., Kelso, J., Prü, K., Meyer, M., Krause, J., Ronan, M.T., Lachmann, M., Pä, S., 2007. Patterns of damage in genomic DNA sequences from a Neandertal. Proc. Natl. Acad. Sci. 104, 14616–14621. https://doi.org/10.1073/pnas.0704665104.

Brotherton, P., Endicott, P., Sanchez, J.J., Beaumont, M., Barnett, R., Austin, J., Cooper, A., 2007. Novel high-resolution characterization of ancient DNA reveals C > U-type base modification events as the sole cause of post mortem miscoding lesions. Nucleic Acids Res. 35, 5717–5728. https://doi.org/10.1093/nar/gkm588.

Cadle, J.E., Dessauer, H.C., Gans, C., Gartside, D.F., 1990. Phylogenetic relationships and molecular evolution in uropeltid snakes (Serpentes: Uropeltidae): allozymes and albumin immunology. Biol. J. Linn. Soc. 40, 293–320. https://doi.org/10.1111/j.1095-8312.1990.tb00541.x.

Carstens, B.C., Pelletier, T.A., Reid, N.M., Satler, J.D., 2013. How to fail at species delimitation. Mol. Ecol. 22, 4369–4383. https://doi.org/10.1111/mec.12413.

Carter, J.D., 2003. The effects of preservation and conservation treatments on the DNA of museum invertebrate fluid preserved collections. National Museum of Wales. M.Phil. thesis.

Castresana, J., 2000. Selection of Conserved Blocks from Multiple Alignments for Their Use in Phylogenetic Analysis. Mol. Biol. Evol. 17, 540–552. https://doi.org/10.1093/oxfordjournals.molbev.a026334.

Cyriac, V.P., Kodandaramaiah, U., 2017. Paleoclimate determines diversification patterns in the fossorial snake family Uropeltidae Cuvier, 1829. Mol. Phylogenet. Evol. 116, 97–107. https://doi.org/10.1016/j.ympev.2017.08.017.

Cyriac, V.P., Kodandaramaiah, U., 2021. Warning signals promote morphological diversification in fossorial uropeltid snakes (Squamata: Uropeltidae). Zool. J. Linn. Soc. 191, 468–481. https://doi.org/10.1093/zoolinnean/zlaa062.

Cyriac, V.P., Narayanan, S., Sampaio, F.L., Umesh, P., Gower, D.J., 2020. A new species of *Rhinophis* Hemprich, 1820 (Serpentes: Uropeltidae). from the Wayanad region of peninsular India 4778, 329–342.

Dabney, J., Meyer, M., Pääbo, S., 2013. Ancient DNA damage. Cold Spring Harb. Perspect. Biol. 5, a012567 https://doi.org/10.1101/cshperspect.a012567.

Dayrat, B., 2005. Towards integrative taxonomy. Biol. J. Linn. Soc. 85, 407–415. https://doi.org/10.1111/j.1095-8312.2005.00503.x.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 36, W465–W469. https://doi.org/10.1093/nar/gkn180.

Drummond, A.J., Suchard, M.A., Xie, D., Rambaut, A., 2012. Bayesian Phylogenetics with BEAUti and the BEAST 1.7. Mol. Biol. Evol. 29, 1969–1973. https://doi.org/10.1093/molbev/mss075.

Flouri, T., Jiao, X., Rannala, B., Yang, Z., 2018. Species Tree Inference with BPP Using Genomic Sequences and the Multispecies Coalescent. Mol. Biol. Evol. 35, 2585–2593. https://doi.org/10.1093/molbev/msy147.

Ganesh, S.R., Achyuthan, N.S., 2020. A new species of shieldtail snake (Reptilia: Squamata: Uropeltidae) from Kolli Hill complex, southern Eastern Ghats, peninsular India. J. Threat. Taxa 12, 15436–15442. https://doi.org/10.11609/JOTT.5680.12.4.15436-15442.

Ganesh, S.R., Murthy, B.C., 2022. Taxonomy of the shieldtail snake genus *Teretrurus* Beddome, 1886 (Serpentes: Uropeltidae) with a revised key and remarks on the geographic gaps in the Western Ghats. Peninsular India. Rec. Zool. Surv. India 122, 25–35.

Ganesh, S.R., Punith, K.G., Adhikari, O.D., Achyuthan, N.S., 2021. A new species of shieldtail snake (Squamata: Uropeltidae: *Uropeltis*) from the Bengaluru uplands. India. J. Threat. Taxa 13, 18508–18517.

Gans, C., 1966. Liste der rezenten Amphibien und Reptilien. Das Tierreich. Eine Zusammenstellung Und Kennzeichnung Der Rezenten Tierformen, Uropeltidae, in, pp. 1–29.

Gehara, M., Barth, A., de Oliveira, E.F., Costa, M.A., Haddad, C.F.B., Vences, M., 2017. Model-based analyses reveal insular population diversification and cryptic frog species in the *Ischnocnema parva* complex in the Atlantic forest of Brazil. Mol. Phylogenet. Evol. 112, 68–78. https://doi.org/10.1016/J.YMPEV.2017.04.007.

Gilbert, M.T.P., Haselkorn, T., Bunce, M., Sanchez, J.J., Lucas, S.B., Jewell, L.D., van Marck, E., Worobey, M., 2007. The Isolation of Nucleic Acids from Fixed, Paraffin-Embedded Tissues-Which Methods Are Useful When? PLoS ONE 2. https://doi.org/10.1371/journal.pone.0000537.

Gower, D.J., 2020. A new species of *Rhinophis* Hemprich, 1820 (Serpentes: Uropeltidae) from the central hills of Sri Lanka. Zootaxa 4810, 495–510. https://doi.org/10.11646/zootaxa.4263.1.7.

Gower, D.J., Captain, A., Thakur, S.S., 2008. On the taxonomic status of *Uropeltis bicatenata* (Günther) (Reptilia: Serpentes: Uropeltidae). Hamadryad 33, 64–82.

Gower, D.J., Giri, V., Captain, A., Wilkinson, M., 2016. A reassessment of *Melanophidium* Günther, 1864 (Reptilia: Serpentes: Uropeltidae) from the Western Ghats region of peninsular India, with the description of a new species 4085, 481–503. https://doi.org/10.11646/zootaxa.4085.4.2.

Gower, D.J., Wickramasinghe, L.J.M., 2016. Recharacterization of *Rhinophis dorsimaculatus* Deraniyagala, 1941 (Serpentes: Uropeltidae), including description of new material. Zootaxa 4158, 203–212. https://doi.org/10.11646/zootaxa.4158.2.3.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O., 2010. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. Syst. Biol. 59, 307–321. https://doi.org/10.1093/sysbio/syq010.

Hykin, S.M., Bi, K., McGuire, J.A., 2015. Fixing formalin: A method to recover genomic-scale DNA sequence data from formalin-fixed museum specimens using high-

throughput sequencing. PLoS ONE 10, 1–16. https://doi.org/10.1371/journal.pone.0141579.
Jins, V.J., Sampaio, F.L., Gower, D.J., 2018. A new species of *Uropeltis* Cuvier, 1829 (Serpentes: Uropeltidae) from the Anaikatty Hills of the Western Ghats of India. Zootaxa 4415, 401–422. https://doi.org/10.11646/zootaxa.4415.3.1.
Jónsson, H., Ginolhac, A., Schubert, M., Johnson, P.L.F., Orlando, L., 2013. mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics 29, 1682–1684. https://doi.org/10.1093/bioinformatics/btt193.
Kalyaanamoorthy, S., Minh, B., Wong, T., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589.
Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A., Flouri, T., 2017. Multi-rate Poisson Tree Processes for single-locus species delimitation under Maximum Likelihood and Markov Chain Monte Carlo. Bioinformatics 33, 1630–1638. https://doi.org/10.1093/bioinformatics/btx025.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.
Lajmi, A., Karanth, P.K., 2019. Eocene-Oligocene cooling and the diversification of *Hemidactylus* geckos in Peninsular India. Mol. Phylogenet. Evol. 106637 https://doi.org/10.1016/j.ympev.2019.106637.
Lanfear, R., Calcott, B., Ho, S.Y.W., Guindon, S., 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol. Biol. Evol. 29, 1695–1701. https://doi.org/10.1093/molbev/mss020.
Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T., Calcott, B., 2016. PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. Mol. Biol. Evol. 34, 772–773. https://doi.org/10.1093/molbev/msw260.
Leaché, A.D., Fujita, M.K., 2010. Bayesian species delimitation in West African forest geckos (*Hemidactylus fasciatus*). Proc. R. Soc. B Biol. Sci. 277, 3071–3077. https://doi.org/10.1098/rspb.2010.0662.
Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754–1760. https://doi.org/10.1093/bioinformatics/btp324.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 1000 Genome Project Data Processing Subgroup, 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
Luo, A., Ling, C., Ho, S.Y.W., Zhu, C.D., 2018. Comparison of methods for molecular species delimitation across a range of speciation scenarios. Syst. Biol. 67, 830–846. https://doi.org/10.1093/sysbio/syy011.
McDiarmid, R.W., Campbell, J.A., Toure, T., 1999. Snake Species of the World: A Taxonomic and Geographic Reference. Volume 1.
McGuire, J.A., Cotoras, D.D., O'Connell, B., Lawalata, S.Z.S., Wang-Claypool, C.Y., Stubbs, A., Huang, X., Wogan, G.O.U., Hykin, S.M., Reilly, S.B., Bi, K., Riyanto, A., Arida, E., Smith, L.L., Milne, H., Streicher, J.W., Iskandar, D.T., 2018. Squeezing water from a stone: high-throughput sequencing from a 145-year old holotype resolves (barely) a cryptic species problem in flying lizards. PeerJ 6, e4470.
Meegaskumbura, M., Senevirathne, G., Manamendra-Arachchi, K., Pethiyagoda, R., Hanken, J., Schneider, C.J., 2019. Diversification of shrub frogs (Rhacophoridae, *Pseudophilautus*) in Sri Lanka – Timing and geographic context. Mol. Phylogenet. Evol. 132, 14–24. https://doi.org/10.1016/J.YMPEV.2018.11.004.
Miller, M.A., Pfeiffer, W., Schwartz, T., 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: 2010 Gateway Computing Environments Workshop (GCE). IEEE, pp. 1–8. https://doi.org/10.1109/GCE.2010.5676129.
Minh, B., Nguyen, M.A.T., von Haeseler, A., 2013. Ultrafast Approximation for Phylogenetic Bootstrap. Mol. Biol. Evol. 30, 1188–1195.
Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B., Kent, J., 2000. Biodiversity hotspots for conservation priorities. Nature 403, 853–858. https://doi.org/10.1038/35002501.
Nguyen, L.T., Schmidt, H.A., Von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32, 268–274.
O'Connell, K.A., Mulder, K.P., Wynn, A., de Queiroz, K., Bell, R.C., 2022. Genomic library preparation and hybridization capture of formalin-fixed tissues and allozyme supernatant for population genomics and considerations for combining capture-and RADseq-based single nucleotide polymorphism data sets. Mol. Ecol. Resour. 22, 487–502.
Okonechnikov, K., Conesa, A., García-Alcalde, F., 2015. Qualimap 2: advanced multi-sample quality control for high-throughput sequencing data. Bioinformatics 32, btv566. https://doi.org/10.1093/bioinformatics/btv566.
Pääbo, S., 1989. Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. Proc. Natl. Acad. Sci. 86, 1939–1943. https://doi.org/10.1073/pnas.86.6.1939.
Padial, J.M., Miralles, A., De la Riva, I., Vences, M., 2010. The integrative future of taxonomy. Front. Zool. 7, 16. https://doi.org/10.1186/1742-9994-7-16.
Paradis, E., Schliep, K., 2019. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics 35, 526–528. https://doi.org/10.1093/bioinformatics/bty633.
Pethiyagoda, R., Gunatilleke, N., de Silva, M., Kotagama, S., Gunatilleke, S., de Silva, P., Meegaskumbura, M., Fernando, P., Ratnayeke, S., Jayewardene, J., Raheem, D., 2007. Science and biodiversity: the predicament of Sri Lanka. Curr. Sci. 92, 426.
Prathapan, K.D., Rajan, P.D., Poorani, J., 2009. Protectionism and natural history research in India. Curr. Sci. 97, 1411–1413.
Pyron, R.A., Kandambi, H.K.D., Hendry, C.R., Pushpamal, V., Burbrink, F.T., Somaweera, R., 2013. Genus-level phylogeny of snakes reveals the origins of species richness in Sri Lanka. Mol. Phylogenet. Evol. 66, 969–978. https://doi.org/10.1016/j.ympev.2012.12.004.
Pyron, R.A., Ganesh, S.R., Sayyed, A., Sharma, V., Wallach, V., Somaweera, R., 2016. A catalogue and systematic overview of the shield-tailed snakes (Serpentes: Uropeltidae). Zoosystema 38, 453–506. https://doi.org/10.5252/z2016n4a2.
Pyron, R.A., Beamer, D.A., Holzheuser, C.R., Lemmon, E.M., Lemmon, A.R., Wynn, A.H., O'Connell, K.A., 2022. Contextualizing enigmatic extinctions using genomic DNA from fluid-preserved museum specimens of Desmognathus salamanders. Conserv. Genet. 23, 375–386.
R Development Core Team, 2019. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.
Rajendran, M.V., 1985. Studies in Uropeltid Snakes. Madurai Kamaraj University. PhD thesis.
Rambaut, A., Drummond, A.J., Xie, D., Baele, G., Suchard, M.A., 2018. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. Syst. Biol. 67, 901–904. https://doi.org/10.1093/sysbio/syy032.
Rannala, B., Yang, Z., 2013. Improved reversible jump algorithms for Bayesian species delimitation. Genetics 194, 245–253. https://doi.org/10.1534/genetics.112.149039.
Rasmussen, S., Allentoft, M.E., Nielsen, K., Orlando, L., Sikora, M., Sjögren, K.-G., Pedersen, A.G., Schubert, M., Van Dam, A., Kapel, C.M.O., Nielsen, H.B., Brunak, S., Avetisyan, P., Epimakhov, A., Khalyapin, M.V., Gnuni, A., Kriiska, A., Lasak, I., Metspalu, M., Moiseyev, V., Gromov, A., Pokutta, D., Saag, L., Varul, L., Yepiskoposyan, L., Sicheritz-Pontén, T., Foley, R.A., Lahr, M.M., Nielsen, R., Kristiansen, K., Willerslev, E., 2015. Early divergent strains of Yersinia pestis in Eurasia 5,000 years ago. Cell 163, 571–582. https://doi.org/10.1016/j.cell.2015.10.009.
Reid, N.M., Carstens, B.C., 2012. Phylogenetic estimation error can decrease the accuracy of species delimitation: a Bayesian implementation of the general mixed Yule-coalescent model. BMC Evol. Biol. 12, 196. https://doi.org/10.1186/1471-2148-12-196.
Revell, L.J., 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol. Evol. 3, 217–223. https://doi.org/10.1111/j.2041-210X.2011.00169.x.
Rieppel, O., Zaher, H., 2002. The skull of the Uropeltinae (Reptilia, Serpentes), with special reference to the otico-occipital region. Bull. Nat. Hist. Museum. Zool. Ser. 68, 123–130. https://doi.org/10.1017/s0968047002000146.
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. Syst. Biol. 61, 539–542. https://doi.org/10.1093/sysbio/sys029.
Ruane, S., Austin, C.C., 2017. Phylogenomics using formalin-fixed and 100+ year old intractable natural history specimens. Mol. Ecol. Resour. 17, 1003–1008. https://doi.org/10.1111/1755-0998.12655.
Saint, K.M., Austin, C.C., Donellan, S.C., Hutchinson, M.N., Donnellan, S.C., Hutchinson, M.N., 1998. C-mos, a nuclear marker useful for squamate phylogenetic analysis. Mol. Phylogenet. Evol. 10, 259–263. https://doi.org/10.1006/mpev.1998.0515.
Sampaio, F.L., Narayanan, S., Cyriac, V.P., Govindappa, V., Gower, D.J., 2020. A new Indian species of Rhinophis Hemprich, 1820 closely related to R. sanguineus Beddome, 1863 (Serpentes: Uropeltidae). Zootaxa 4881, 1–24. https://doi.org/10.11646/zootaxa.4881.1.1.
Schubert, M., Ginolhac, A., Lindgreen, S., Thompson, J.F., AL-Rasheid, K.A.S., Willerslev, E., Krogh, A., Orlando, L.,, 2012. Improving ancient DNA read mapping against modern reference genomes. BMC Genomics 13. https://doi.org/10.1186/1471-2164-13-178.
Schubert, M., Lindgreen, S., Orlando, L., 2016. AdapterRemoval v2: Rapid adapter trimming, identification, and read merging. BMC Res. Notes 9, 1–7. https://doi.org/10.1186/s13104-016-1900-2.
Seitz, A., Nieselt, K., 2017. Improving ancient DNA genome assembly. PeerJ 5, e3126.
Simmons, J.E., 2014. Fluid preservation: a comprehensive reference. Rowman & Littlefield.
Smith, M.A., 1943. Fauna of British India, Vol. III. Taylor and Francis, Serpentes.
Sukumaran, J., Knowles, L.L., 2017. Multispecies coalescent delimits structure, not species. Proc. Natl. Acad. Sci. U. S. A. 114, 1607–1611. https://doi.org/10.1073/pnas.1607921114.
Talavera, G., Dincă, V., Vila, R., 2013. Factors affecting species delimitations with the GMYC model: insights from a butterfly survey. Methods Ecol. Evol. 4, 1101–1110. https://doi.org/10.1111/2041-210X.12107.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680. https://doi.org/10.1093/nar/22.22.4673.
Townsend, T.M., Alegre, R.E., Kelley, S.T., Wiens, J.J., Reeder, T.W., 2008. Rapid development of multiple nuclear loci for phylogenetic analysis using genomic resources: an example from squamate reptiles. Mol. Phylogenet. Evol. 47, 129–142. https://doi.org/10.1016/j.ympev.2008.01.008.
Turvey, S.T., Marr, M.M., Barnes, I., Brace, S., Tapley, B., Murphy, R.W., Zhao, E., Cunningham, A.A., 2019. Historical museum collections clarify the evolutionary history of cryptic species radiation in the world's largest amphibians. Ecol. Evol. 9, 10070–10084. https://doi.org/10.1002/ece3.5257.

Uetz, P., Hošek, J., 2022. The Reptile Database [WWW Document]. Available from. http://www.reptile-database.org.

Vijayakumar, S.P., Dinesh, K.P., Prabhu, M.V., Shanker, K., 2014. Lineage delimitation and description of nine new species of bush frogs (Anura: Raorchestes, Rhacophoridae) from the Western Ghats Escarpment. Zootaxa 3893, 451–488. https://doi.org/10.11646/zootaxa.3893.4.1.

Wickramasinghe, L.J.M., Vidanapathirana, D.R., Wickramasinghe, N., Ranwella, P.N., 2009. A new species of *Rhinophis* Hemprich, 1820 (Reptilia: Serpentes: Uropeltidae) from Rakwana massif, Sri Lanka. Zootaxa 2044, 1–22. https://doi.org/10.11646/zootaxa.2044.1.1.

Wickramasinghe, L.J.M., Vidanapathirana, D.R., Wickramasinghe, N., Gower, D.J., 2020. A new species of *Rhinophis* Hemprich, 1820 (Reptilia: Uropeltidae), from cloud forest of the Knuckles massif of Sri Lanka. Zootaxa 4810, 65–80. https://doi.org/10.11646/zootaxa.4810.1.3.

Williams, C., Pontén, F., Moberg, C., Söderkvist, P., Uhlén, M., Pontén, J., Sitbon, G., Lundeberg, J., 1999. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am. J. Pathol. 155, 1467–1471. https://doi.org/10.1016/S0002-9440(10)65461-2.

Wong, S.Q., Li, J., Tan, A.Y., Vedururu, R., Pang, J.M.B., Do, H., Ellul, J., Doig, K., Bell, A., McArthur, G.A., Fox, S.B., Thomas, D.M., Fellowes, A., Parisot, J.P., Dobrovic, A., 2014. Sequence artefacts in a prospective series of formalin-fixed tumours tested for mutations in hotspot regions by massively parallel sequencing. BMC Med. Genomics 7, 23. https://doi.org/10.1186/1755-8794-7-23.

Yang, Z., 2015. The BPP program for species tree estimation and species delimitation. Curr. Zool. 61, 854–865.

Yang, Z., Rannala, B., 2010. Bayesian species delimitation using multilocus sequence data. Proc. Natl. Acad. Sci. 107, 9264–9269. https://doi.org/10.1073/pnas.0913022107.

Zerbino, D.R., Birney, E., 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18, 821–829. https://doi.org/10.1101/gr.074492.107.

Zhang, J., Kapli, P., Pavlidis, P., Stamatakis, A., 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29, 2869–2876. https://doi.org/10.1093/bioinformatics/btt499.

Zhang, C., Zhang, D.-X., Zhu, T., Yang, Z., 2011. Evaluation of a Bayesian Coalescent Method of Species Delimitation. Syst. Biol. 60, 747–761. https://doi.org/10.1093/sysbio/syr071.