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# The complete mitochondrial genome of the broad-winged damselfly *Mnais costalis* Selys (Odonata: Calopterygidae) obtained by next-generation sequencing

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**Running head:** Complete mitogenome of *Mnais costalis*

**Abstract**

We used next-generation sequencing to characterise the complete mitochondrial genome of the damselfly *Mnais costalis* (Odonata, Calopterygidae). Illumina paired end reads were mapped against COI and 16S sequences from *M. costalis* and then extended using an iterative *de novo* map procedure. The final assembly was a contiguous sequence of 15,487 bp, which contained all standard mitochondrial coding regions and the putative A+T rich region. The gene configuration of the *M. costalis* mitogenome is similar to that of other odonates, comprising 13 protein-coding genes, large and small rRNA genes, and 22 tRNA genes. We found three intergenic spacers that are also present in all available whole odonate mitogenomes. Base composition of the *M. costalis* mitogenome is 40% (A), 20% (C), 14% (G) and 26% (T), with a high A+T content (66%). The characterisation the complete mitochondrial genome of *M. costalis* adds to the growing list of mitogenomes currently available for odonates, and will help to improve primer design for future population genetic studies. A phylogenetic analysis including the complete mitochondrial genome sequences of odonates support the inclusion of *Epiophlebia superstes* within the suborder Zygoptera.

**Keywords:** mitogenome, dragonfly, *de novo* map, phylogeny

## Introduction

Mitochondrial sequence data continue to provide with the majority of genetic markers used in phylogenetic studies of odonate species (Ballare & Ware 2011), despite the problems that may arise from the use of mitochondrial markers in instances of introgression or hybridization (e.g. Hayashi et al 2005). This widespread use of mitochondrial markers for such studies is mainly due to the limited number of alternative nuclear genes that could be amplified with nearly-universal primers across different odonate species (but see Ferreira et al 2014). Having full mitochondrial genomes available provides the opportunity of improving primer design, and also to increase the number of target genes used in phyologenetic inference, therefore providing more robust phylogenetic reconstructions (e.g. Lin et al 2010). Also, mitochondrial genomes may allow to answer diverse comparative and evolutionary genomics questions in insects, such as the evolution of genome size or the study of gene rearrangements (Cameron 2014), or to analyze the evoutionary processes that underly the evolution of synonimous and non-synonimous codon usage (e.g. Kumar et al 2012).

In the last couple of years, there has been a notable increase in the number of mitochondrial genome data available for odonates; and currently the complete (or nearly complete) mitochondrial genomes of 15 odonates species belonging to nine families are available (Table 1). However, and despite it covers nearly a third of the families currently described within the order, this constitutes yet a small fraction of the extant odonate diversity (~6,000 species in 30 families; Dijkstra et al. 2013).

Here, we report the complete mitochondrial genome of the damselfly *Mnais costalis* (Zygoptera, Calopterygidae) from Japan, obtained by next generation sequencing. Members of the Calopterygidae, commonly known as broad-winged damselflies, demoiselles or jewelwings; have metallic-coloured bodies and some species also have conspicuously pigmented wings. They have been used as models for research into reproductive behaviour, interspecific interactions, character displacement and sperm competition (Córdoba-Aguilar 2008), with *Mnais* damselflies being particularly important as they are one of the few species that have a male-linked colour polymorphism that is associated with behavioural phenotype (Plaistow & Tsubaki 2000, Tsubaki et al 1997, Tsubaki & Okuyama in press). Finally, we reconstruct the phylogenetic relationships among odonates, based on the complete mitochondrial genomes currently available.

## Material and methods

*Sample collection, DNA extraction and sequencing*

Five adult *Mnais costalis* males (2 orange and 3 clear winged) were collected at Togichi Prefecture (36°42' N, 140°13' E) and used to extract genomic DNA from the thoracic muscle. For DNA

extraction, we used the Qiagen Genomic-Tip 500/G, following manufacturer's instructions. Samples were pooled for sequencing (pool 1 – clear and pool 2 – orange) on a HiSeq 2000 (Illumina), generating 2x100bp paired end reads (approximate fragment size 550-750bp), with chemistry v.3, at the Centre for Genomic Research (www.liverpool.ac.uk/genomic-research/). Information on the number of reads obtained and data quality control is provided in the Supplementary Information.

*Obtaining and anotating the mitochondrial genome*

To obtain the mitochondrial genome sequence, we used the iterative fine-tuning option within the map to reference as implemented in Geneious v.9.0.5 (www.geneious.com). This allows read mapping to extend past the ends of the reference sequence on each iteration. COI and 16S sequences of *Mnais costalis* (GenBank AB708347 and AF170952, respectively) were used as seeds against which the Illumina reads from both pools were mapped in a first step, and the obtained contigs were used to seed subsequent mapping, with the procedure repeated for a total of 425 iterations to recover the entire mitogenome.

Mitochondrial genome annotation was done in Geneious v.9.0.5. First, open reading frames (ORFs) were identified, and the putative ORFs were then compared with the odonate mitogenomes listed above. Gene identity was confirmed by BLAST search (Altschul et al. 1990) against Genbank's nr database (www.ncbi.nih.gov; date accessed 17 February 2016). Transfer RNA genes were identified using ARWEN (Lasslett & Canbäck, 2008; http://mbio-serv2.mbioekol.lu.se/ARWEN/).

*Phylogenetic analyses*

Sequences of 13 protein-coding genes (PCGs), two rRNA genes and 22 tRNA genes from the currently available odonate mitogenomes (Table 1), were used to perform a phylogenetic analysis. Sequences from the same genes were extracted from the mitochondrial genome available for *Ephemera orientalis* (Ephemeroptera, GenBank Accession EU591678; Lee et al 2009), *Parafronurus youi* (Ephemeroptera, GenBank Accession EU349015; Zhang et al 2008) and Pteronarcys princeps (Plecoptera, GenBank Accession NC_006133; Stewart & Beckenbach 2006); to be used as outgroups in the phylogenetic analyses.

PCGs were aligned using Muscle (Edgar 2004) as implemented in Geneious v.7.0.1 (www.geneious.com). For the aligment of the 16S and the 12s sequences, we used Mafft v.7 (Katoh & Standley 2013; http://mafft.cbrc.jp/alignment/server/index.html), with the the option Q-INS-I, which considers the secondary structure information of the RNA. Transfer RNA sequences

were aligned using the software LocARNA (Will et al 2007, Smith et al 2010, Will et al 2012; http://rna.informatik.uni-freiburg.de/LocARNA/Input.jsp), which aligns tRNA sequences based on their sequence and structure features. Regions with ambiguities, as well as variable regions within each alignment, were recognized and excluded using Gblocks (Castresana 2000, Talavera & Castresana 2007; http://molevol.cmima.csic.es/castresana/Gblocks_server.html), using the relaxed parameters (*i.e.* allow smaller final blocks, allow gap positions within the final blocks and allow less strict flanking positions). Alignments of individual genes were concatenated as two datasets: (1) PCG; comprising the 13 PCGs with 11,707 nucleotides and (2) PCG+RNA: 13 PCGs, two rRNAs and 22 tRNAs; with 15,110 nucleotides. Both datasets were used in phylogenetic analyses. Phylogenetic relationships were reconstructed using Maximum Likelihood (ML). Heuristic searches were carried out using the Randomized Axelerated Maximum Likelihood algorithms implemented in RaxML-HPC2 (Stamatakis 2006; Stamatakis et al. 2008), through the CIPRES web portal (http://www.phylo.org). The analysis was run under de GTRCAT model, and bootstrap support values were generated with a rapid bootstrapping algorithm under the auto Majority Rule Criterion (autoMRE; Stamatakis et al., 2008).

**Results and Discussion**

Here, we provide the third complete mitochondrial genome for a member of the family Calopterygidae, and further confirm the utility of next-generation sequencing to obtain whole odonate mitogenomes (see also Lorenzo-Carballa et al. 2014). A total of 2,813,625 short-sequence reads were mapped to obtain a single contig 15,487 bp long. The mean coverage for the contig was ~9,000 reads.

The size of the complete mitochondrial DNA genome of *M. costalis* (GenBank accession number KU871065) is within the size range of the other 14 complete odonate mitochondrial genomes (15,056–16,685 bp). It comprises the standard metazoan panel of 13 protein-coding genes, 2 rRNA genes (12S and 16S rRNA) and 22 tRNA genes (Figure1, Table 1). Protein-coding genes employ the typical invertebrate mitochondrial start codons: *cox1, cox2, cox3, atp6, nad4, nad4L, cob* and *nad1* use ATG; *nad2, nad3, nad6* and *atp8* use ATC, and *nad5* uses ATT. Eight protein-coding genes have the standard stop codons TAA (*nad1, nad2, nad6, nad4L, atp6* and *atp8)* and TAG (*nad3* and *cob*); *cox1, cox2, cox3* and *nad5* have an incomplete stop codon of a single T, while *nad4* has an incomplete TA stop codon (Table 1). Base frequency of the whole mtDNA genome is A=40%, T=26.2%, C=19.5% and G=14.2%4%, with an overall A+T content of 66.2% that is within the range of other odonate mitogenomes (A+T=64.1-73.1%), including the calopterygids *Vestalis melania* (64%) and *Atrocalopteryx atrata* (70%). All protein-coding genes have a high AT content,

which varies between 69.2% (*nad4L*) and 58.5% (*cox3*) AT content. All 22 tRNA-coding sequences of *M. costalis* can be folded into the characteristic clover-leaf secondary structure, and they range in size from 62 bp in *trnC* to 76 bp in *trnY* (Supplemental Information Figure 1; Table 1).

The mtDNA genome of *M. costalis* is identical in gene number and gene arrangement to that of the other fourteen complete odonate mitogenomes. Sixteen gene junctions in the mtDNA of *M. costalis* have short overlaps, with the largest junction having 13 nucleotides overlap (between *atp8* and *atp6*). There are three non-coding intergenic spacers (s1-s3), which are characteristic of other odonate mitogenomes. The s5 spacer located between *trnL* and *nad1* in anisopterans (dragonflies) is absent in *M. costalis* and all other zygopteran (damselflies) mitogenomes, further supporting the suggestion that this trait is a synapomorphy for the Zygoptera and Anisoptera (Lin et al., 2010, Lorenzo-Carballa et al 2014).

Only three SNPs (three Rs in positions 5,085 [*cox3*], 9,636 and 9,638 [*nad4L*]) and two ambiguities (Ns) in positions 8,901 [*nad4*] and 14,732 [A+T rich region] were found in the sequence, which indicates that some variability is expected between different individuals/morphs of *M. costalis*. Thus, having the complete mitochondrial genome available will help to improve primer design for future population genetic studies on this interesting species.

Our ML analyses using both datasets (PCG and PCG+RNA) recovered the same topology, and support the inclusion of *Epiophlebia superstes* within the suborder Zygoptera, although this relationship is supported with a higher bootstrap value by the analysis of the PCG dataset (Figure 2). The inclusion of *E. superstes* within the Zygoptera would be in agreement with the finding that the mitochondrial genome of this species also lacks the intergenic spacer s5 between *nad1* and *trnL2,* and thus the mitogenomic organization of this relict dragonfly would be more similar to that of the Zygoptera (Wang et al 2015).

A surprising result of our analyses is that the monophyly of the suborder Anisoptera is not well supported by any of the datasets (Figure 2), which could be due to the fact that available data for two anisopteran species (*Orthetrum triangulare melania* and *Cordulia aenea*) are incomplete. Obtaining more complete mitochondrial genomes for other representatives of the Anisoptera in the future could help to improve the resolution of the analyses. Also, combining whole mitogenome data with nuclear markers could help not only to obtain more robust phylogenies, but also to clarify the position of the Epiophlebidae within the order.

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**Figure 1:** Genetic map of the mitochondrial genome of *Mnais costalis,* showing the location of the protein coding genes (green), transfer RNAs (pink), ribosomal RNAs (red), the intergenic spacers s1-s3 and the A+T rich region (grey). Picture of *M. costalis* by Stewart J. Plaistow.

**Figure 2:** Maximum likelihood (ML) tree obtained when analyzing nucleotides from protein coding genes, 16S and 12S, and tRNAs; after ambiguous regions were excluded from all the genes. Values above branches represent boopstrap support values for the analyses with each dataset (PCG+RNA / PCG).

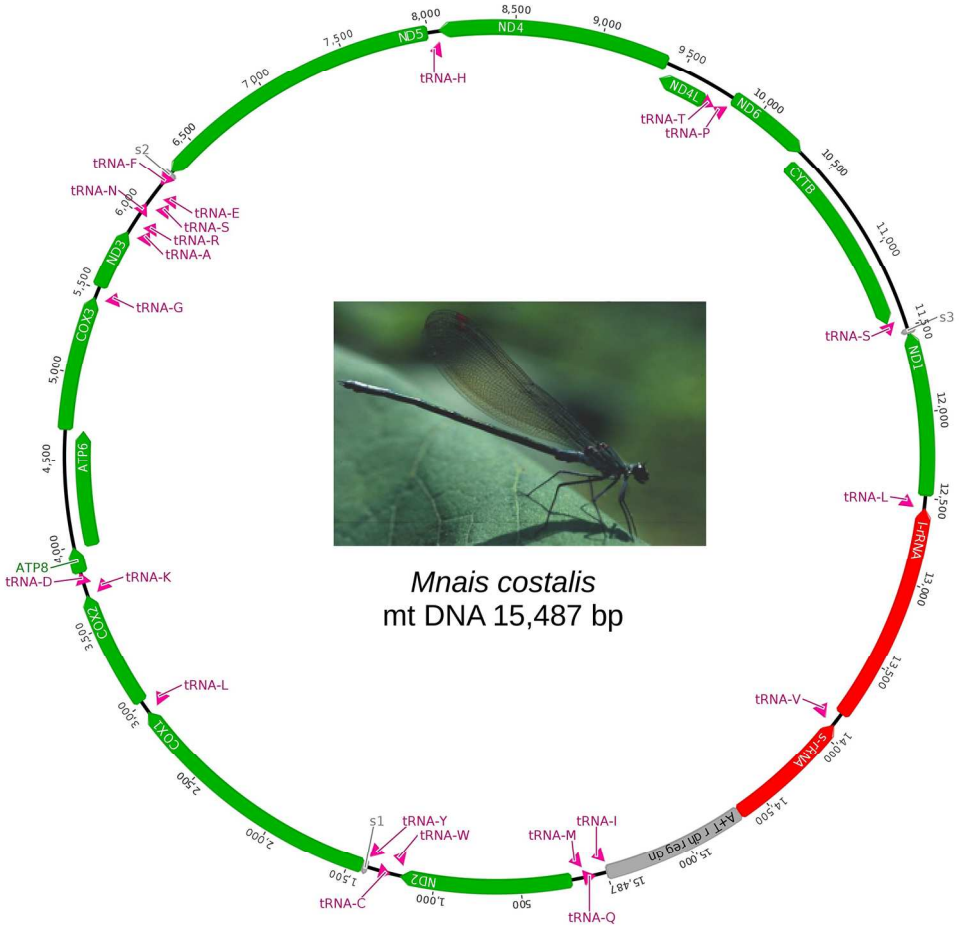
**Table 1.** Mitochondrial genomes currently available for Odonata.

| Suborder | Family | Species | Mitogenome size (bp) | GenBank Accession | Reference |
|---|---|---|---|---|---|
| Anisoptera | Libellulidae | *Orthetrum triangulare melania* | 14,033* | AB126005 | Yamauchi et al. 2004 |
| | | *Hydrobasileus croceus* | 15,088 | KM244659 | Tang et al. 2014 |
| | | *Brachythemis contaminata* | 15,056 | KM658172 | Yu et al. 2015 |
| | Corduliidae | *Cordulia aenea* | 14,448* | JX963627 | Simon & Hadrys 2013 |
| | Gomphidae | *Davidius lunatus* | 15,913 | EU591677 | Lee et al. 2009 |
| | | *Ictinogomphus* sp. | 15,393 | KM244673 | Tang et al 2014 |
| Anisozygoptera | Epiophlebidae | *Epiophlebia superstes* | 15,435 | JX050223 | Wang et al. 2015 |
| Zygoptera | Platycnemidae | *Platycnemis foliacea* | 15,382 | NC027180 | unpublished |
| | Coenagrionidae | *Ischnura pumilio* | 15,250 | KC878732 | Lorenzo-Carballa et al. 2014 |
| | Euphaeidae | *Euphaea formosa* | 15,700 | HM126547 | Lin et al. 2010 |
| | | *E. yayeyamana* | 15,709 | KF718293 | unpublished |
| | | *E. ornata* | 15,863 | KF718295 | unpublished |
| | | *E. decorata* | 15,861 | KF718294 | unpublished |
| | Pseudolestidae | *Pseudolestes mirabilis* | 15,122 | FJ606784 | unpublished |
| | Calopterygidae | *Atrocalopteryx atrata* | 15,424 | KP233805 | unpublished |
| | | *Vestalis melania* | 16,685 | JX050224 | Chen et al. 2015 |

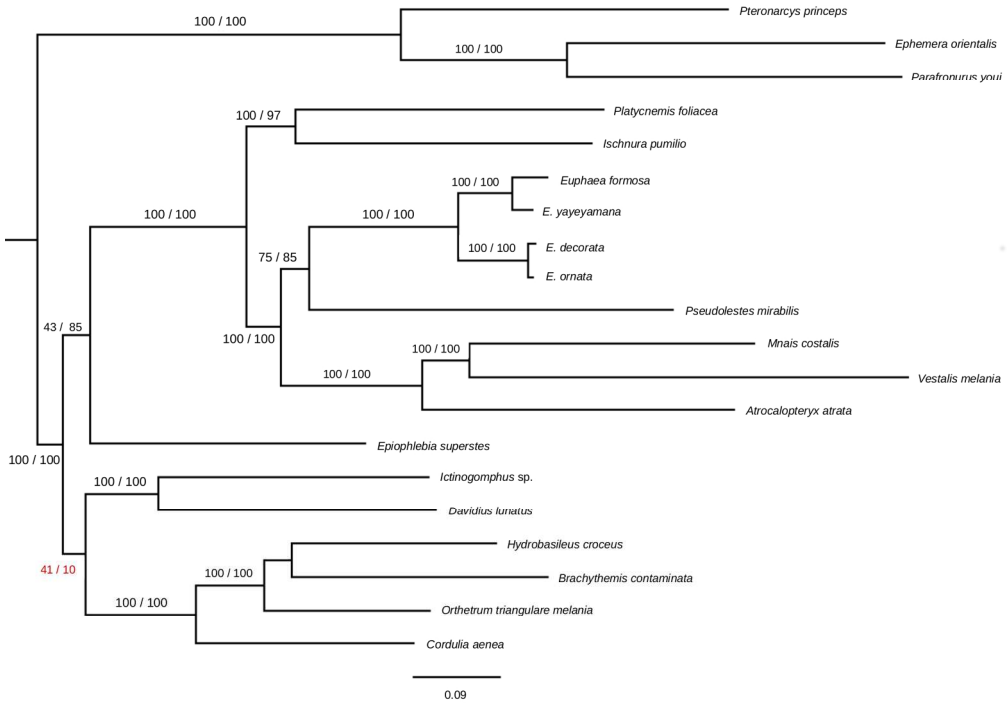
* indicates that the mitogenome sequence is incomplete

**Table 2.** Organisation of the mitochondrial genome of the damselfly *Mnais costalis.* Incomplete stop codons are indicated with parentheses. *s1-s3* are intergenic spacers.

| Feature | Position | Length | A+T (%) | Strand | Start/Anti codon | Stop codon |
|---|---|---|---|---|---|---|
| ***trnI*** | 1-72 | 72 | 65.3 | + | GAT | - |
| ***trnQ*** | 68-137 | 70 | 68.5 | - | TTG | - |
| ***trnM*** | 138-206 | 69 | 66.7 | + | CAT | - |
| ***nad2*** | 207-1196 | 990 | 67.9 | + | ATC | TAA |
| ***trnW*** | 1194-1263 | 70 | 72.9 | + | TCA | - |
| ***trnC*** | 1255-1316 | 62 | 74.2 | - | GCA | - |
| ***trnY*** | 1317-1392 | 76 | 72.4 | - | GTA | - |
| ***s1*** | 1393-1417 | 25 | 68 | n.a. | - | - |
| ***cox1*** | 1418-2951 | 1534 | 60.4 | + | ATG | T(aa) |
| ***trnL2*** | 2951-3019 | 69 | 65.2 | + | TAA | - |
| ***cox2*** | 3019-3706 | 688 | 63.2 | + | ATG | T(aa) |
| ***trnK*** | 3706-3779 | 74 | 64.9 | + | CTT | - |
| ***trnD*** | 3778-3842 | 65 | 78.5 | + | GTC | - |
| ***atp8*** | 3843-4001 | 159 | 68.6 | + | ATC | TAA |
| ***atp6*** | 3989-4672 | 684 | 64.6 | + | ATG | TAA |
| ***cox3*** | 4672-5458 | 787 | 58.5 | + | ATG | T(aa) |
| ***trnG*** | 5458-5522 | 65 | 76.9 | + | TCC | - |
| ***nad3*** | 5522-5875 | 354 | 67.1 | + | ATC | TAG |
| ***trnA*** | 5874-5938 | 65 | 75.4 | + | TGC | - |
| ***trnR*** | 5939-6005 | 67 | 61.2 | + | TCG | - |
| ***trnN*** | 6003-6069 | 67 | 73.1 | + | GTT | - |
| ***trnS1*** | 6070-6138 | 69 | 56.5 | + | GCT | - |
| ***trnE*** | 6141-6205 | 65 | 81.5 | + | TTC | - |
| ***trnF*** | 6203-6268 | 66 | 63.6 | + | GAA | - |
| ***s2*** | 6269-6293 | 25 | 80 | n.a. | - | - |
| ***nad5*** | 6294-7994 | 1701 | 65.1 | - | ATT | T(aa) |
| ***trnH*** | 7995-8058 | 64 | 68.7 | - | GTG | - |
| ***nad4*** | 8059-9398 | 1339 | 67.9 | - | ATG | TA(a) |
| ***nad4L*** | 9392-9685 | 294 | 69.2 | - | ATG | TAA |
| ***trnT*** | 9687-9755 | 69 | 65.2 | + | TGT | - |
| ***trnP*** | 9759-9827 | 69 | 68.1 | - | TGG | - |
| ***nad6*** | 9829-10323 | 495 | 62.8 | + | ATC | TAA |
| ***cob*** | 10323-11451 | 1129 | 61 | + | ATG | TAG |
| ***trnS2*** | 11455-11519 | 65 | 58.5 | + | TGA | - |
| ***s3*** | 11520-11536 | 17 | 88.2 | n.a. | - | - |
| ***nad1*** | 11537-12487 | 951 | 66.1 | - | ATG | TAA |
| ***trnL1*** | 12489-12553 | 65 | 69.2 | - | TAG | - |
| ***l-rRNA*** | 12554-13844 | 1291 | 71 | - | - | - |
| ***trnV*** | 13845-13916 | 72 | 61.1 | - | TAC | - |
| ***s-rRNA*** | 13917-14653 | 737 | 69.2 | - | - | - |
| ***A+T rich region*** | 14654-15487 | 834 | 79.7 | n.a. | - | - |




170x165mm (300 x 300 DPI)




280x209mm (181 x 181 DPI)