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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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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 of 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 currently available mitochondrial genome sequences of odonates suggests that *Epiophlebia superstes* is more closely related to the Zygoptera than to the Anisoptera.

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

Introduction

Despite the problems that may arise from the use of mitochondrial markers in instances of introgression or hybridisation (e.g. Hayashi, Dobata, & Futahasi, 2005), mitochondrial sequence data continue to provide the majority of the genetic markers used in phylogenetic studies on odonates (Ballare & Ware, 2011). 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 species (but see Ferreira *et al*., 2014). Having full mitochondrial genomes available provides the opportunity to improve primer design, and also to increase the number of target genes used in phylogenetic inference, therefore providing more robust phylogenetic reconstructions (e.g. Lin, Chen, & Huang, 2010). Also, mitochondrial genomes may allow diverse comparative and evolutionary genomics questions to be answered, not only in odonates, but in insects in general; such as the evolution of genome size, the study of

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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, Miya, M. U., & Nishida, 2004
		Hydrobasileus croceus	15,088	KM244659	Tang *et al*., 2014
		Brachythemis contaminata	15,056	KM658172	Yu, Cheng, Ma, Yu, & Zhang, 2016
	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.

gene rearrangements (Cameron, 2014), or to analyse the evolutionary processes that underlie the evolution of synonymous and non-synonymous 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 16 odonate species belonging to nine families are available (Table 1). However, and despite it covering nearly a third of the families currently described within the order, this constitutes yet a small fraction of the extant odonate diversity (~6000 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 a behavioural phenotype (Plaistow & Tsubaki, 2000; Tsubaki, Hooper, & Siva-Jothy, 1997; Tsubaki & Okuyama, 2016). Finally, we reconstruct the phylogenetic relationships among odonates, based on the mitochondrial genomes currently available.

Material and methods

Sample collection, DNA extraction and sequencing

Five adult *Mnais costalis* males (two orange and three 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 (Venlo, The Netherlands), following

the manufacturer's instructions. Samples were pooled for sequencing (pool 1 – clear and pool 2 – orange) on a HiSeq 2000 (Illumina, California, USA), generating 2 × 100 bp paired end reads (approximate fragment size 550–750 bp), 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 annotating 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, Gish, Miller, Myers, & Lipman, 1990) against Genbank's nr database (www.ncbi.nih.gov; date accessed 17 February 2016). Transfer RNA genes were identified using ARWEN (Laslett & 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 genomes available for *Ephemera orientalis* (Ephemeroptera, GenBank Accession EU 591678; Lee *et al*., 2009), *Parafronurus youi* (Ephemeroptera, GenBank Accession EU 349015; Zhang, Zhou, Gai, Song, & Zhou, 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 alignment of the 16S and 12S sequences we used Mafft v.7 (Katoh & Standley, 2013; http://mafft.crc.jp/), with the option Q-INS-I, which considers the secondary structure information of the RNA. Transfer RNA sequences were aligned using the software LocARNA (Smith, Heyne, Richter, Will, & Backofen, 2010; Will, Joshi, Hofacker, Stadler, & Backofen, 2012; Will, Reiche, Hofacker, Stadler, & Backofen, 2007; http://rna.informatik.uni-freiburg.de/LocARNA/), which aligns tRNA sequences based on their sequence and structure features. Regions with ambiguities, as well as variable regions within each alignment, were recognised 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 position 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; comprising the 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 randomised accelerated maximum likelihood algorithms implemented in RAxML-HPC2 (Stamatakis, 2006; Stamatakis, Hoover, & Rougemont, 2008), through the CIPRES web portal (http://www.phylo.org). The analyses were run under the

GTRCAT model, and bootstrap support values were generated with a rapid bootstrap algorithm under the 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, Thompson, Cordero-Rivera, & Watts, 2014). A total of 2,813,625 short-sequence reads were mapped to obtain a single contig of 15,487 bp long with a mean coverage of ~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, two rRNA genes (12S and 16S rRNA) and 22 tRNA genes (Figure 1, Table 2). Protein-coding genes employ the typical invertebrate mitochondrial start codons: *cox1*, *cox2*, *cox3*, *atp6*,

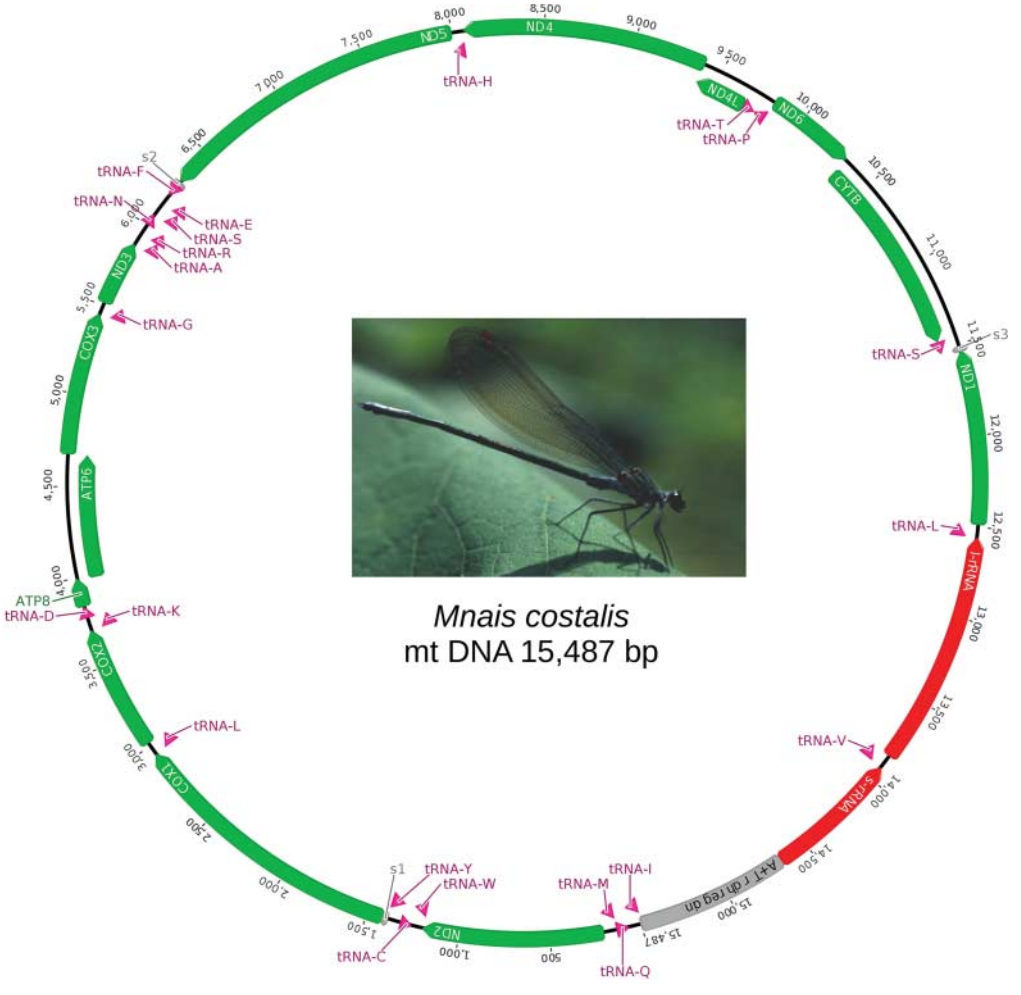

Figure 1. Genetic map of the mitochondrial genome of *Mnais costalis*, showing the location of the protein coding genes (green), transfer RNAs (pink), ribosomal RNAs (red), the intergenic spacers s1–s3 and the A + T rich region (grey). Picture of *M. costalis* by Stewart J. Plaistow.

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	na	–	–
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	na	–	–
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	10,323–11,451	1129	61	+	ATG	TAG
trnS2	11,455–11,519	65	58.5	+	TGA	–
s3	11,520–11,536	17	88.2	na	–	–
nad1	11,537–12,487	951	66.1	–	ATG	TAA
trnL1	12,489–12,553	65	69.2	–	TAG	–
l-rRNA	12,554–13,844	1291	71	–	–	–
trnV	13,845–13,916	72	61.1	–	TAC	–
s-rRNA	13,917–14,653	737	69.2	–	–	–
A + T rich region	14,654–15,487	834	79.7	na	–	–

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). The base frequency of the whole mtDNA genome is A = 40%, T = 26.2%, C = 19.5% and G = 14.2%, 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 S1; Table 1).

The mtDNA genome of *M. costalis* is identical in gene number and gene arrangement to that of the other 14 complete odonate mitogenomes. Sixteen gene junctions in the mtDNA of

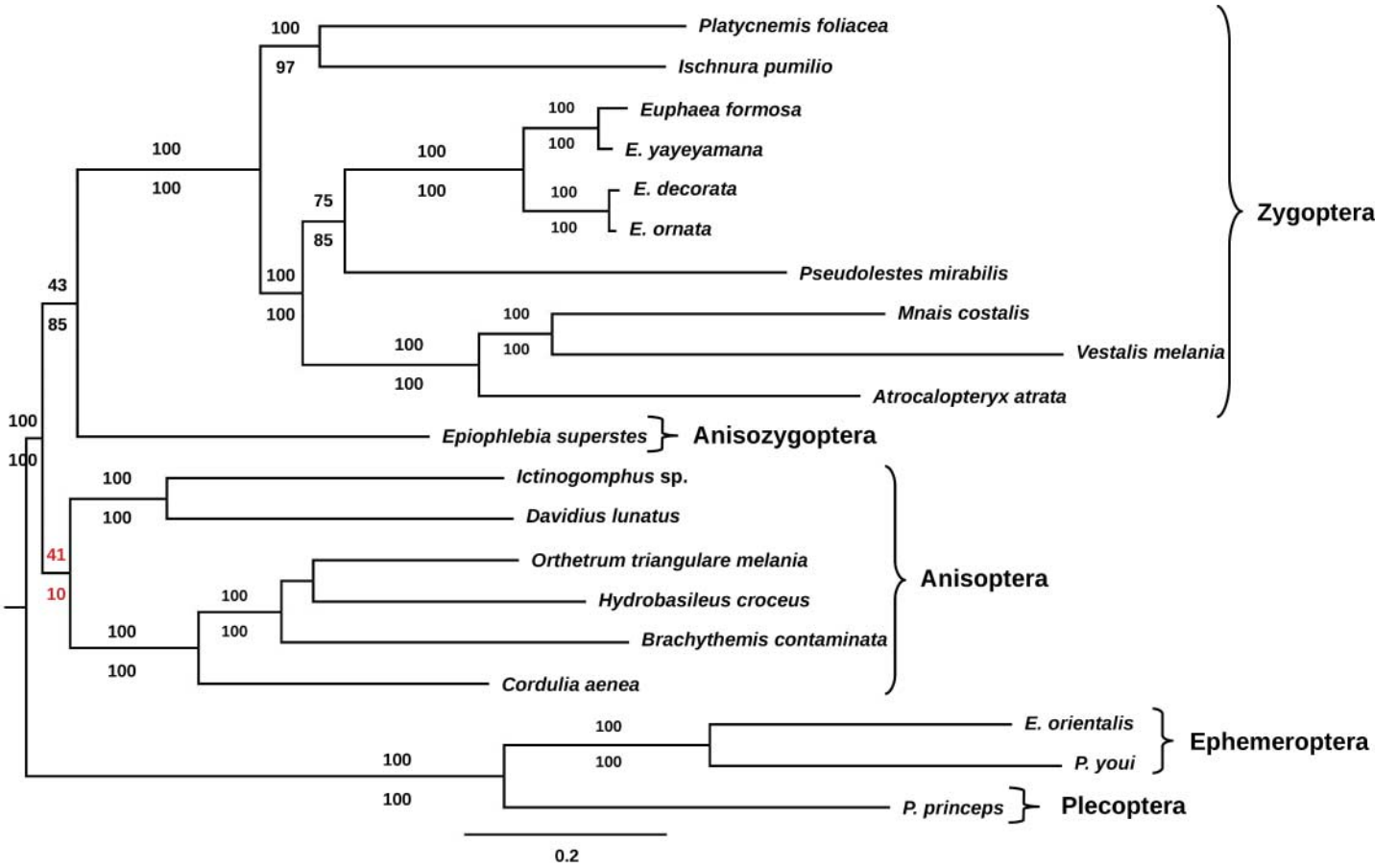

Figure 2. Maximum likelihood (ML) tree obtained when analysing nucleotides from protein coding genes, 16S and 12S and tRNAs; after ambiguous regions were excluded from all the alignments. Values above and below branches represent bootstrap support values for the analyses with the PCG + RNA and PCG; respectively (see main text for details).

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 5085 [*cox3*]; 9638 [*nad4L*]) and two ambiguities (Ns in positions 8901 [*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 for this species will help to improve primer design for future population genetic studies.

Our ML analyses using both datasets (PCG and PCG + RNA) recovered the same topology. Contrary to the current accepted classification of the suborder Anisozygoptera as the extant sister-group of the Anisoptera (Dijkstra *et al*., 2013), our results place *E. superstes* as a basal group to the Zygoptera, although this relationship is supported with a higher bootstrap value by the analysis of the PCG dataset (Figure 2). These results would be in agreement with the finding that the mitochondrial genome of this species lacks the intergenic spacer s5 between *nad1* and *trnL2*, and therefore the mitogenomic organisation of this relict odonate would be more similar to that of the Zygoptera (Wang *et al*., 2015).

A surprising result from 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 the available mitogenomes for to anisopteran species are incomplete (*Orthetrum triangulare melania* and *Cordulia aenea*, Table 1). Obtaining more complete mitochondrial genomes for other representatives of the Anisoptera, as well as for more *Epiophlebia* species in the future, would help to improve the resolution of the analyses and to clarify relationships between extant odonate groups. Also, combining complete mitogenome data with nuclear markers would help to obtain more robust phylogenies for the order.

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Supplemental data

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/13887890.2016.1234980

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