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Short Communication

Resolution of the enigmatic phylogenetic relationship of the critically endangered Western Swamp Tortoise *Pseudemydura umbrina* (Pleurodira: Chelidae) using a complete mitochondrial genome

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ABSTRACT

Pseudemydura umbrina is one of the most endangered turtle species in the world, and the imperative for its conservation is its distinctive morphology and relict status among the Chelidae. We use Illumina sequencing to obtain the complete mitogenome for resolving its uncertain phylogenetic position. A novel nuclear paralogue confounded the assembly, and resolution of the authentic mitogenome required further Sanger sequencing. The *P. umbrina* mitogenome is 16,414 bp comprising 37 genes organized in a conserved pattern for other vertebrates. The nuclear paralogue is 547 bp, 97.8% identity to the corresponding mitochondrial sequence. Particular features of the mitogenome include an *nd3* 174 + 1A frameshift, loss of DHC loop in $tRNA^{Ser}$ (AGN), and a light-strand replication initiation site in Wancy region that extends into an adjacent tRNA gene. Phylogenetic analysis showed that *P. umbrina* is the monotypic sister lineage to the remaining Australasian Chelidae, a lineage probably dating back to the Cretaceous.

1. Introduction

Despite the development of approaches to phylogenetic analysis based on multiple nuclear markers, mitochondrial sequences remain of value as an independent line of evidence. This is in part because the mitochondrial genome is maternally inherited and so effectively haploid, with an effective population size one fourth that of the autosomal nuclear genes. As such the mitochondrial phylogeny has a substantially higher probability of tracking the species tree, because lineage sorting of mitochondrial haplotypes is more likely to resolve along a given internal branch of the phylogeny than is lineage sorting of nuclear genes (Moore, 1995). Other attributes such as a more rapid rate of evolution, high copy number, and almost complete lack of recombination, mean that mitochondrial sequences will remain a workhorse for phylogenetic and phylogeographic analysis to complement the new developments using nuclear genes. The ease with which whole mitochondrial genomes can now be sequenced has caused a move from sequencing small fragments of the mitochondrial genome to sequencing whole genomes. This innovation takes advantage of high-throughput parallel sequencing technologies (Illumina), high representation of mitochondrial sequence in reads generated by these technologies, and sophisticated bioinformatics software for extracting and assembling the mitochondrial genomes from the volumous NGS data (Smith, 2015). Here we apply a combination of NGS and Sanger sequencing to obtain and to validate the whole mitochondrial sequence of the threatened Western Swamp Tortoise (*Pseudemydura umbrina* Siebenrock, 1901).

The Western Swamp Tortoise is a critically endangered freshwater chelid turtle found only in a very restricted region near Perth in Western Australia (Burbidge, 1981; Kuchling et al., 1992). The species is the smallest of Australian chelid turtles, and has an exceptionally low fecundity – one clutch per year of three to five eggs – which adds to its vulnerability (Burbidge, 1981). It is a relict lineage extending back, with little evident morphological change, to at least the early Miocene (Gaffney et al., 1989), and its affinities among the Chelidae are not well established. Early work using serological comparisons revealed that *Pseudemydura*, *Emydura-Elseya*, and *Chelodina* formed an unresolved trichotomy (Burbidge et al., 1974). Many of the defining morphological characters of *Pseudemydura* have been regarded as autapomorphies, and so not useful for phylogenetic analysis (Gaffney, 1977). Gaffney favoured an hypothesis that placed *Pseudemydura* as the sister taxon to the remaining extant chelids of Australasia and South America, albeit admitting that his case, based on a single retained primitive character, was weak. A number of morphological similarities with the South American chelid genus *Platemys* (now including *Acanthochelys*)

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suggested that its affinities lie there (Legler, 1981). DNA sequence data have been unable to resolve the conundrum. 12S mitochondrial rRNA data were unable to establish the relationships of *Pseudemydura*, but did suggest that it was the sister taxon to the other Australasian short-necked genera – *Emydura*, *Elseya*, *Myuchelys*, *Rheodytes* and *Elusor* (Seddon et al., 1997). Additional sequence from mt *16S rRNA* and nuclear *c-mos* supported this arrangement, but bootstrap support remained poor (Georges et al., 1998). A more recent analysis, using the same data drawn from Genbank, has *Pseudemydura* as the sister taxon to the *Chelodina* (Guillon et al., 2012). Clearly the matter remains unresolved.

We sequenced the complete mitogenome for *Pseudemydura umbrina* to conduct a phylogenetic analysis with other published whole mitogenomes for turtles in the suborder Pleurodira to clarify the affinities of the species that have so long eluded resolution. We identified and accommodated a novel nuclear paralogue from the very region for which partial mitochondrial sequences have been drawn in past studies (*12S* and *16S rRNA*) (Georges et al., 1998; Guillon et al., 2012; Seddon et al., 1997).

2. Materials and methods

The sequenced specimen (AA65990) was a female *Pseudemydura umbrina* that hatched on 01 May 1995 at Perth Zoo from an egg, incubated at 29 °C, and laid by a female marked as an adult in 1963 in Ellen Brook Nature Reserve, near Perth, Western Australia (31° 45′ S, 116° 02′ E). The sample originated from its left front leg amputated by zoo veterinarians on 08 January 2014, owing to severe osteomyelitis.

Genomic DNA was isolated from skin according to protocols of the DNeasy Tissue Kit (Qiagen Pty Ltd, Chadstone Centre, Australia). Three pairs of primers were designed from conserved regions of pleurodiran mitogenomes downloaded from GenBank, to amplify PCR products of 7063 bp, 7851 bp and 5595 bp, together encompassing the whole mitogenome with overlaps of 325 bp, 3453 bp and 288 bp respectively (see Supplementary Table 1 for primer pairs). These long-range PCRs were performed using Ranger polymerase (Bioline, Eveleigh, Australia) according to manufacturer protocols. Purified PCR products were sequenced by BGI (Shenzhen, China) using a shotgun library on a HiSeq 2000 (Illumina, San Diego, USA) to yield 150.68 Mbp of data with paired reads of length of 95 bp. Assembly was undertaken against reference genomes, *Elusor macrurus* (KU736930, Schmidt et al., 2016) and *Chelodina longicollis* (KJ713173, Zhang and Georges, 2014), using Geneious v6.1 (Kearse et al., 2012). However, no continuous MT genome was produced and chromatograms revealed extremely high peaks and ambiguities in the 3′-12s – $tRNA^{Val}$ – 5′-16S region. Instead, paired end sequences were assembled *de novo* in Geneious v6.1 (Kearse et al., 2012) with default medium-sensitivity settings. Gene start and end sites were visually examined in Geneious and adjusted where necessary. Ambiguities and confirmation of particular features of the mitogenome were addressed with Sanger sequencing undertaken through Macrogen Corporation (Seoul, Korea) (for primer sequences, refer to Supplementary Table 1). MITOS (Bernt et al., 2013) was used with the standard vertebrate mitochondrial code to annotate the resultant genome. Putative secondary structure of tRNAs was modelled using MITOS, and putative secondary structure of replication-initiation sites was modelled using Mfold (Zucker, 2003).

For phylogenetic analysis, we retrieved complete mitogenomes for turtles of sub-order Pleurodira from GenBank, including the Australia longneck chelids *Chelodina longicollis* (KJ713173, Zhang and Georges, 2014) and *C. oblonga* (formerly *rugosa*) (HQ172157, Wang et al., 2012), the Australasian shortneck chelids *Emydura subglobosa* (KC692462, Nie, L.W. and Hou, H.Z., unpublished), *Elseya branderhorsti* (KC692461, Nie, L.W. and Hou, H.Z., unpublished) and *Elusor macrurus* (KU736930, Schmidt et al., 2016), the South American chelids *Chelus fimbriata* (NC_015989, Wang et al., 2012) and *Platemys platycephala* (KC692464, Nie, L.W. and Hou, H.Z., unpublished), the two African pelomedusoid turtles *Pelusios castaneus* (KC692463, Nie, L.W. and Hou, H.Z., unpublished) and *Pelomedusa subrufa* (AF039066, Zardoya and Meyer, 1998a), now *Pelomedusa variabilis* (Petzold et al., 2014), and one South American pelomedusoid *Podocnemis unifilis* (JF802204, Nie, L.W. and Hou, H.Z., unpublished). The mitogenome for *Chrysemys picta* (Cryptodira, Emydidae) (AF069423, Mindell et al., 1999) was used as an outgroup taxon.

Sequences were aligned with the online version of MAFFT 7.046 (Katoh and Standley, 2013) using the G-INS-i algorithm with the scoring matrix for nucleotide sequences set to 1PAM/K = 2, a gap opening penalty of 1.53, and an offset value of 0.5. Phylogenetic analysis was performed with maximum likelihood (ML) using GARLI 2.0 (Zwickl, 2006). We identified the best-fitting model of molecular evolution using the Akaike Information Criterion (AIC) in Modeltest 3.7 (Posada and Crandall, 1998) using PAUP* 4.0b10 (Swofford, 2002). Modeltest identified GTR + I + G as the best model. GARLI was implemented with 10 search replicates with the following default setting values changed: streefname = random; attachmentspertaxon = 24; genthreshfortopoterm = 100,000; significanttopochange = 0.00001. For bootstrapping, we ran 1000 replicates with the previous settings with the following changes: genthreshfortopoterm = 10,000; significanttopochange = 0.01; treerejectionthreshold = 20, as suggested in the GARLI manual to speed up bootstrapping. Trees were rooted with *Chrysemys picta*.

3. Results

The *de novo* assembly of long-range PCR products yielded a contig of 12,696 bp with a putative 3′-12s – $tRNA^{Val}$ – 5′-16S repeat but missing the control region and the 5′ portion of the *12s rRNA* gene. Sanger sequencing by primer walking (Supplementary Table 1) along the missing segments yielded the complete mitogenome of 16,414 bp and suggested that the putative 3′-12s – $tRNA^{Val}$ – 5′-16S repeat was an artefact. Using this genome sequence in a reference-guided assembly yielded the whole mitogenome of 16,416 bp. However, chromatograms revealed high peaks and some ambiguities in the 3′-12s – $tRNA^{Val}$ – 5′-16S region suggesting that two sequences were present. Such a result can arise from contamination, mitochondrial heteroplasmy, duplication, and/or nuclear paralogues (Spinks and Shaffer, 2007). To eliminate the possibility of contamination during extraction and subsequent amplification, we re-extracted gDNA and re-amplified with primers CEE12sF and pum8354R (Supplementary Table 1). This produced two PCR products that could be clearly distinguished when run on a gel, one of expected size (*ca* 7063 bp) and the other much smaller (*ca* 547 bp). Gel extraction and Sanger sequencing of the two products yielded sequences corresponding to the ambiguous 3′-12s – $tRNA^{Val}$ – 5′-16S region. BLAST searches using the two resultant fragments and the high level of sequence similarity between the two fragments suggested that contamination was not the cause.

To eliminate the possibility of a duplication, confirming the result of the primer walking, additional PCR amplifications flanking the 12s – $tRNA^{Val}$ – 16s region each produced unique PCR products of expected size, with sequence length consistent with the 7063 bp product and resolving the double-peaks. We regard this as the authentic mtDNA sequence. The second sequence was 547 bp with 12 nucleotide differences (97.8% identity) from the authentic mitochondrial sequence. A 5′ portion of 302 bp overlapped with and matched the previously sequenced *P. umbrina 12s rRNA* gene (GenBank Accession U40650) with 98% (296/302) identity. We regard this 547 bp sequence to be a nuclear paralogue (Supplementary Table 2).

The *Pseudemydura umbrina* mitogenome (Fig. 1, GenBank accession number KY486272) is 16,414 bp comprising 32.0% adenine, 25.4% cytocine, 11.3% guanine and 31.4% thymine in the H-strand. All 37 genes (13 protein coding genes, two ribosomal RNAs, 22 tRNAs) and the D-loop region were arranged in the same order as in other chelid turtles (Wang et al., 2012; Zhang and Georges, 2014). Also, consistent

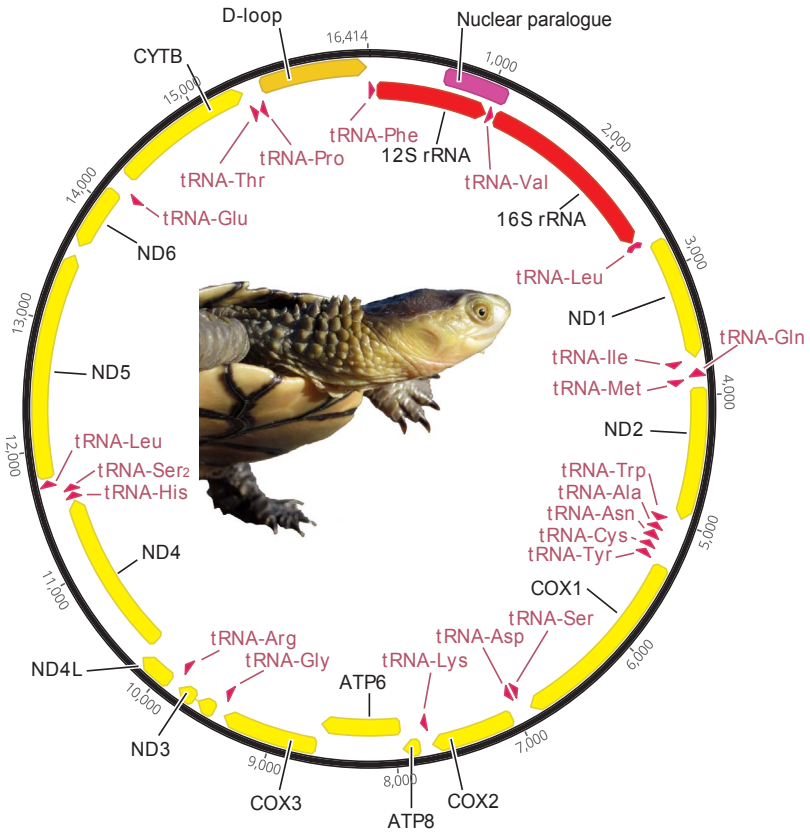

Fig. 1. Gene composition of the mitochondrial sequence for *Pseudemydura umbrina* including the location of the nuclear paralogue (NuMt).

with the general vertebrate pattern, the mt genes are transcribed from the heavy (H) strand with the exception of *nd6* and 8 tRNAs. All protein-coding genes start with ATG or ATA. Stop codons were TAA, TAG, or T, for which TAA the stop codon is completed with the normal addition of 3′ adenine residues to the mRNA following transcription.

An extra A-T base pair occurred at position 174 of *nd3,* which would be expected to throw this coding sequence out of frame. This is presumably accommodated by a translational frameshift to effect a complete translation, as has been identified at this position in other chelids (Russell and Beckenbach, 2008).

All 22 tRNAs fold into clover-leaf secondary structure with the exception of $tRNA^{Ser2}$ (anticodon AG*Y*) – it lacks the dihydrouridine stem (D-stem), as formerly reported for animal mitochondrial genomes (de Bruijn et al., 1980). As in *Chelodina* and *Elusor* (Schmidt et al., 2016; Zhang and Georges, 2014), *P. umbrina* has a DNA fragment of 51 bp (CCAACAGGCTTTTATCTAGCATCTTAAACATAATTCAAAGCTCTGATATCA) in the WANCY region, including the 5′ portion of $tRNA^{Asn}$, the 3′ portion of $tRNA^{Cys}$ and the intervening sequence between the two. When modelled, this region folds into a stem-loop secondary structure, which potentially serves as a replication-initiation site for the light strand.

The phylogenetic analysis used an alignment that excluded the Control Region because of alignment ambiguities, of 16,063 bp per taxon of which 7451 bp were constant, 2322 were variable but parsimony uninformative, and 6290 characters were parsimony informative. ML recovered one tree with a −ln score of −106130.40 (Fig. 2). All nodes had strong support (> 90%), with most nodes having 100% bootstrap support. The phylogeny, for the first time, clearly places *Pseudemydura umbrina* as sister lineage to the remaining Australasian chelid turtles (Fig. 2), to the exclusion of the South American chelid taxa.

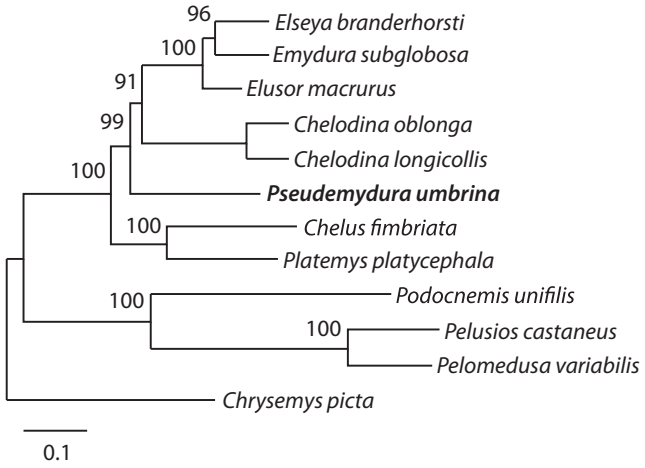

Fig. 2. Maximum likelihood tree for *Pseudemydura umbrina* and other Pleurodiran mitochondrial genomes available from genebank (see Section 2). Bootstrap values were estimated from 1000 replicates. Scale: Nucleotide substations per site.

4. Discussion

The mitochondrial genome of *Pseudemydura* is unremarkable in many respects, with the arrangement of genes and direction of transcription following the general vertebrate pattern. There are no novel duplications as have been found in other turtles (Zheng et al., 2013), no unusual microsatellites (Zardoya and Meyer, 1998a), and the programmed frameshift in *nd3* has been reported before in turtles (Russell and Beckenbach, 2008; Zardoya and Meyer, 1998b). The presence of this particular frameshift in *Pseudemydura* suggests that it is an ancient feature of the chelid mitogenome. The presence of a putative light-strand replication initiation site in the WANCY region, extending into the coding region of the adjacent tRNAs, challenges the proposition that

absence of such a replication site is a feature of the Pleurodira (Wang et al., 2012).

The identification of a nuclear paralogue to the mitochondrial 12s – $tRNA^{Val}$ – 16s region throws into doubt phylogenies that have relied on sequence from this region, and may in part explain the difficulty that prior researches have encountered in resolving the phylogenetic relationships of *Pseudemydura umbrina* (Georges et al., 1998; Guillon et al., 2012; Seddon et al., 1997). However, the differences between the nuclear paralogue and the authentic mitochondrial sequence are relatively minor (12 bp), and it is more likely that failure to resolve the phylogeny was because of the limited sequence data available through traditional approaches. Using the whole mitochondrial genome for *Pseudemydura umbrina*, we were able to resolve its phylogentic relationships, as an ancient relict lineage that is the sister taxon to the remaining Australasian Chelidae. This finding supports the contention that *Pseudemydura umbrina*, for which the fossil record shows little morphological change since the early Miocene (Gaffney et al., 1989), dates back to the Cretaceous (Burbidge et al., 1974). Insofar as phylogenetic distinctiveness is a consideration in setting conservation priorities (Crozier, 1997; Faith, 1992; Vane-Wright et al., 1991), our analysis confirms that not only is *Pseudemydura umbrina* a critically endangered species, but one of special value in representing diversity that has accumulated in a relict lineage of considerable antiquity. In recognition of this distinction, and to clarify the phylogenetic relationship of *Pseudemydura umbrina* in taxonomy, we remove *Pseudemydura* from the subfamily Chelodininae and resurrect for it, the subfamily Pseudemydurinae Gaffney, 1977.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2017.07.019.

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