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RESEARCH NOTE

The first Margaritiferidae male (M-type) mitogenome: mitochondrial gene order as a potential character for determining higher-order phylogeny within Unionida (Bivalvia)

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The unionid family Margaritiferidae, comprising 12 extant species, is widely distributed across the northern hemisphere in North America, Europe and Asia (Bolotov *et al.*, 2016). Most species in this family have dramatically declined over the last century, with nine of the 12 species assessed as threatened in the most recent IUCN Red List (IUCN, 2016). Among these is the Moroccan pearl mussel *Margaritifera marocana* (Pallary, 1918), considered one of the 100 most threatened species on the planet (Baillie & Butcher, 2012). This species is now restricted to two small streams in the Oum Er Rbia and Sebou basins and conservation measures are urgently needed (Sousa *et al.*, 2016). Beyond the conservation concern, Unionida are also biologically interesting. They present an unusual mechanism of mitochondrial inheritance called doubly uniparental inheritance (DUI), in which all individuals have the typical maternally transmitted mtDNA (F-type), but the males possess in their germ cells a paternally inherited mtDNA instead (M-type) (Zouros *et al.*, 1994; Breton *et al.*, 2009). So far, DUI has been observed in over 100 species from four bivalve orders (Gusman, Azuelos & Breton, 2017), including three families within Unionida, i.e. Unionidae, Hyriidae and Margaritiferidae (Walker *et al.*, 2006). However, to date, no whole M-type mitogenome has been published for any species belonging to the last two of these families.

The gene arrangement within mitogenomes is highly conserved in many taxonomic groups. For example, most vertebrates share the same gene order (Pereira, 2000). In other faunal groups, like Bivalvia, the mitochondrial genome arrangement is more variable, although not many distinct gene orders have been described so far (Serb & Lydeard, 2003). Still, in unionoids, mitogenome rearrangements seem to be rare events that are unlikely to be homoplastic. In this context, mitogenome gene order might be used as an additional character for phylogenetic inference. However, its utility for the Unionida phylogeny has never been tested.

The order Unionida has 6 recognized families with around 800 species (Lopes-Lima *et al.*, 2014), but the phylogenetic relationships among these families are still not fully resolved (Graf, 2013). This lack of coherence among studies has been consistently attributed to the low number of molecular markers used and insufficient taxon sampling (Bogan & Roe, 2008; Graf, 2013; Fonseca *et al.*, 2016).

Under the above-mentioned assumptions the aims of the present study are to (1) sequence and analyse the whole M- and F-type mitogenomes of *M. marocana*, (2) infer the phylogenetic relationships among Unionoidea species using all both the F- and M-type mtDNA sequences publicly available and (3) determine the gene order of all analysed mitogenomes and evaluate its phylogenetic utility.

One male specimen deposited in the Muséum d'Histoire Naturelle de Marrakech (voucher MHNM16ZMB23) from the Laabid River (GPS WGS84: 32.142334, −7.027595) was dissected for sampling of gonadal and mantle tissue. DNA extractions followed Froufe *et al.* (2016). The complete M- and F-type mitogenomes were then sequenced, assembled and annotated using an established pipeline (Gan, Schultz & Austin, 2014). The F and M mitogenomes have been deposited in GenBank database under the accession numbers KY131953 and KY131954, respectively.

The two newly obtained *M. marocana* mitogenome sequences were aligned with all (43) M- and F-type Unionida mitogenome

sequences available on GenBank as of March 2016, as well as with the F- and M-type mitogenomes of *Mytilus galloprovincialis* as outgroup (list of genomes and respective accession numbers used supplied on request). DNA (NUC) and amino acid (AA) sequences of all mtDNA protein-coding genes (PCGs) except ATP8, and the gender-specific open reading frames (M-ORF, H-ORF and F-ORF) were used in the phylogenetic analyses. The sequences of each gene were aligned using MAFFT v. 7.304 (Katoh & Standley, 2013) and trimmed with GUIDANCE v. 1.5 (Penn *et al.*, 2010; see Froufe *et al.*, 2016, for parameters used). The gene alignments were then concatenated, resulting in two alignments with the following length: 14,350 aligned nucleotide positions or 6,246 aligned amino acid + nucleotide positions (4,085 aligned amino acids positions and 2,161 aligned nucleotide positions from the rRNAs genes). The optimal partitioning scheme (i.e. the best set of nonoverlapping partitions that cover the whole alignment) for each alignment was selected using PartitionFinder v. 1.1.1 (Lanfear *et al.*, 2012) under the greedy algorithm with proportional branch lengths across partitions. The best substitution models of DNA and protein evolution for each partition were selected under the BIC ranking method (Schwarz, 1978). The codon positions of the PCG and each rRNA were defined as the initial data blocks for the partitioning schemes search. Maximum likelihood (ML) phylogenetic inference was performed using RAxML v. 8.0.0 (Stamatakis, 2014) with 100 rapid bootstrap replicates and 20 ML searches. Bayesian Inference (BI) was applied using MrBayes v. 3.2.1 (Ronquist *et al.*, 2012) with two independent runs (1×10^7 generations with a sampling frequency of 1 tree for every 100 generations), each with four chains (three hot and one cold). All runs reached convergence (average standard deviation of split frequencies <0.01). The posterior distribution of trees was summarized in a 50% majority rule consensus tree (burn-in of 25%).

The length of the two newly sequenced mitogenomes of *M. marocana*, 16,001 bp for the female haplotype and 17,562 bp for the male haplotype, is within the typical range for each sex-specific haplotype within Unionida. The sequenced haplotypes include the 13 PCGs typically found in metazoan mitochondrial genomes, the sex-specific ORF described for all Unionida mitogenomes with

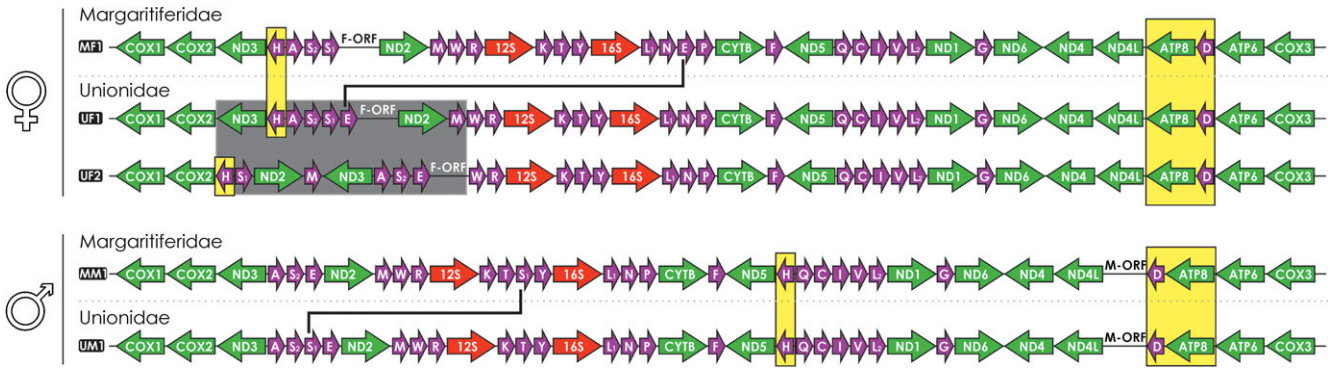

Figure 1. Diagrams of the five distinct gene orders detected in Unionida. In the female F-type lineage, three gene orders are depicted: Unionidae F-type 1 (UF1), Unionidae F-type 2 (UF2) and Margaritiferidae F-type 1 (MF1). In the male M-type lineage, two gene arrangements are shown: Unionidae M-type 1 (UM1) and Margaritiferidae M-type 1 (MM1). Continuous lines indicate different locations of genes between mitogenomes. Grey box highlights gene rearrangement region between UF1 and UF2. Yellow boxes indicate main differences in gene arrangement between female and male mitogenomes, tRNA (H) location and rearrangement of ATP8-tRNA(D) region.

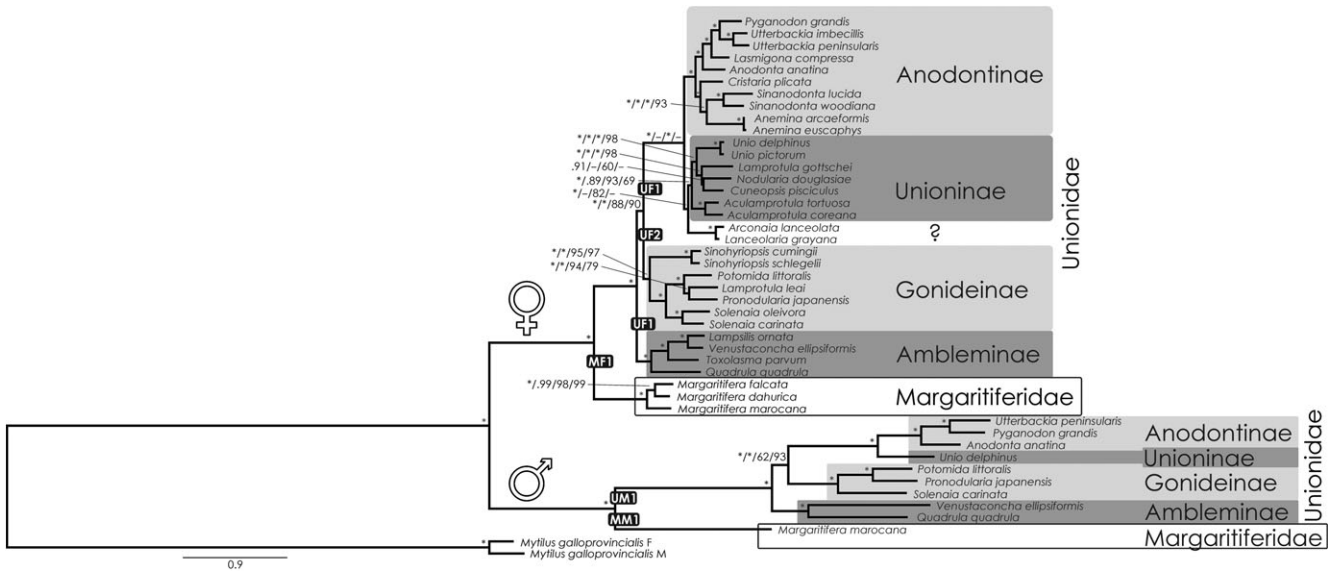

Figure 2. Phylogenetic (BI-NUC) tree of Unionida estimated from 14 concatenated individual mtDNA gene sequences (12 protein-coding and 2 rRNA genes). Values for branch support are represented in the following order: (1) Bayesian posterior probabilities (PP) for BI-NUC tree, (2) Bayesian PP for BI-AA tree, (3) ML bootstrap support (BS) values for ML-NUC and (4) ML BS values for ML-AA tree. Maximum support values (PP = 1, BS = 100) are represented by asterisks. All five distinct detected gene orders are mapped on the phylogeny branches (see Fig. 1 for gene order codes).

DUI system (Breton *et al.*, 2009, 2011a) and 22 transfer RNA (tRNA) and two ribosomal RNA (rRNA) genes. The M-type genome is the largest sequenced to date within the Unionida. M-type genomes are generally larger than F-type genomes due to the larger size of the PCG COX2 and M-ORF in M-type genomes compared with COX2 and F-ORF in F-type genomes (Breton *et al.*, 2009). Four intergenic regions were identified in the *M. marocana* M-type genome between the following gene pairs: NAD3–tRNA(A) 106 bp, tRNA(H)–tRNA(Q) 411 bp, ND4L–tRNA(D) 255 bp and tRNA(D)–ATP8 498 bp. These regions were analysed to search for the M-ORF. The results of the blast search (Altschul *et al.*, 1997) retrieved a significant hit with another Margaritiferidae M-ORF (*Margaritifera monodonta*, *E*-value = 4e–34) and a Fickett test score of 1.201 (Fickett, 1982; a score > 0.95 means the sequence is probably coding), suggesting that the M-ORF is located in the region between the genes ND4L and tRNA(D).

The M-type mitogenome of *M. marocana* presents a novel gene order within Unionida (Fig. 1). The F-type mitogenome gene order is the same as already observed for the two previously available Margaritiferidae F-type mitogenomes (Breton *et al.*, 2011b; Yang *et al.*, 2015).

All the phylogenies inferred in this study support the reciprocal monophyly of both (Unionidae + Margaritiferidae) F- and M-type lineages (Fig. 2 shows the topology of the BI-NUC tree; all other phylogenetic trees figures supplied on request). Additionally, the monophyly of Unionidae (both F- and M-type), Margaritiferidae (F-type) and all represented Unionidae subfamilies are well supported in all inferred mtDNA trees, with the exception of the Unioninae, for which monophyly was only well supported in the BI-NUC tree. The remaining phylogenetic trees (BI-AA, ML-NUC and ML-AA) showed conflicting results regarding the position of the clade comprising *Arconaia lanceolata* and *Lanceolaria grayana* (Fig. 2). These conflicting results have also been found in previous studies where different mitogenome phylogenetic methodologies revealed distinct tree topologies (Huang *et al.*, 2013; Fonseca *et al.*, 2016).

Five distinct mtDNA gene orders have been detected in the present dataset, three in the F-type lineage and two in the M-type lineage. In the F-type lineage, gene order UF1 is shared by the Unionidae subfamilies Anodontinae, Ambleminae and Unioninae, whereas gene orders UF2 and MF1 are found in the represented species of the subfamily Gonideinae and the family Margaritiferidae, respectively (Figs 1, 2). In the female lineage, there is only a difference between UF1 and MF1 in the location of tRNA(E) (Fig. 1). The gene order of UF2 is more distinct and might have resulted from a tandem duplication of the gene region between COX2 and tRNA(W) followed by random deletion of segments of the duplicated gene region (Doucet-Beaupré *et al.*, 2010). Between the M and F mitogenomes the differences are in the location of tRNA(H) and the inversion of the ATP8-tRNA(D) region. An additional distinct location of tRNA(S1) is also found in margaritiferid M mitogenomes (Fig. 1). Mapping gene orders over the inferred mtDNA phylogeny suggests that UF1 might be ancestral within Unionidae and UF2 derived in the ancestral lineage of the Gonideinae. However, these hypotheses have limited support, because no mitogenome sequences, and therefore no gene order information, are available for three of the seven presently recognized unionid subfamilies. Future inclusion of mtDNA gene orders of these currently unrepresented subfamilies could change the inference of the ancestral and derived mtDNA gene orders within Unionidae. In the M-type, only one gene arrangement per family is obtained: UM1 for the Unionidae and MM1 for the Margaritiferidae. Due to the fact that the Unionida are a very old order (Graf & Cummings, 2007), and as a consequence of the several distinct mitogenome gene arrangements already found, it is likely that as novel mitogenomes from additional unionoid families and subfamilies become available, the corresponding gene orders might be useful to resolve their phylogenetic relationships within the order.

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