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

This submission is intended as a Research Note

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Short Running Head: *MARGARITIFERA MAROCANA* MITOGENOMES AND UNIONIDA PHYLOGENY

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The family Margaritiferidae (Bivalvia: Unionida), comprising 12 extant species, is widely distributed across the northern hemisphere of North America, Europe and Asia (Bolotov *et al*., 2016). Most species in this family have dramatically declined over the last century, with nine out of the 12 Margaritiferidae 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). The species is now restricted to two small streams in the Oum Er Rbia and Sebou basins, and conservation measures are urgently needed (Lopes-Lima, M. pers. obs.).

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; Plazzi *et al*., 2015). So far, DUI has been observed in species from three families of Unionida, i.e., Unionidae, Hyriidae and Margaritiferidae. However, to date, no whole M-type mitogenome has been published for either species belonging to the latter two families.

The gene arrangement within mitogenomes is conserved in many taxonomic groups. For example, most vertebrates share the same gene order (Pereira *et al*., 2000). In other groups, like Bivalvia, the mitochondrial genome arrangement is more variable, although not many distinct gene orders have been described so far (Plazzi *et al*., 2013). Due to this fact, mitogenome rearrangements seem to be rare events that are difficult to replicate. In this context, mitogenome gene order might be used as an additional character for phylogenetic inference. However, its utility for Bivalvia and particularly in the Unionida phylogeny has never been tested.

The order Unionida has six recognized families with around 800 species (Lopes-Lima *et al*., 2014). However, the phylogenetic relationships among these families have been highly contentious with the available studies presenting conflicting results (*e.g.*, Bogan & Hoeh, 2000; Graf & Cummings, 2006). This lack of coherence among studies has been consistently attributed to the low number of molecular markers and insufficient taxon sampling (Bogan & Roe, 2008; Fonseca *et al*., 2016).

Under the above mentioned assumptions the aims of the present study are to: i) sequence and analyse the whole M- and F-type mitogenomes of *Margaritifera marocana*, ii) infer the phylogenetic relationships among Unionida species using all both the F- and M-type mtDNA sequences publicly available, and iii) 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 gonadal and mantle tissue collections. DNA extractions followed Froufe *et al.* (2016). The complete M- and F-type mitogenomes of *M. marocana* were then sequenced, assembled and annotated using an established pipeline (Gan *et al*., 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 outgroups (list of genomes and respective accession numbers used supplied on request). DNA (NUC) and amino acid (AA) sequences of all mtDNA protein-coding genes (PCG) 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 software (version 7.304, Katoh & Standley, 2013) and trimmed with GUIDANCE (version 1.5, Penn *et al*., 2010; see Froufe *et al*., 2016 for the 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 non-overlapping partitions that cover the whole alignment) for each alignment was selected using PartitionFinder v. 1.1.1 software (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 BIC ranking method (Schwarz, 1978). The codon positions of the protein-coding genes and each rRNA were defined as the initial data blocks for the partitioning schemes search. The Maximum Likelihood (ML) phylogenetic inference was performed using RAxML (v. 8.0.0, Stamatakis, 2014) with 100 rapid bootstrap

replicates and 20 ML searches. The Bayesian methodology (BI) was applied using MrBayes v. 3.2.1 (Ronquist *et al*., 2012) with two independent runs ($1x10^7$ generations with a sampling frequency of 1 tree for every 100 generations), each with four chains (3 hot and 1 cold). All runs reached convergence (average standard deviation of split frequencies below 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 gender specific haplotype within Unionida. The sequenced haplotypes include the 13 protein-coding genes typically found in metazoan mitochondrial genomes, the gender-specific ORF described for all Unionida mitogenomes with DUI system (Breton *et al*., 2009, 2011a), and 22 transfer RNA (tRNA) and 2 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 protein-coding genes COX2 and M-ORF in M-type genomes compared to the orthologous genes in the F-type genomes (Breton *et al*., 2009). The four intergenic regions identified in the *M. marocana* M-type genome were analysed in the 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*, evalue= 4e-34), and the 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 tRNA-D and ND4L.

The M-type mitogenome of *M. marocana* presents a novel gene order within the 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).

Figure 1 to be presented here

All the phylogenies inferred in this study support the reciprocal monophyly of both (Unionidae + Margaritiferidae) F- and M-type lineages (Fig. 2 represents 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) and Margaritiferidae (F-type) and all 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 already been found in previous studies where distinct mitogenome phylogenetic methodologies revealed distinct tree topologies (Huang *et al*., 2013; Fonseca *et al*., 2016).

Figure 2 to be presented here

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 Gonideinae subfamily and the family Margaritiferidae, respectively (Figs. 1 and 2). Mapping gene orders over the inferred mtDNA phylogeny suggests that UF1 might be ancestral within the Unionidae and UF2 derived in the ancestral lineage of the Gonideinae. However, this result has limited supporting value because no mitogenome sequences, and therefore no gene order information, are available for three out of the seven presently recognised Unionidae 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 evolutionary history. 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 is a very old order (Graf & Cummings, 2007), and as a consequence of the number of distinct mitogenome gene arrangements already found, it is likely that as novel mitogenomes from additional Unionida families and subfamilies became available, more arrangements will be found. This may help to clarify the higher order phylogenetic relationships within the freshwater mussels.

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Figure 1. Diagrams of the five distinct gene orders detected on all Unionida from the present dataset. 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).

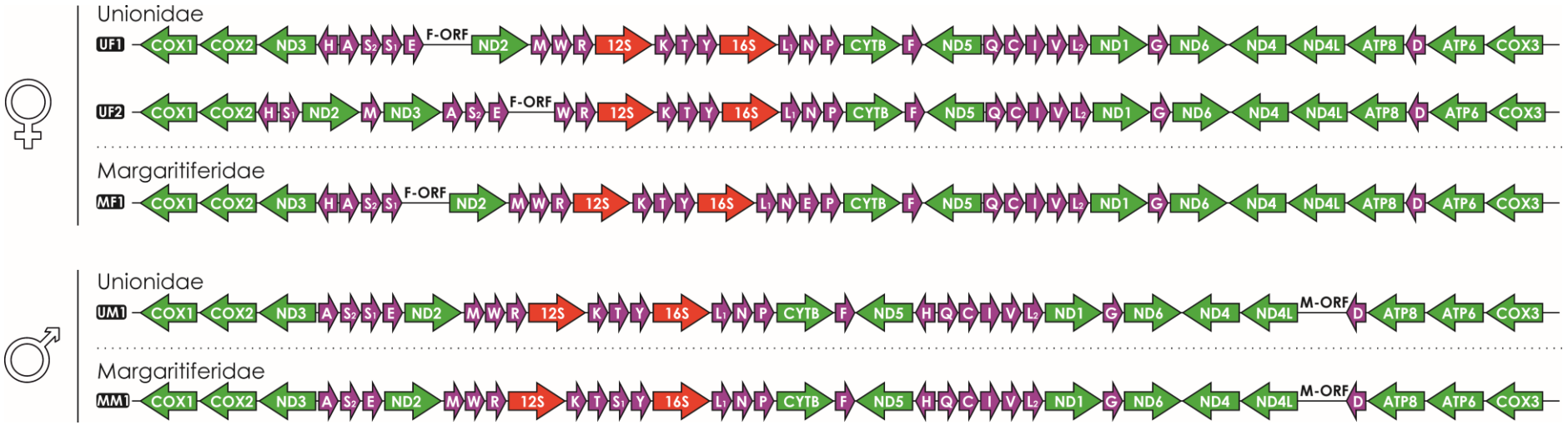
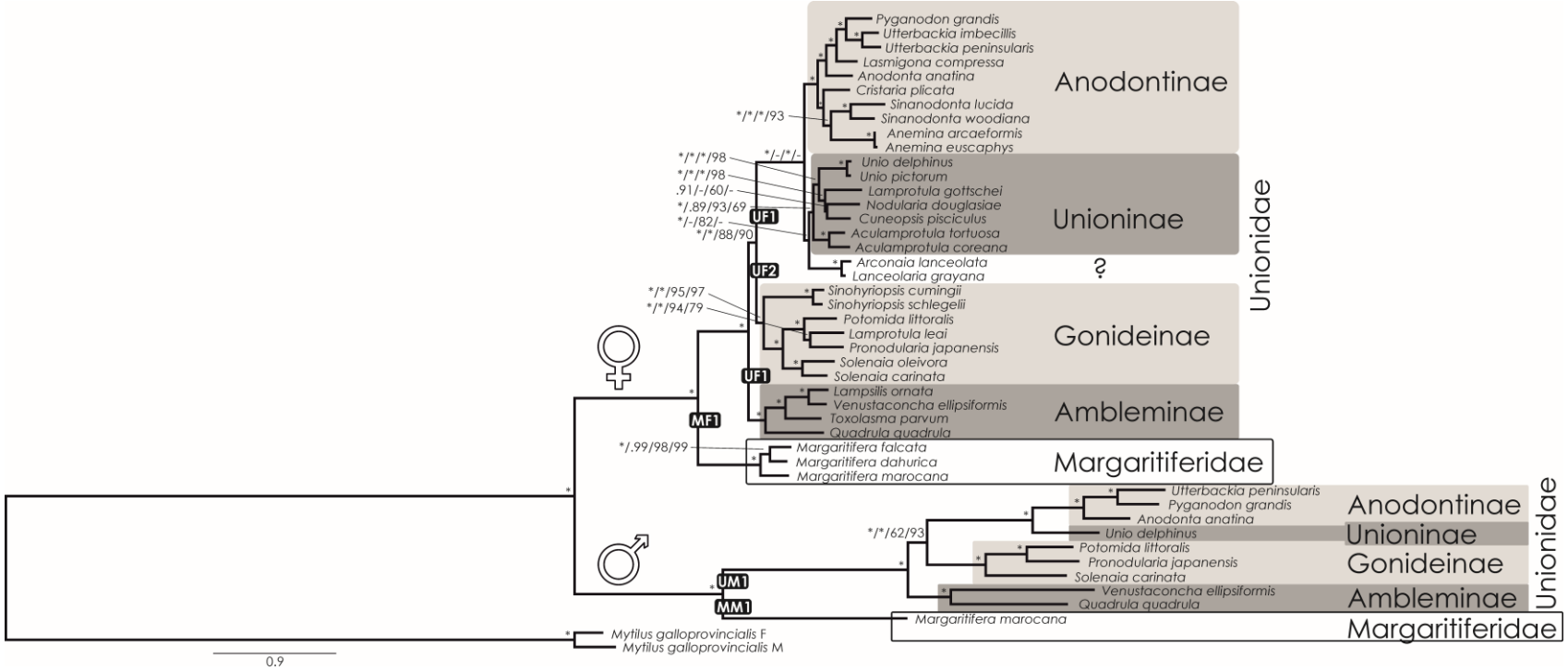


Figure 2. Phylogenetic (BI-NUC) tree of Unionida (freshwater mussels) estimated from 14 concatenated individual mtDNA gene sequences (12 protein-coding and 2 rRNA genes). The values for branch support are represented in the following order: 1) Bayesian Posterior Probabilities for BI-NUC tree, 2) Bayesian Posterior Probabilities for BI-AA tree, 3) ML bootstrap support values for ML-NUC, and 4) ML bootstrap support values for ML-AA tree. Maximum supporting values (BI = 1 and ML = 100) are represented with "*". All five distinct detected gene orders are mapped in the phylogeny branches (see Fig. 1 for gene order codes).



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