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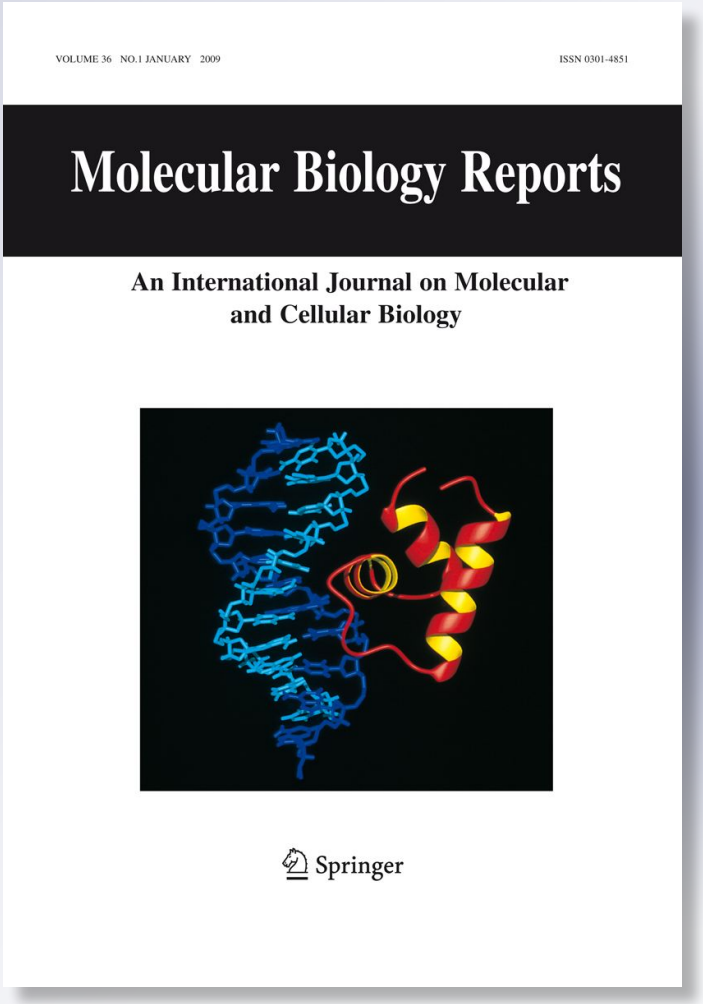

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The complete mitochondrial genome of two recently derived species of the fish genus *Nannoperca* (Perciformes, Percichthyidae)

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Abstract Here we report the complete sequence of mitochondrial genomes for two sister taxa of freshwater teleosts, the recently derived Yarra pigmy perch *Nannoperca obscura* and the southern pigmy perch *Nannoperca australis*. These represent the first complete mitochondrial genomes for *Percichthyidae* (Perciformes), a family mostly distributed in Australia. The de novo genome assembly of 316,430 pyrosequencing reads from 454 libraries has produced the entire mitochondria for *N. obscura* and a nearly complete version for *N. australis*. The mtDNA genome from the latter was completed through the design of one primer set and standard Sanger sequencing for genome finishing, followed by the hybrid assembly of reads with MIRA software using *N. obscura* sequence as reference genome. The complete mitogenomes of *N. obscura* and *N. australis* are 16,496 and 16,494 bp in size, respectively. Both genomes contain 13 protein-coding genes, two ribosomal RNA genes, 22 transfer RNA genes and a control region. Several characteristics of mitochondria typically found in teleost fishes were detected, such as: (i) most genes found in the heavy strand, with the exception of ND6 and eight tRNA genes; (ii) avoidance of G as the third base of codons; (iii) presence of gene overlapping; (iv) percentage of bases usage. We found only eight indels and 197 nucleotide substitutions between these *Nannoperca* mitogenomes, consistent with a previous hypothesis of recent speciation. The data reported here provide a resource for comparative analysis of recent evolution of mitochondrial genomes.

Keywords Mitochondrial genome · Fish · Pyrosequencing · Nannoperca

Introduction

The mitochondrial genome of vertebrates consists of a circular molecule with size varying among 16–19 kb. It usually contains 37 genes encoding 13 protein-coding genes, 2 ribosomal RNAs, 22 transfer RNAs and a variable control region (CR) or D-loop. Mitochondrial DNA (mtDNA) is a useful tool in studies of phylogenetics, phylogeography, molecular evolution, and population and conservation genetics due to its relatively simple structure, predominant female inheritance, and high rate of evolution [2]. The complete analysis of fish genomes has been extensively used for the study of fish evolution e.g., [22–24].

The teleost family Percichthyidae comprises eight genera and approximately 22 species of fish mostly found in freshwater and estuarine environments of Australia [15]. The Australian percichthyds include several species of significant conservation concern, such as the Yarra pygmy perch (*Nannoperca obscura*) and the southern pygmy perch (*Nannoperca australis*). These small sized freshwater fish

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(<100 mm total length) are endemic to south-eastern Australia and threatened due to river regulation, introduction of exotic species, habitat degradation and severe droughts [6, 29]. *Nannoperca obscura* is also listed as critically endangered in South Australia [10] and as vulnerable by the IUCN [12] red list. Population genetic studies have detected strong geographic structure and distinct evolutionary units in both species [6, 10]. Molecular phylogenetic analysis of Percichthyidae strongly supports a sister relationship and recent speciation between *N. obscura* and *N. australis* [13], a hypothesis consistent with morphological specializations in *Nannoperca* [15].

This is the first study to report complete mitochondrial genome sequences for Percichthyidae. Our results suggest close evolutionary relationship between the two species and represent an important resource for comparative analysis of recent evolution of mitochondrial genomes.

Materials and methods

Sample collection and DNA extraction

A total of 10 μg of genomic DNA was extracted from muscle tissues kept in −80°C from one specimen of *N. obscura* and one of *N. australis* using a modified salting-out method [30]. Our sample of *N. obscura* was collected at the Lake Alexandrina and *N. australis* at Tookayerta Creek (SA), in the southern end of the Murray-Darling Basin.

Next-generation sequencing

Samples were sent to the Australian Genome Research Facility (www.agrf.com.au) in order to obtain DNA sequences. The DNA was nebulised and ligated to 454 sequencing primers. Each fragment was tagged with a common sequence used further to separate the pooled *Nannoperca* genome sequences. Samples were then subjected to high throughput DNA sequencing on 1/8 of a 70 9 75 PicoTiterPlate using the Roche GS FLX (454) system as described elsewhere [21].

Sanger sequencing

In order to confirm the sequence of specific regions with low coverage and produce a better version of mitochondria finished data, a set of primers were used to amplify genome regions of the *N. australis* mitochondrial genome. Primers were designed using NCBI primer-BLAST on pre-assembled mtDNAs (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and further verified using OligoAnalyser 3.1 (http://www.idtdna.com/analyzer/applications/oligoanalyzer/). The set of primers used for genome finishing in *N. australis* was 5′-GTTCACTCCACCGGGGCAGC-3′/5′-TGTTCCCGCGGTCGCC-3′.

Sequence extraction, cleaning and removal of contaminants

Reads were extracted from SFF 454 format and original FASTA format sequencing reads were cleaned using Seqclean script from the TGICL package [25]. Seqclean script performs automated trimming and validation of sequencing reads, screening for a number of contaminants, low quality and low-complexity sequences.

Sanger reads were extracted from the AB1 files using PHRED algorithm [8, 9] with modified parameters. The usage of PHRED algorithm trim_alt and trim_cutoff 0.10 parameters were chosen following Prosdocimi et al. [27].

Selection of mitochondrial genome reads

Since the assembly of original sequencing reads did not allow for the complete sequence of mtDNA, we used local BLAST searches [1] to select sequencing reads specific for fish mitochondrial genome. BLAST was run using default parameters.

Sequence assembly of reads

The sequence assembly of genomic reads obtained from 454 machines was performed using two methods, and results were compared. The classical software for genome and transcriptome building CAP3 [11] was run using default parameters and also more stringent parameters for assembly, as described elsewhere [26]. We also used the algorithm MIRA [5] running with default parameters for "de novo assembly" and for "reference-based genome assembly" according to the software's manual (http://mira-assembler.sourceforge.net/docs/mira.html; accessed December 2010).

Annotation of mitochondrial genomes

The annotation of mitochondrial genomes for both species was conducted using the Artemis [3, 28] and Artemis comparison tool (ACT) software [4]. BLAST searches against mitochondrial genomes of Percoidei fishes were used to confirm manual annotations. tRNAs were annotated using the software tRNAscan-SE 1.21 [20] in default search, and using vertebrate mitochondrial genetic code for tRNA structure prediction. The rRNA gene boundaries were estimated from nucleotide sequence alignments with other Percoidei fish.

Table 1 Summary of cleaning procedure on pyrosequencing data for *Nannoperca* genomes

Fish mitogenome	Total number of reads	Number of short reads[a]	Number of dust reads[a]	Number of low quality reads[a]	Total number of clean reads	Number of mtDNA related reads[b]	Number of NGS bases used in assembly
N. obscura	145,071	16,823	761	3	127,484	684	236,018
N. australis	171,359	16,159	773	1	154,426	403	158,716

[a] Seqclean data

[b] Retrieved by BLAST analysis against Percoidei mitochondrias

Results and discussion

Sequence cleaning and selection of mitochondrial specific reads

The genomes of the two species of *Nannoperca* studied here were sequenced in 454 machines, producing more than 300,000 sequencing reads (Table 1). After sequence cleaning, putative mitochondrial reads were selected by similarity searches against a specially built BLAST database containing all published mitochondrias of Percoidei fishes. As expected, only a small fraction of the sequencing reads was tagged as putative mitochondrial reads, corresponding to 0.5 and 0.26% of the total number of bases sequenced for *N. obscura* and *N. australis*, respectively (Table 1). Nonetheless, the overall sum of putative mitochondrial bases allowed a mitogenome coverage of ~14-fold for *N. obscura* and ~9.5-fold for *N. australis*.

Mitochondrial genome assembly

The genome of both mitochondria's was successfully assembled using the MIRA assembly algorithm [5]. The classical CAP3 software for sequence assembly was not capable to assembly mitochondrial mapped reads due to problems on building slightly repetitive sequences present in the 12S rRNA and 16S rRNA (data not shown). The mitogenome of *N. obscura* was built based in the pyrosequencing reads alone and the MIRA software was run with a parameter set for "de novo" assembly. The mitogenome of *N. australis* could not be assembled using only the original pyrosequencing reads. Some small genomic regions of the *N. australis* mitochondria were not represented in the 454 dataset probably due to the randomness of sequences sampled and at least one sequence gap was observed in the bioinformatics assembly of its mitogenome. In order to produce the complete version of the mitochondrial genome for this species we developed two primers by observing the flanking regions of the gap found in the assembly. The size of the gap region was measured by comparative genomic analysis to the *N. obscura* assembled data. Primers were used to amplify a *N. australis* genomic library and generated sequences that allowed genome finishing using a mixed assembly of Sanger reads produced in ABI machines and 454 pyrosequencing data. However, even when Sanger's sequencing reads were available, the mitogenome of *N. australis* could not be assembled using MIRA software with parameters for "de novo" assembly. Therefore, MIRA parameters were set for the usage of a reference genome and the already assembled *N. obscura* mitochondria was used as a reference for the genome assembly of *N. australis* mitochondria. This allowed us to obtain a complete sequence for the mitogenome of *N. australis*. Manual curation and annotation confirmed the correct assembly and organization of both mitochondrial genomes (Supplementary Information Tables S1 and S2).

Genomic organization

The complete sizes of the whole mitochondrias in *N. obscura* and *N. australis* are, respectively, 16,496 and 16,494 bp. The mitogenome content, the gene order and the gene coding strands of *Nannoperca* sequenced here is in accordance with the vertebrate consensus [33, 37]. The overall nucleotide base composition was: A, 27.78%; C, 28.61%; G, 16.55% and T, 27.06% for *N. obscura* and A, 27.77%; C, 28.70%; G, 16.55% and T, 26.98% for *N. australis*. Complete data on nucleotide usage is shown in Table S3 (Supplementary Information). The AT content was slightly higher than the GC content, as observed in other teleosts [16, 32].

Protein-coding genes

The number and size of the protein-coding genes found in both *N. obscura* and *N. australis* is highly similar to their orthologs in other Percoidei. We observed several overlapping genes amongst the 13 protein-coding genes. For example, in *N. obscura* ATPase8 and ATPase6 overlapped by ten nucleotides, ATPase6 and COX3 overlapped by 1 bp, ND4L and ND4 overlapped by seven nucleotides and ND5 and ND6 overlapped by 4 bp. The results for coding sequence overlaps observed in *N. australis* were identical to that of *N. obscura*, suggesting recent common ancestry and putative genera-specific pattern.

All genes for both genomes started with the standard codon (ATG) for Methionine, with the exception of COX1, which presented the alternative starting codon GTG. In regard to stop codons, *N. obscura* had most genes ending with the TAA codon (COX1, COX3, ATPase8, ATPase6, ND3, ND5 and ND6) and the TAG codon (ND1, ND2, ND4 and CYTB). One gene ended with AGA (COX2) and another with AGG mitochondrial specific stop codons (ND4). Two genes ended with different stop codons in the *N. australis* mitogenome: ND3 with a TAG instead of TAA and ND4L with a TAA instead of TAG. Despite the close evolutionary relationship of these species [13], fish mitochondrial genomes are known to show variance in stop codons [14, 36].

Codon usage for the whole set of mitochondrial genes annotated are presented in Table S4 (Supplementary Information). With the exception of Valine, all the other fourfold degenerated amino acids used C in the ending codon position and avoided G in the same site (except for Glycine) for both mitogenomes. These features are similar to results observed in mitochondrias from other vertebrates [17, 34, 35].

Non-coding regions

The mitochondrial genomes of *N. obscura* and *N. australis* have shown a non-coding region of, respectively, 819 and 820 bp. The CR presented a higher A + T content (~61%) than the average value for the whole mitogenome (~54%), a feature reported in all Percoidei [35].

Transfer and ribosomal RNA genes

The mitochondrial genomes of *N. obscura* and *N. australis* both encode 22 tRNA genes, ranging from 66 to 75 bp. Among these tRNAs, two forms of tRNA-Leu and tRNA-Ser were identified. Ribosomal genes were predicted based on sequence similarity with other mitochondrias of Percoidei. As in other mitogenomes, rRNA genes were located between tRNA-Phe and tRNA-Val (rRNA 12S); and between tRNA-Val and tRNA-Leu (rRNA 16S). The lengths and base compositions of rRNA are shown in Tables S1, S2 and S3 (Supplementary Information). The overall A + T contents of ribosomal RNAs are 53.82 for *N. obscura* and 53.70 for *N. australis*, which is consistent with the number found for other bony fishes [19, 35].

SNP characterization between Nannoperca fishes

The Clustalw global sequence alignment [31] between the entire mitochondrias of the two species studied here showed 205 differences: eight single-nucleotide indels and 197 substitution mismatches. In comparison to *N. australis*

Table 2 Number of nucleotidic differences found for mtDNA complete genomes of fishes belonging to the same genus

Genus	Mitogenome 1	Mitogenome 2	Number of differences[a]
Lutjanus	*Lutjanus_bengalensis*	*Lutjanus_kasmira*	649
Lutjanus	*Lutjanus_bengalensis*	*Lutjanus_malabaricus*	1948
Lutjanus	*Lutjanus_bengalensis*	*Lutjanus_rivulatus*	1360
Lutjanus	*Lutjanus_bengalensis*	*Lutjanus_russellii*	1537
Lutjanus	*Lutjanus_bengalensis*	*Lutjanus_sebae*	1886
Lutjanus	*Lutjanus_kasmira*	*Lutjanus_malabaricus*	1890
Lutjanus	*Lutjanus_kasmira*	*Lutjanus_rivulatus*	1296
Lutjanus	*Lutjanus_kasmira*	*Lutjanus_russellii*	1506
Lutjanus	*Lutjanus_kasmira*	*Lutjanus_sebae*	1860
Lutjanus	*Lutjanus_malabaricus*	*Lutjanus_rivulatus*	1849
Lutjanus	*Lutjanus_malabaricus*	*Lutjanus_russellii*	1873
Lutjanus	*Lutjanus_malabaricus*	*Lutjanus_sebae*	1358
Lutjanus	*Lutjanus_rivulatus*	*Lutjanus_russellii*	1412
Lutjanus	*Lutjanus_rivulatus*	*Lutjanus_sebae*	1816
Lutjanus	*Lutjanus_russellii*	*Lutjanus_sebae*	1849
Micropterus	*Micropterus_dolomieu*	*Micropterus_floridanus*	1049
Micropterus	*Micropterus_dolomieu*	*Micropterus_salmoides*	1087
Micropterus	*Micropterus_dolomieu*	*Micropterus_salmoides_salmoides*	1091
Micropterus	*Micropterus_floridanus*	*Micropterus_salmoides*	481
Micropterus	*Micropterus_floridanus*	*Micropterus_salmoides_salmoides*	479
Micropterus	*Micropterus_salmoides*	*Micropterus_salmoides_salmoides*	112
Nannoperca	*Nannoperca obscura*	*Nannoperca australis*	205

[a] Indels + base substitutions

data, *N. obscura* mitogenome presented three deletions and five insertions. The CR in *N. obscura* had two deletions of an A and a G bases at positions 6 and 680, and an insertion of an A at base 580. The third deletion in the *N. obscura* genome was found at position 3525, inside the 16S rRNA gene. The four insertions in *N. obscura* mitogenome were observed at alignment positions 1340, 1908, 1940 and 1941; the first of them being internal to the 12S rRNA gene and the others at the beginning of the 16S rRNA gene. No indels were found in protein-coding sequences.

In regard to nucleotide substitutions, 85.8% substitutions were transitions (169 out of a total of 197). For substitutions happening within protein-coding genes, we verified 20 substitutions in ND1, 21 in ND2, 9 in COX1, 5 in COX2, 1 in ATPase8, 13 in ATPase6, 7 in COX3, 2 in ND3, 4 in ND4L, 18 in ND4, 30 in ND5, 9 in ND6 and 25 in the CYTB gene.

General features of Nannoperca mitogenomes

The comparison of complete *Nannoperca* mitogenomes provides evidence for high similarity in DNA content between these teleosts. We recovered eight indels (all in non protein-coding regions) and only 205 nucleotide substitutions between *N. australis* and *N. obscura*. Table 2 shows the number of differences between the *Nannoperca* mitogenomes and those of other published Percoidei mitogenomes. The sequence divergence between *N. obscura* and *N. australis* was the lowest reported between Percoidei species and it was only slightly higher than comparisons between subspecies of *Micropterus salmoides* (112 differences). In fact, most congeneric species differed by at least ~450 nucleotide substitutions (Table 2). These results are consistent with molecular phylogenetic [13] and morphological evidence [15], as well as with observation of natural hybridization between *N. australis* and *N. obscura* (Michael Hammer, personal communication), that strongly indicate a very recent (i.e., Pleistocene) split between the two species.

Conclusions

Here we showed that random sequencing of genomes in 1/8 pyrosequencing reactions can enable the complete sequencing of a mitochondrial genome, as observed for *N. obscura*, and a nearly complete mitogenome as observed for *N. australis*. This study represents the first report of mitochondrial genomes for the family Percichthyidae. It generated the complete genomes for two closely related and recently diverged species that might prove useful in comparative evolutionary studies (GenBank accession numbers JF519732 and JF519733). General features as complete size (~16.5 kb), number/order/size of protein and RNA-coding genes were nearly identical to other fish mitochondrial genomes [7, 18, 35]. Other features as (i) most genes found in the heavy strand, with the exception of ND6 and eight tRNA genes; (ii) avoidance of G as the third base of codons; (iii) presence of gene overlapping; and (iv) percentage of bases were also in accordance to patterns observed in teleosts.

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