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Show me your true face: How many *Emydura* species occur in the Mitchell River Drainage, Kimberley, Australia?

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Abstract. We sequenced the mitochondrial genomes (mitogenomes) and ten nuclear loci of 20 *Emydura* samples from the Carson, Mitchell, and Ord River drainages to examine the taxonomic identity of turtles with variable facial coloration from the Mitchell River. We analysed our data together with previously published sequences, including mitogenomes of crucial name-bearing type material. Our results provide evidence for the occurrence of a variably coloured single species in the Mitchell River that harbours two deeply divergent mitochondrial lineages. One of these lineages could originate from an ancient hybridization and mitochondrial introgression from the *E. subglobosa*/*E. tanybaraga* complex. Our results and published evidence suggest that *Emydura* represents a speciation continuum and that the evolutionary history of the genus is characterized by multiple hybridization and introgression events. Based on comparison with previously published mitogenomes of type material and our present results, we conclude that *E. victoriae* (Gray, 1842) is a junior synonym of *E. australis* (Gray, 1841).

Key words. *Emydura australis*, *Emydura victoriae*, hybridization, introgression, turtles.

Introduction

The majority of freshwater turtles in Australia and New Guinea are side-necked turtles (Chelidae) of the suborder Pleurodira. Chelid turtles also occur on the Indonesian islands of Timor and Roti and in South America, representing a radiation of Gondwanan origin (Georges & Thomson 2006, de la Fuente et al. 2014). Across their entire distribution, two general morphotypes exist: short-necked versus long-necked turtles. The latter are represented in Australia and New Guinea by only one genus (*Chelodina*), while the short-necked turtles are assigned to six distinct genera that include two only distantly related clades (Georges & Thomson 2010, TTWG 2021). These two short-necked clades plus a third clade constituted by *Chelodina* represent the sister group of South American chelids (Georges et al. 1999, Zhang et al. 2017, Thomson et al. 2021, TTWG 2021). *Emydura* belongs, together with *Elseya*, *Elusor*, *Myuchelys*, and *Rheodytes*, to one of the Australasian short-necked clades. The second short-necked clade contains only the monotypic genus *Pseudemydura* and represents the sister lineage of all other Australasian chelids (Zhang et al. 2017, TTWG 2021). *Emydura* contains five currently accepted species (*E. gunaleni*, *E. macquarii*, *E. subglobosa*, *E. tanybaraga*, *E. victoriae*), yet with many taxonomic insecurities, both with respect to species number and nomenclature (Kehlmaier et al. 2019, TTWG 2021).

From the Kimberley of northern Western Australia east to the Daly River in the Northern Territory occur *Emydura* generally referred to as "northern red-faced turtles." Two species were described early on by John Edward Gray (1841, 1842) of the British Museum: *Emydura australis* (Gray, 1841), questionably from Western Australia, and *Emydura victoriae* (Gray, 1842) from the Victoria River. Over the following decades, until the late 1870s, Gray caused a series of confusions in the literature regarding recognition and synonymy of these two species and regarding the collection localities of their type specimens (reviewed in detail by Cann & Sadlier 2017). Despite these confusions, Boulenger (1889) recognized *E. australis* as the valid name for the red-faced turtle from "N. W. Australia." This concept, with *E. victoriae* considered a junior synonym of *E. australis*, was generally followed until the late 1980s (e.g., Goode 1967, Burbidge et al. 1974, Cogger 1975, 1986). However, based on Gray's earlier confusions, Cogger et al. (1983) placed *E. australis* into the synonymy of *E. macquarii* and, by default, recognized *E. victoriae* as

the valid name for a single taxon of the northern red-faced turtle. During the 21st century the concept of synonymizing *E. australis* under *E. macquarii* was followed by Georges & Thomson (2006, 2010), Cogger (2014) and TTWG (2017, 2021), who recognized *E. victoriae* as the valid name for a single taxon of northern red-faced turtle and considered *E. australis* a nomen dubium. Other authors maintained a concept of two species of northern red-faced turtles: *E. australis* for populations in the Kimberley and *E. victoriae* for those in the western Northern Territory, e.g., Cann (1998), Cogger (2000), Cann & Sadlier (2017), and Vetter (2018). According to Cann & Sadlier (2017) it remains questionable which of the two taxa is occurring in the Ord River drainage along the Western Australian border with the Northern Territory.

Apart from the convoluted taxonomic and nomenclatural history of the northern red-faced turtle, the differences in shell morphology, face colours and adult size among populations in different river systems of the Kimberley as presented in Cann & Sadlier (2017) may suggest the possibility of additional, still unrecognized taxa. For example, Ward & Morris (2017: 74, 75) published a photo of a yellow-faced *Emydura* from the Mitchell River catchment (previously already presented in Cann 1998) under the heading "Mitchell Plateau short-necked turtle" and state "it is not known why this species has not received a scientific name but it is certainly another Kimberley endemic." Any unrecognized endemic turtle species would obviously have significance and its evaluation and description high priority for biodiversity conservation. Fieldwork by GK in 1998 and 2000 showed, however, that the Mitchell River harbours not only yellow-faced turtles, but also orange- to red-faced *Emydura*, suggestive of two syntopic taxa (Figs 1, 2).

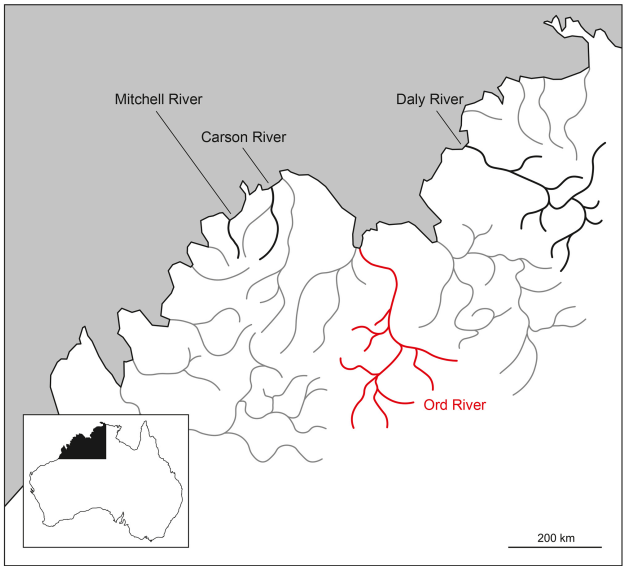

Figure 1. Study region with crucial rivers indicated. Inset shows location of enlarged map sector.

A first step toward resolution of the taxonomic and nomenclatural conundrum of the northern red-faced turtle was taken by Kehlmaier et al. (2019) who demonstrated that, based on mitogenomes, the type specimen of *E. australis* is deeply divergent from *E. macquarii* and, thus, was wrongly synonymized under that taxon. Kehlmaier et al. (2019) further demonstrated that *E. australis* and *E. victoriae* have similar mitogenomes, suggestive of conspecificity or a close relationship. An obvious conclusion could have been that the name *E. australis* has priority over *E. victoriae* whenever a single-species taxonomy is adopted for northern red-faced turtles. However, Kehlmaier et al. (2019) considered the geographic provenance of the *E. australis* holotype to remain uncertain and refrained from revoking the nomen dubium status of *E. australis*, stating this would require wider sampling of fresh material to clarify the distribution of the species. This reasoning was also adopted by TTWG (2021) and we tentatively follow this view.

In the present paper we use tissue and blood samples from vouchered and/or photo-vouchered *Emydura* specimens from three river systems of the northern Kimberley for mitochondrial and nuclear DNA sequencing to examine whether (1) an endemic undescribed yellow-faced *Emydura* species occurs in the Mitchell River syntopically with the red-faced species; and (2) the red-faced Ord River taxon currently identified with *E. victoriae* is genetically distinct from the red-faced turtles in the Mitchell River. Furthermore, based on analyses of our data together with GenBank sequences of all Australian *Emydura* species (*E. macquarii*, *E. subglobosa, E. tanybaraga*, *E. victoriae*) including the mitogenomes of the holotypes of *E. australis* and *E. victoriae* (Kehlmaier et al. 2019), we (3) evaluate the hypothesis that *E. victoriae* is a different taxon from *E. australis*.

Material and methods

Wet lab

Twenty tissue or blood samples of *Emydura* were processed, which were collected 20–25 years ago (Supplementary document S1). Tissues (n = 14) originated either from alcohol-preserved specimens in the Western Australian Museum (WAM), Perth, or from turtles that were released after study at the collection site. The blood samples were drawn during the same fieldwork campaigns.

DNA was extracted from alcohol-preserved tissue or blood samples using the innuPREP DNA Mini Kit 2.0 (Analytik Jena) with a final elution of twice 50 µl milliQ water and incubation at room-temperature for 5 min. DNA concentration and quality were assessed using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and a 4200 TapeStation system (Agilent). Where necessary, DNA was sheared to approximately 150-bp-long fragments using a Covaris M220 ultrasonicator (Covaris) before being converted into single-indexed double-stranded Illumina DNA-libraries following Meyer & Kircher (2010). For

samples GK51–GK57, DNA quality was sufficient to amplify the mitogenome and selected nuclear loci by PCR. Resulting PCR products were sheared to 150 bp before being converted into DNA libraries as described above. Negative controls were processed along with the samples and screened for contamination. To increase the amount of endogenous library molecules, double-stranded DNA libraries were subjected to two rounds of in-solution hybridization capture in a dedicated capture-only workspace using DNA baits generated from PCR products (Maricic et al. 2010, Horn 2012). For details on PCR and bait library preparation, see Supplementary document S2. Sequencing was conducted on an Illumina MiSeq platform generating 75-bp-paired-end raw reads (1.5 million per sample for GK51–57; 3.5 million per sample for others).

Selection of target loci

In addition to the mitogenome, nine out of 15 nuclear loci studied by Thomson et al. (2021) were selected, based on their genetic divergences (Supplementary document S2): Aryl hydrocarbon receptor 1 (AHR, partial, coding), bone morphogenetic protein 2 (BMP2, partial, coding), high mobility group protein B2 (HMGB2, partial, coding, and intron), hepatocyte nuclear factor 1 homeobox A (HNF1A, partial, intron 2), paired box protein (PAX1P1, partial, intron), proteasome 26S subunit (PSMC1, partial, coding and intron), recombination activating protein 1 (RAG-1, partial, coding), and the non-coding anonymous loci TB01 and TB73. The partial ornithine decarboxylase locus (ODC, coding and introns) was added, as it is useful for resolving relationships of turtles (Fritz et al. 2012, 2023, Praschag et al. 2017).

Bioinformatics

After adapter trimming with Skewer 0.2.2 (Jiang et al. 2014), read merging (minimum length 35 bp), quality filtering (minimum Q-score 20), and duplicate removal using BBmap-suite 37.24 (https://sourceforge.net/projects/bbmap/) (Bushnell et al. 2017), the remaining reads were screened for contamination using FastQScreen 0.11.4

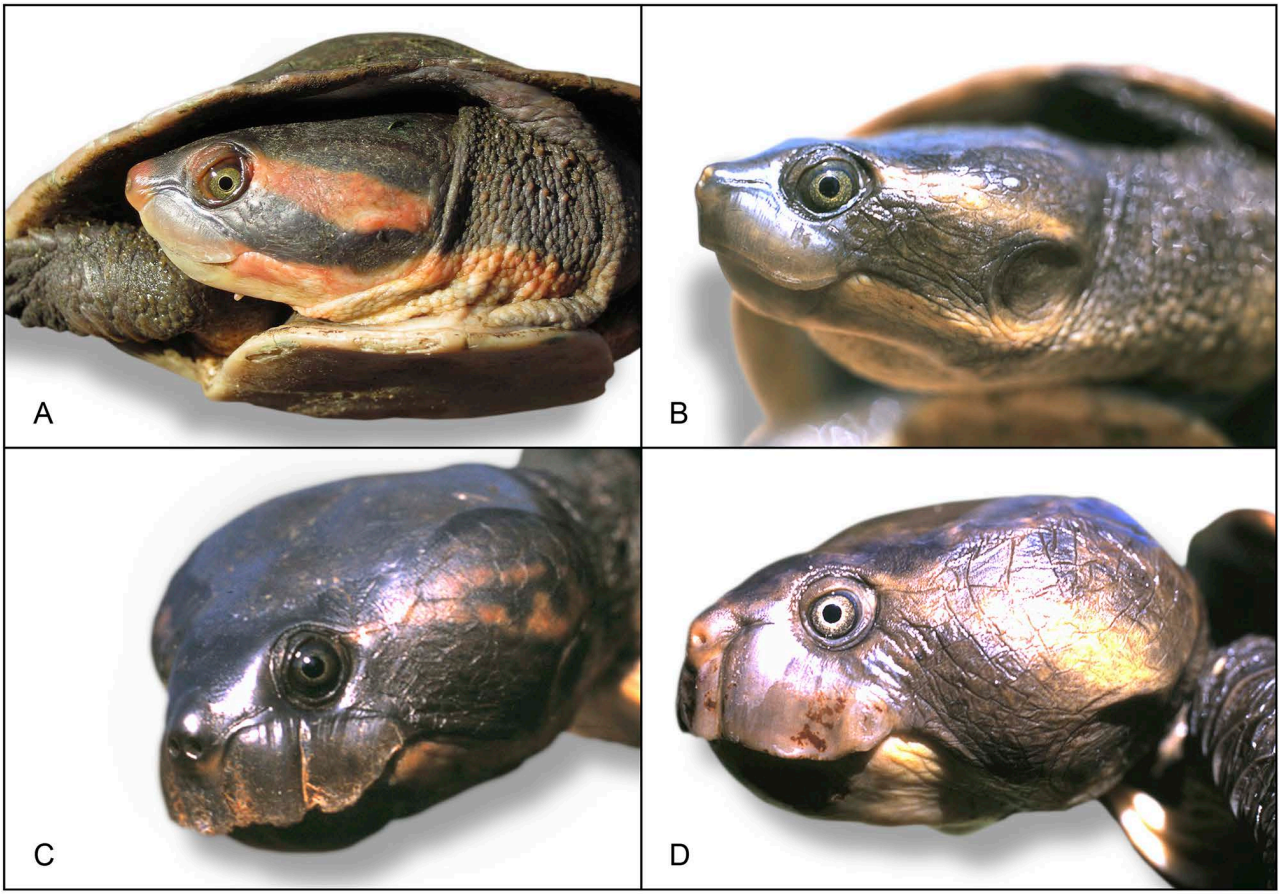

Figure 2. Turtles from the Mitchell River. Top (A, B) young adults, bottom (C, D) aged megacephalic individuals. The big-headed morphotype is related to a molluscivorous diet (Iverson et al. 1989, Cann & Sadlier 2017). Left (A, C) red-faced turtles, right (B, D) yellow-faced turtles.

(Wingett & Andrews 2018) and a set of predefined mitochondrial sequences (*Bacillus*, *Bos*, *Canis*, *Cyprinus*, *Ecoli*, *Felis*, *Gallus*, *Homo*, *Mus*, *Penicillium*, *Sula*, *Sus*, *Ursus*). Mitogenome and nuclear loci were assembled using MITObim (Hahn et al. 2013) and a two-step baiting and iterative mapping approach with an allowed mismatch value of 2, and selected GenBank sequences (depending on the targeted locus) as appropriate starting seeds, e.g., KY857554 (*Emydura victoriae*) for mtDNA. The resulting contigs were visualized and checked for assembly artefacts in Tablet 1.21.02.08 (Milne et al. 2013). Artefacts were manually removed from assembled contigs and all positions with coverage below threefold masked as ambiguous (N) using the maskfasta subcommand of BEDTools 2.29.2 (Quinlan & Hall 2010). Sequence length distribution of mapped reads was calculated with a customized awk command and Microsoft Excel. The contigs were aligned to the complete mitogenome of *Chelodina oblonga* (KY776449) and annotated accordingly.

Phylogenetic analyses

Mitogenome phylogeny was inferred using Maximum Likelihood and Bayesian inference approaches as implemented in RAxML 8.0.0 (Stamatakis 2014) and MrBayes 3.2.6 (Ronquist et al. 2012). The processed alignment (15,851 bp) comprised 35 *Emydura* spp. sequences (15 from GenBank; 20 newly generated) and *Elseya flaviventralis* (KY776454) as the outgroup. Phylogenetic networks for phased nuclear sequences were drawn using SplitsTree4 4.19.0 (Bryant & Huson 2023); DnaSP 6.12 (Rozas et al. 2017) was used to obtain both alleles. The final alignment of the ten concatenated nuclear loci comprised 8,799 bp with 26 *Emydura* spp. samples (10 from GenBank; 16 newly generated). Details on samples, GenBank sequences and individual analyses are explained in the Supplementary documents S1 and S2. The alignments are available as Supplementary documents S3–6.

Results

We obtained mitogenomes for all 20 samples and for 16 of them also nuclear DNA sequences. The sequence lengths of the mitogenomes range from 15,998 bp to 16,009 bp and include 13 protein-coding genes, two rRNA genes, 22 tRNA genes, and the partial control region of which approximately 350 bp are missing at the 3'-end. Sequences can be retrieved from the European Nucleotide Archive (see Supplementary document S1 for accession numbers and lengths of nuclear DNA fragments).

Mitogenome phylogeny

With respect to the well-supported mitochondrial phylogeny, our samples from the Mitchell River represent two deeply divergent clades, which, however, do not match the facial coloration (Fig. 3). Five samples from the Mitchell River (green bar in Fig. 3) cluster together with mitogenomes of *Emydura subglobosa*, *E. tanybaraga*, and a single sample identified as *E. victoriae*. The remaining samples from the Mitchell River (blue bar; Fig. 3) and a sample from the neighbouring Carson River cluster in a deeply divergent second clade that also contains the mitogenome sequences of *E. macquarii*, the holotypes of *E. australis* (Gray, 1841) and *E. victoriae* (Gray, 1842) as well as all turtles from the Ord River (red bar; Fig. 3).

Mitogenome sequences of *E. subglobosa* are paraphyletic with respect to *E. tanybaraga*, the five turtles from the Mitchell River (green bar; Fig. 3), and an *E. victoriae* from the Daly River, Northern Territory. Furthermore, within this clade, the two subspecies of *E. subglobosa* are not reciprocally monophyletic, and a subclade containing sequences of *E. subglobosa*, *E. tanybaraga*, and the sequence of an *E. victoriae* from the Daly River, Northern Territory, is sister to the five Mitchell River turtles.

In the second clade, *E. macquarii* is paraphyletic with respect to our mitogenomes from the Ord River, the eight individuals from the Mitchell and the Carson Rivers (red and blue bars; Fig. 3), and the holotypes of *E. australis* (Gray, 1841) and *E. victoriae* (Gray, 1842). Sequences of *E. macquarii* are not reciprocally monophyletic and represent instead three clades that are subsequent sister taxa to all remaining sequences. Both the holotypes of *E. australis* and *E. victoriae* cluster with maximum support with the mitogenomes from the Ord River; this clade is sister to the mitogenomes from turtles from the Carson and Mitchell Rivers.

Nuclear genomic evidence

We present two SplitsTree calculations, one based on the nuclear data from the Carson, Ord and Mitchell Rivers and another one that includes in addition GenBank sequences for the other northern Australian *Emydura* species and *E. victoriae* (Figs 4, 5). In both calculations, the turtles from the three rivers are weakly differentiated and connected via many reticulations. However, it is noteworthy that the alleles of the turtles from the Mitchell River representing the two deeply divergent mitochondrial clades (blue and green symbols in Figs 4, 5) are completely mixed but slightly differentiated from the turtles from the Ord River (red symbols). In the calculation including GenBank sequences, *E. macquarii* is most differentiated from all other taxa, but also the alleles of *E. subglobosa* and *E. tanybaraga* represent distinct clusters. The alleles from two *E. victoriae* on GenBank are little differentiated from our sequences (Fig. 5).

Discussion

Emydura turtles in the Mitchell River have pronounced differences in facial coloration (red- or orange-faced ver-

sus yellow-faced; Fig. 2). Our study shows that there are two deeply divergent mitochondrial lineages present, but there is no match between facial coloration and mitochondrial identity (Fig. 3). Furthermore, the lacking evidence for nuclear genomic differentiation (Figs 4, 5) provides unambiguous evidence that only one morphologically variable species lives in the Mitchell River.

The two mitochondrial lineages suggest, however, that the Mitchell River has been originally colonized by two genetically distinct source populations that subsequently amalgamated on the nuclear genomic level. The presence of two distinct mitochondrial lineages in such situations following range expansions is rather exceptional. Currat et al. (2008) found in the majority of cases of secondary contact a clear dominance of one organelle lineage, typically of the resident taxon, that introgressed the invader; this situation is often related to sex-biased dispersal and mainly male-mediated gene flow. However, in turtles the home ranges (Slavenko et al. 2016) and most likely the dispersal abilities of both sexes are similar, which may explain the presence of two deeply divergent mitochondrial lineages in the Mitchell River. When it is considered that one of the mitochondrial clades clusters with *E. subglobosa* and *E. tanybaraga* (Fig. 3), two yellow-faced species distributed east of the Mitchell River drainage (TTWG 2021), it seems possible that the "green clade" (Fig. 3) of the Mitchell River reflects an ancient introgression from *E. subglobosa* or *E. tanybaraga* into *E. victoriae*. That multiple hybridizations and introgressions occurred is supported by another mitogenome of *E. victoriae* (KY857554) from the Daly River, which is genetically distinct but clusters in the same clade (Fig. 3). In the Daly River, located approximately 500 km NE of our study region (Fig. 1), both *E. tanybaraga* and *E. subglobosa* occur together with *E. victoriae* (TTWG 2021).

Figure 3. Maximum Likelihood tree based on near-complete sequences of mitochondrial genomes (15,851 bp) from *Emydura* from the Carson, Mitchell, and Ord Rivers including GenBank sequences of the holotypes of *Emydura australis* (Gray, 1841), *E. victoriae* (Gray, 1842) and the remaining Australian *Emydura* species, rooted with *Elseya flaviventralis*. Numbers at nodes are bootstrap values and posterior probabilities from a Bayesian tree of the same topology; asterisks represent maximum support under both approaches. Coloured squares indicate facial coloration; coloured bars on the right highlight different mitochondrial clades in the study region.

This pattern of hybridization resembles the situation revealed further east for different genetic lineages of *E. macquarii* (Georges et al. 2018), suggestive of a speciation continuum with persisting gene flow where ever and whenever possible. The non-reciprocally monophyletic topology of the mitochondrial genomes of *E. subglobosa* and *E. tanybaraga* (Fig. 3), two largely parapatric species from Queensland, the Northern Territory and southern New Guinea (TTWG 2021), suggests the same for these species (but see below). To explore the complicated pattern of the mitogenomic tree topology for *E. macquarii* is beyond the scope of the present study, but it seems likely that mitochondrial introgression and hybridization are involved here, too. Georges et al. (2018) examined the *E. macquarii* complex using a much larger sample, mtDNA sequences and genome-wide SNP markers, albeit without including other species. However, recent studies on Palearctic grass and water snakes (*Natrix* spp.) have shown that it is crucial to include congeneric species in phylogeographic and genomic investigations to detect current gene flow and past hybridization (Asztalos et al. 2021, Schöneberg et al. 2023). This integrative approach could also enhance future studies on *Emydura* and other Australasian chelids, in particular since mitochondrial introgression and mitochondrial capture seem to be widespread in these taxa (Hodges 2015, Kehlmaier et al. 2019).

In this context it is relevant that *E. macquarii* was inferred as the most basal species of the genus, sister to a clade comprised of *E. victoriae* and the sister species *E. subglobosa* and *E. tanybaraga* (Thomson et al. 2021, phylogeny based on 15 nuclear loci). However, according to allozyme results (54 loci) and morphological evidence, Georges & Adams (1992) suggested a somewhat conflicting branching pattern. *Emydura macquarii* was again the most basal taxon, but *E. tanybaraga* and *E. victoriae* together were the sister group of another clade containing *E. subglobosa worrelli* (a taxon not mentioned by Thomson et al. 2021) and *E. s. subglobosa*. In any case, these topologies reveal that the reported mitochondrial mismatches are not restricted to sister taxa, in which ancestral polymorphism could also be responsible for shared mitochondrial lineages.

We conclude that *Emydura* represents another case of a speciation continuum and that the evolutionary history of the genus is characterized by multiple hybridization and introgression events, most likely paired with incomplete lineage sorting in recently diverged taxa. Our results (Figs 3–5) support that the short-necked turtles in the Mitchell and Ord River drainages are conspecific and it seems that only Mitchell River turtles have signatures for

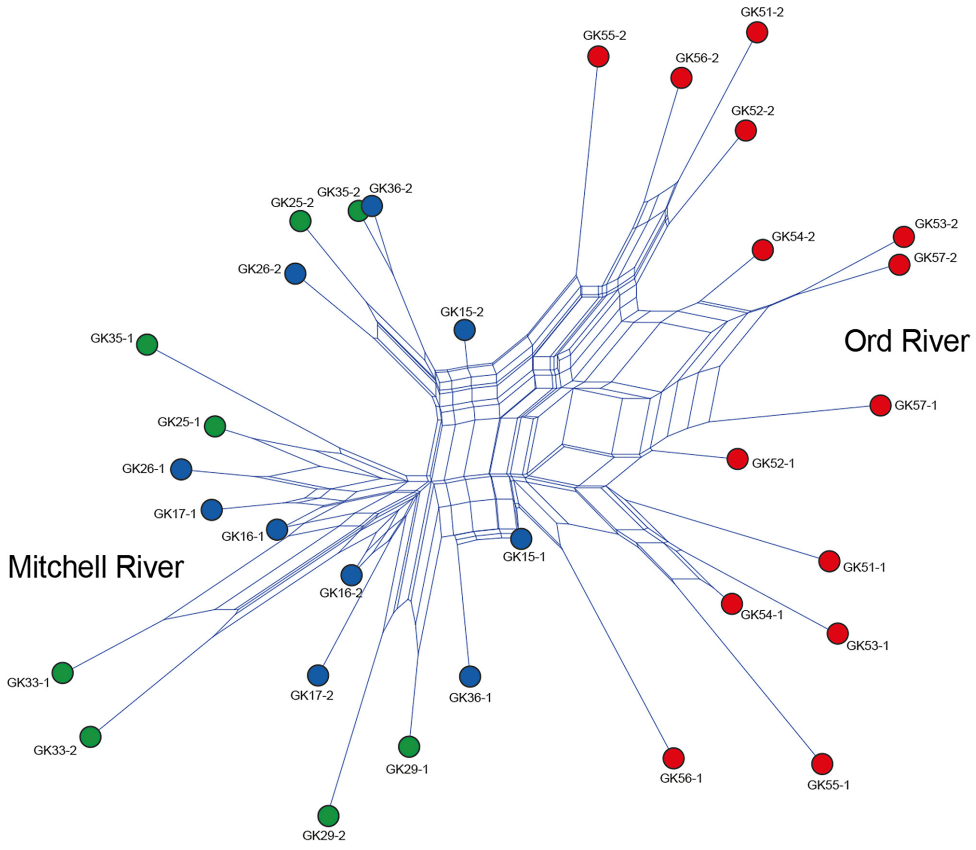

Figure 4. SplitsTree analysis for concatenated phased DNA sequences of ten nuclear loci (8,799 bp) from the Mitchell and Ord Rivers. Coloured circles correspond to coloured bars in Figure 3 and display mitochondrial identity of the respective individual.

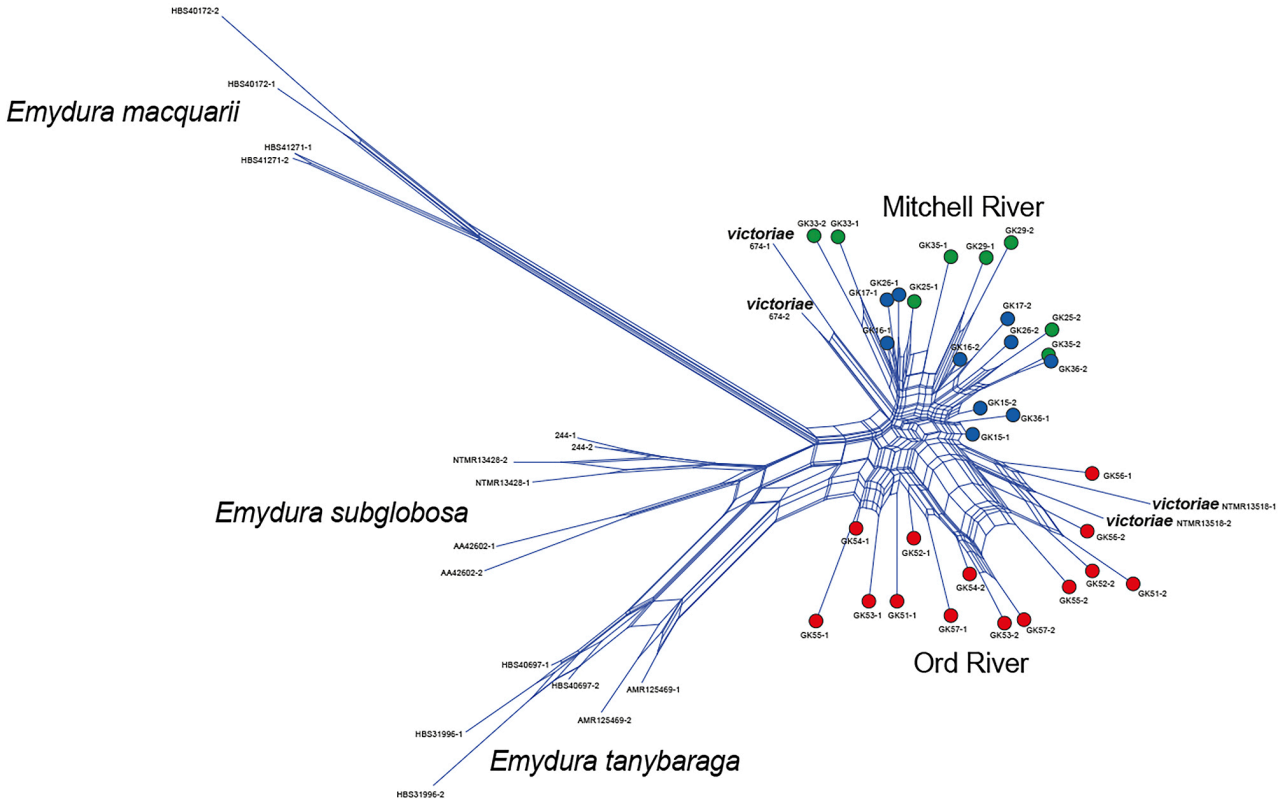

Figure 5. SplitsTree analysis for concatenated phased DNA sequences of ten nuclear loci (8,799 bp) from the Mitchell and Ord Rivers and GenBank sequences for the other northern Australian *Emydura* species and *E. victoriae*. Coloured circles correspond to coloured bars in Figure 3 and display mitochondrial identity of the respective individual.

past hybridization with the *E. subglobosa*/*E. tanybaraga* complex. With respect to nomenclature, our mitogenomic data (Fig. 3) strongly suggest that the name-bearing holotypes of *E. australis* (Gray, 1841) and *E. victoriae* (Gray, 1842) represent the same taxon. Since there is no prevailing usage of either name (see introduction and ICZN 1999, article 23.9), the valid name for northern red-faced turtles is *Emydura australis* (Gray, 1841).

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Supplementary data

The following data are available online:

Supplementary document S1. Studied material and used GenBank sequences and their accession numbers.

Supplementary document S2. Supplementary text, Tables S2–S7.

Supplementary document S3. Alignment of mitogenomes.

Supplementary document S4. Edited alignment of mitogenomes used for Figure 3.

Supplementary document S5. Alignment of concatenated new nuclear DNA sequences (phased) used for Figure 4.

Supplementary document S6. Alignment of concatenated new nuclear DNA sequences plus GenBank sequences (phased) used for Figure 5.