The complete mitochondrial genome of the grooved carpet shell, *Ruditapes decussatus* (Bivalvia, Veneridae)

Fabrizio Ghiselli Corresp., [1] , **Liliana Milani** [1] , **Mariangela Iannello** [1] , **Emanuele Procopio** [1] , **Peter L Chang** [2] , **Sergey V Nuzhdin** [2] , **Marco Passamonti** [1]

[1] Biological, Geological and Environmental Sciences, University of Bologna, Italy, Bologna, BO, Italy

[2] Department of Biological Sciences, Program in Molecular and Computational Biology, University of Southern California, Los Angeles, CA, United States

Corresponding Author: Fabrizio Ghiselli
Email address: fabrizio.ghiselli@unibo.it

Despite a large number of animal complete mitochondrial genomes being available in public databases, the current knowledge about mitochondrial genomics in invertebrates is sketchy. This paper reports, for the first time, the complete mitochondrial genome of the grooved carpet shell, *Ruditapes decussatus*, also known as European clam. *R. decussatus* is morphologically and ecologically similar to the Manila clam *Ruditapes philippinarum*, which has been recently introduced for aquaculture in the very same habitats of *R. decussatus*, and that is replacing the native species. Currently the production of the European clam is almost insignificant, nonetheless it is considered a high value product, and therefore it is an economically important species, especially in Portugal, Spain and Italy.In this work we: i) assembled *R. decussatus* mitochondrial genome from RNA-Seq data, and validated it by Sanger sequencing, ii) analyzed and characterized *R. decussatus* mitochondrial genome, comparing its features with those of other venerid bivalves; iii) assessed mitochondrial genetic polymorphism, both from a nucleotide (SNPs) and a structural (CNV of tandem repeats) point of view, across 26 samples.Despite using high-throughput approaches we did not find evidence of sex-linked mitochondrial genomes, so it seems that *R. decussatus* does not have Doubly Uniparental Inheritance of mitochondria, a phenomenon known in ~100 bivalve species. According to our analyses, *R. decussatus* is more genetically similar to species of the Genus Paphia than to the congeneric *R. philippinarum*, a finding that bolster the already proposed need of a taxonomic revision. We also found a quite low genetic variability across the examined samples, with a very low number of SNPs and a low polymorphism of the hypervariable sequences flanking the control region (Largest Unassigned Regions, LURs). Strikingly, contrasting with the low nucleotide variability along the entire mitochondrial genome, we observed a pretty high polymorphism in LUR length due to CNV of tandem repeats, and even a LUR length heteroplasmy in two samples. It is not clear if the lack of genetic variability in the

mitochondrial genome of *R. decussatus* is a cause or an effect of the ongoing replacement of *R. decussatus* with the invasive *R. philippinarum*, and more analyses, especially on nuclear sequences, are required to assess this point. In the coding regions we found putative indications for RNA editing.

AUTHOR COVER PAGE

The Complete Mitochondrial Genome of the Grooved Carpet Shell, *Ruditapes decussatus* (Bivalvia, Veneridae).

Fabrizio Ghiselli[1,*,º], Liliana Milani[1,º], Mariangela Iannello[1], Emanuele Procopio[1], Peter L. Chang[2], Sergey V. Nuzhdin[2], and Marco Passamonti[1]

[1] Department of Biological, Geological and Environmental Sciences - University of Bologna, Italy.

[2] Program in Molecular and Computational Biology, Department of Biological Sciences, University of Southern California, Los Angeles, CA, USA.

º Equal contribution

*Corresponding Author: Fabrizio Ghiselli fabrizio.ghiselli@unibo.it

Abstract

Despite a large number of animal complete mitochondrial genomes being available in public databases, the current knowledge about mitochondrial genomics in invertebrates is sketchy. This paper reports, for the first time, the complete mitochondrial genome of the grooved carpet shell, *Ruditapes decussatus*, also known as European clam. *R. decussatus* is morphologically and ecologically similar to the Manila clam *Ruditapes philippinarum*, which has been recently introduced for aquaculture in the very same habitats of *R. decussatus*, and that is replacing the native species. Currently the production of the European clam is almost insignificant, nonetheless it is considered a high value product, and therefore it is an economically important species, especially in Portugal, Spain and Italy.

In this work we: *i)* assembled *R. decussatus* mitochondrial genome from RNA-Seq data, and validated it by Sanger sequencing, *ii)* analyzed and characterized *R. decussatus* mitochondrial genome, comparing its features with those of other venerid bivalves; *iii)* assessed mitochondrial genetic polymorphism, both from a nucleotide (SNPs) and a structural (CNV of tandem repeats) point of view, across 26 samples.

Despite using high-throughput approaches we did not find evidence of sex-linked mitochondrial genomes, so it seems that *R. decussatus* does not have Doubly Uniparental Inheritance of mitochondria, a phenomenon known in ~100 bivalve species. According to our analyses, *R. decussatus* is more genetically similar to species of the Genus Paphia than to the congeneric *R. philippinarum*, a finding that bolster the already proposed need of a taxonomic revision. We also found a quite low genetic variability across the examined samples, with a very low number of SNPs and a low polymorphism of the hypervariable sequences flanking the control region (Largest Unassigned Regions, LURs). Strikingly, contrasting with the low nucleotide variability

along the entire mitochondrial genome, we observed a pretty high polymorphism in LUR length due to CNV of tandem repeats, and even a LUR length heteroplasmy in two samples. It is not clear if the lack of genetic variability in the mitochondrial genome of *R. decussatus* is a cause or an effect of the ongoing replacement of *R. decussatus* with the invasive *R. philippinarum*, and more analyses, especially on nuclear sequences, are required to assess this point. In the coding regions we found putative indications for RNA editing.

Keywords: (3-10)

complete mitochondrial genome; mitochondrial length polymorphism; mitochondrial repeats; mitochondrial RNA editing; codon usage; bivalve molluscs; European clam; comparative mitochondrial genomics.

Introduction

Despite a large number of animal complete mitochondrial genomes (mtDNAs) being available in public databases (>55,000 in GenBank), up to now sequencing has been focused mostly on vertebrates (~50,000 in GenBank), and the current knowledge about mitochondrial genomics in invertebrates—with the notable exception of few model organisms (e.g. Drosophila and *Caenorhabditis elegans*)—is sketchy. To better understand invertebrate mitochondrial biology—and, most importantly, mitochondrial biology and evolution in general—it is necessary to adopt a more widespread approach in gathering and analyzing data. Failing to do so would bias our knowledge toward a few taxonomic groups, with the risk of losing a big part of the molecular and functional diversity of mitochondria. Actually, despite maintaining its core features in terms of genetic content, mtDNA in Metazoa shows a wide range of variation in some other traits such as, for example, genome architecture, abundance of unassigned regions (URs)—namely regions with no assigned product (protein, RNA)—repeat content, gene duplications, introns, UTRs, and even additional coding genes (see Breton et al., 2014 for a review) or genetic elements (e.g, small RNAs, see Pozzi et al., 2017) . All this emerging diversity is in sharp contrast with the—at this point outdated—textbook notion about mtDNA role being limited to the production of a few subunits of the protein complexes involved in oxidative phosphorylation (OXPHOS).

This paper reports, for the first time, the complete mitochondrial genome of the grooved carpet shell, *Ruditapes decussatus* (Linnaeus, 1758). *R. decussatus*—also known as European clam—is distributed all over the Mediterranean coasts, as well as on the Atlantic shores, from Lofoten Islands (Norway) to Mauritania, including the British Isles. *R. decussatus* lives in warm coastal waters, especially in lagoons, and it is morphologically and ecologically similar to the Manila

clam *Ruditapes philippinarum*, which has been recently introduced for aquaculture in the very same habitats of *R. decussatus*. *R. philippinarum*, native from the Philippines, Korea, and Japan, was accidentally introduced into North America in the 1930s, and from there it was purposely introduced in France (1972), UK (1980), and Ireland (1982) for aquaculture purposes (Gosling, 2003). According to historical records, *R. decussatus* was one of the most important species for aquaculture in Europe, but overfishing, irregular yields, recruitment failure, and outbreaks of bacterial infection pushed the producers to introduce *R. philippinarum*; Italy imported large quantities of *R. philippinarum* seed from UK in 1983 and 1984. Compared to the European clam, the Manila clam turned out to be faster growing, more resistant to disease, to have a more extended breeding period and a greater number of spawning events, and to begin sexual maturation earlier (i.e. at a smaller size). The unavoidable consequence was the replacement of *R. decussatus* with *R. philippinarum*, and currently the production of the European clam is almost insignificant. Nonetheless the grooved carpet shell is considered a high value product, and therefore it is an economically important species, especially in Portugal, Spain and Italy (Gosling, 2003; Leite et al., 2013; de Sousa et al., 2014).

Molluscs in general, and bivalves in particular, exhibit an extraordinary degree of mtDNA variability and unusual features, such as: large mitochondrial genomes (up to ~47Kb), high proportion of URs, novel protein coding genes with unknown function, frequent and extensive gene rearrangement, and differences in strand usage (Gissi, Iannelli & Pesole, 2008; Breton et al., 2011; Ghiselli et al., 2013; Milani et al., 2014b; Plazzi, Puccio & Passamonti, 2016). Moreover, mitochondrial genome size varies among bivalves because of gene duplications and losses (Serb & Lydeard, 2003; Passamonti et al., 2011; Ghiselli et al., 2013), and sometimes genes are fragmented as in the case of ribosomal genes in oysters (Milbury et al., 2010). The

most notable feature of bivalves is the Doubly Uniparental Inheritance (DUI) of mitochondria (Skibinski, Gallagher & Beynon, 1994a,b; Zouros et al., 1994a,b). Under DUI, two different mitochondrial lineages (and their respective genomes) are transmitted to the progeny: one is inherited from the egg (female-transmitted or F-type mtDNA), the other is inherited from the spermatozoon (male-transmitted or M-type mtDNA). Following fertilization, the early embryo is heteroplasmic, but the type of mitochondria present in the adult is tightly linked to its sex. Females are commonly homoplasmic for F, while males are heteroplasmic with the following distribution of mtDNA types: the germ line is homoplasmic for the M-type (which will be transmitted via sperm to male progeny), the soma is heteroplasmic to various degrees, depending on tissue type and/or species (Ghiselli, Milani & Passamonti, 2011; Zouros, 2013). To date, the only known animals exhibiting DUI are about 100 species of bivalve molluscs (Gusman et al., 2016). This natural and evolutionarily stable heteroplasmic system can be extremely useful to investigate several aspects of mitochondrial biology (see Passamonti & Ghiselli, 2009; Breton et al., 2014; Milani & Ghiselli, 2015; Milani, Ghiselli & Passamonti, 2016). Indeed, despite many aspects of DUI are still unknown, there is evidence that DUI evolved from a strictly maternal inheritance (SMI) system (Milani & Ghiselli, 2015; Milani, Ghiselli & Passamonti, 2016), by modifications of the molecular machinery involved in mitochondrial inheritance, through as-yet-unknown specific factors (see Diz, Dudley & Skibinski, 2012; and Zouros, 2013 for proposed models). The detection of DUI is not a straightforward process, especially using PCR-based approaches: given that the divergence between F and M genomes is comparable to the distance between mtDNAs of different classes of Vertebrates, primers may fail to amplify one of the two mtDNAs, yielding a false-negative result. Moreover, M-type mtDNA can be rare in somatic tissues, so it may be difficult to amplify from animals sampled outside of the reproductive

season, when gonads are absent (thoroughly discussed in Theologidis et al., 2008). High-throughput sequencing (HTS) approaches can overcome such problems, because a prior knowledge of the mtDNA sequence is not needed, and low-copy variants can be easily unveiled. Until now, HTS has been scarcely utilized to study mitochondrial transcriptomes and genomes (Pesole et al., 2012; Smith, 2013), even if it showed very good potential (Lubośny et al., 2017/2; see for example Yuan et al., 2016). In this work we: *i)* assembled *R. decussatus* mitochondrial genome from RNA-Seq data, and validated it by Sanger sequencing, *ii)* analyzed and characterized *R. decussatus* mitochondrial genome, comparing its features with those of other venerid bivalves; *iii)* assessed mitochondrial genetic polymorphism among the sampled animals, both from a nucleotide (SNPs) and a structural (CNV of tandem repeats) point of view.

Materials & Methods

Sampling

The 26 *Ruditapes decussatus* specimens used in this study were collected from the Northern Adriatic Sea (Goro) during the spawning season. Each individual was sexed, frozen in liquid nitrogen, and stored at -80ºC. Supplementary Table 1 shows the sample list, and details about data availability.

RNA-Seq

12 samples (6 males and 6 females) were used for RNA-Seq. Total RNA extraction and library preparation were performed following the protocol described in Mortazavi et al. (2008), with the modifications specified in Ghiselli et al. (2012). The 12 samples were indexed, pooled and

153 sequenced in two lanes (two technical replicates) of Illumina GA IIx, using 76bp paired-end
154 reads.
155
156 *De Novo Assembly*
157 The mitochondrial genome of *R. decussatus* was not available in the databases, so we
158 used the transcriptome data to generate a draft to be used as a guide for Sanger sequencing. The
159 mtDNA *de novo* assembly followed this pipeline: *i)* the reads of the 12 individuals of *R.*
160 *decussatus* were combined; *ii)* an alignment against 20 complete mitochondrial genomes of
161 Veneridae species (retrieved from GenBank) was performed using BLASTN, with cutoff E-
162 value ≤1E-5; *iii)* the aligned reads were assembled using the A5 pipeline (Andrew And Aaron's
163 Awesome Assembly pipeline, Tritt et al., 2012); *iv)* the obtained contigs were joined into
164 scaffolds using CAP3 (Huang & Madan, 1999). A5 is an assembly pipeline that reduces the
165 entire genome assembly process by automating stage (data cleaning, error correction, assembly,
166 and quality control). A5 can produce quality assemblies without any prior knowledge of the
167 particular genome being assembled and without the extensive parameter tuning required by the
168 other assembly algorithms. A5 can operate directly on FASTQ format data generated by illumina
169 sequencing. CAP3 clips 5' and 3' low-quality regions of reads, and uses base quality values in
170 computation of overlaps between reads, construction of multiple sequence alignments of reads,
171 and generation of consensus sequences. The program also uses forward-reverse constraints to
172 correct assembly errors and link contigs.
173

Sanger Validation

14 *R. decussatus* samples from the same collection campaign—sexed, and stored at -80°C—were used for DNA extraction. DNA from the gonadic tissue was extracted using the Qiagen DNeasy kit. Primers for mtDNA amplification were designed based on contigs obtained from RNA-Seq matching venerid mtDNA sequences, then the "primer walking" method was used to Sanger-sequence the complete mitochondrial genome of *R. decussatus*. The primers were designed with the software Primer3 (Rozen & Skaletsky, 2000) and tested on several samples, then a female (F4) was chosen as reference sample for Sanger validation of mtDNA *de novo* assembly. In addition, we amplified the Largest Unassigned Region (LUR) of 13 females to assess its variability (see Results and Discussion). The list of the primers and their sequences are reported in Supplementary Table 2. PCR reactions were performed in a final volume of 50µl using the GoTaq Flexi DNA Polymerase Kit (Promega), on a 2720 Thermal Cycler (Applied Biosystem). The PCR reactions were set as follows: initial denaturation 95°C for 1 min, then 30 cycles of amplification (denaturation 95°C for 1 min, annealing 48- 60°C for 1 min, extension 72°C for 1 min/kb), then the final extension at 72°C for 5 min. PCR products were checked by electrophoretic run on 1% agarose gel, and then purified using the DNA Clean & Concentrator-25 kit (Zymo Research).

Sanger sequencing was performed by Macrogen Inc. (http://www.macrogen.com). Sequences were aligned with the software MEGA 6.0 (Tamura et al., 2013), using the contigs obtained by RNA-seq as a reference.

Annotation

Open Reading Frames (ORFs) were identified with ORF finder (Wheeler et al., 2005). Alternative start codons were also used because they are common in Bivalvia. ORFs were annotated starting from the first available start codon (ATG, ATA or ATC) downstream of the preceding gene, and ending with the first stop codon in frame (TAA or TAG). tRNA genes and their structure were identified with MITOS (Bernt et al., 2013) and ARWEN (Laslett & Canback, 2008). Secondary structures were predicted using the RNAFold Server, included in the ViennaRNA Web Services (http://rna.tbi.univie.ac.at/; Gruber et al., 2008); the folding temperature was set at 16°C which is the average annual temperature of the water from which the *R. decussatus* specimens used in this work were fished (results available on figshare: https://doi.org/10.6084/m9.figshare.4970762.v1). tRNAs and other secondary structures were drawn with the software Varna GUI (Darty, Denise & Ponty, 2009). Ribosomal small subunit (*rrnS*) and large subunit (*rrnL*) were identified with BLASTN, and annotated considering the start and the end of the adjacent genes as the boundaries of the rRNA genes. Non-genic regions were annotated as Unassigned Regions (URs). In order to identify the putative D-loop/control region (CR), we analyzed the LUR with the MEME suite (Bailey et al., 2009) to find DNA motifs using the following bivalve species as comparison: *Acanthocardia tuberculata*, *Arctica islandica*, *Coelomactra antiquata*, *Fulvia mutica*, *Hiatella arctica*, *Loripes lacteus, Lucinella divaricata, Lutraria rhynchaena, Meretrix lamarckii* (F-type), *Meretrix lamarckii* (M-type), *Meretrix lusoria, Meretrix lyrata, Meretrix meretrix, Meretrix petechialis, Moerella iridescens, Nuttallia olivacea, Paphia amabilis, Paphia euglypta, Paphia textile, Paphia undulata, Ruditapes philippinarum* (F-type), *Ruditapes philippinarum* (M-type*), Semele scabra, Sinonovacula constricta, Solecurtus divaricatus, Solen grandis, Solen strictus, Soletellina diphos*

and the sea urchin *Strongylocentrotus purpuratus* (Echinoidea, Strongylocentrotidae). The list of the species used in the phylogenetic analysis and in the comparative analyses of DNA motifs, sequence similarity, and gene order are available in Supplementary Table 3. The GOMo (Gene Ontology for Motifs; Buske et al., 2010) tool of the MEME suite was used to assign GO terms to the found motifs.

The number of repeats in the LUR of the reference sample (F4) was calculated with tandem repeat finder (http://tandem.bu.edu/trf/trf.html), since the complete LUR sequence was available (results available on figshare: https://doi.org/10.6084/m9.figshare.4970762.v1). In the other cases, in which the LUR could not be sequenced without gaps, the number of repeats was inferred from agarose gel electrophoresis.

Other Analyses

Comparisons among venerid complete mtDNAs were performed with BLAST Ring Image Generator (BRIG, Alikhan et al., 2011) and Easyfig (Sullivan, Petty & Beatson, 2011). Descriptive statistics were obtained with MEGA v6.0 (Tamura et al., 2013), except for the codon usage table, which was obtained with the Sequence Manipulation Suite (Stothard, 2000). SNP calling was performed with the Genome Analysis Toolkit (GATK, McKenna et al., 2010), with the Sanger-sequenced mtDNA as reference. For SNP/INDEL discovery and genotyping we used standard hard filtering parameters or variant quality score recalibration (DePristo et al., 2011). The MitoPhast pipeline (Tan et al., 2015) was used to obtain the Maximum Likelihood (ML) tree, which was visualized with Evolview v2 (He et al., 2016). Briefly, MitoPhast takes as input GenBank files (.gb), extracts the coding sequences, profiles the sequences with Pfam (Finn et al., 2016) and PRINTS (Attwood et al., 2003), performs a multiple sequence alignment with Clustal

241 Omega (Sievers et al., 2011), removes poorly aligned regions with trimAl (Capella-Gutiérrez,
242 Silla-Mart'inez & Gabaldón, 2009), concatenates the coding sequences, performs data
243 partitioning and model selection, and then carries out a ML analysis using RAxML (Stamatakis,
244 2014). The species used in the ML analysis, and their GenBank Accession Numbers are listed in
245 Supplementary Table 3.
246
247 **Results**
248 The RNA-Seq of 12 *R. decussatus* mature gonads (6 males and 6 females) has not retrieved sex-
249 linked mitochondrial haplotypes.
250 The mitochondrial genome contains 13 protein-coding genes, and in the reference female is
251 18,995 bp long (Figure 1); the gene arrangement and other details are shown in Table 1. All
252 genes are located on the heavy strand, and in addition to the classic start codon ATG (Met), the
253 alternative start codons ATA (Met) and ATC (Ile) are present. The most frequently used start
254 codons are: ATA (*cox1, nd1, nd4L, cox2, cob, atp8, nd4*), and ATG (*nd2, atp6, nd3, nd5, nd6,*
255 *cox3*). The stop codons found are TAG (*cox1, nd2, nd4L, cox2, cytb, nd4*) and TAA (*nd1, atp6,*
256 *nd3, atp8, nd6*). The *nd4* gene has an incomplete stop codon (TA-). 22 tRNA genes were
257 identified, including two tRNAs for leucine, tRNA-Leu1(TAG) and tRNA-Leu2(TAA), and two
258 for serine, tRNA-Ser1(TCT) and tRNA-Ser2(TGA), both showing a degenerate D-arm branch.
259 tRNA structures are shown in Supplementary Figure 1. The two rRNAs, *rrnS* and *rrnL*, were
260 both identified: the *rrnS* is located between *cox3* and *cox1*, while *rrnL* is between *cytb* and *atp6*.
261 Unassigned Regions (URs) were identified on the basis of unannotated spaces between different
262 genes; we found 24 URs (Table 2).

263 The analysis of the nucleotide composition points out that the mitochondrial genome of this
264 bivalve species presents a high A+T content, totalling 63% versus 37% of G+C. The minimum
265 values of A+T are found in *cytb* (60.1%) and *nd4* (61%). The nucleotide composition of every
266 gene is shown in Table 3. According to the analysis above, both A and T occur very frequently at
267 the third base of the codons (64.6% on average of A+T), while the less frequent base in third
268 position is C (12%). The most used codons are UUU (Phe), counted 269 times, and UUA (Leu)
269 counted 210 times (6.78% and 5.29% of the total, respectively), while the less used codons are
270 CGC (Arg) counted 6 times (0.15%), ACC (Thr) and CCG (Pro) each counted 16 times (0.4%)
271 (Table 4). Only in 4 cases over 20 (Lys, Leu, Gln, Val), the most frequently used codon matches
272 the correspondent mitochondrial tRNA anticodon.
273 The UR11 is the Largest Unassigned Region (LUR) and is located between *atp8* and *nd5*
274 (Figures 1 and 2A). The LUR of the female used for whole mtDNA Sanger sequencing (i.e. the
275 reference female, F4) is 2,110 bp long, and includes 6.5 repeated sequences—each repeat having
276 a length of 54 bp—localized in the 3' region of the LUR, just upstream the *atp8* gene (Figure
277 2A). DNA secondary structure analysis predicted 3 stem-loop structures in such region (Figure
278 2B and supplementary files on figshare: https://doi.org/10.6084/m9.figshare.4970762.v1), with a
279 change in Gibbs free energy (ΔG) of -71.38 Kcal/mol. We also amplified and sequenced the
280 LUR of 13 more females. We were not able to completely sequence LURs longer than 2,110 bp,
281 because of the known difficulties in Sanger sequencing of regions including multiple repeats.
282 The sequence alignment of the 13 LURs is available for download from figshare
283 (https://doi.org/10.6084/m9.figshare.4970762.v1). LUR lengths, inferred from gel
284 electrophoresis, are reported in Table 5, and they ranges from 2,000 to 5,000 bp. Two females
285 (F3 and F17) showed length heteroplasmy of the LUR. The portion of the genome occupied by

286 URs varies between 14.11% and 29.38%, depending on LUR length. The analysis with MEME
287 (output shown in Supplementary Figures 3 and 4) unveiled two motifs (Figure 2C) that show a
288 strong conservation within the Veneridae family, and with *S. purpuratus*. The sea urchin was
289 included in the analysis because Cao et al. (2004) reported a match between some motifs found
290 in the CR of the marine mussels *Mytilus edulis* and *Mytilus galloprovincialis* with regulatory
291 elements of the sea urchin CR. Accordingly, the search with GOMo assigned a series of GO
292 terms related to transcription to the two motifs (Table 6).
293 Table 7 shows the statistics associated with the SNP calling performed with GATK on the 12
294 samples used for RNA-Seq, with the Sanger-sequenced mtDNA as reference. Overall, 257 SNPs
295 were called, of which 145 (56.4%) are located in CDS. Interestingly, most of the SNPs (103 over
296 145, that is, 71% of the SNPs in CDS) were called because of private alleles of one single male
297 specimen (mRDI01). If we exclude the SNPs associated with this male, the genetic
298 polymorphism drops to 42 SNPs over 14,920 bp of coding mtDNA (GATK output in VCF
299 format and a detailed list of SNPs in tabular format is available on figshare:
300 https://doi.org/10.6084/m9.figshare.4970762.v1). 18 SNPs are indels, 6 of which are located in 4
301 different coding genes: one each in *cox1*, *cytb*, and *nd5*, plus 3 in *cox3* (see Table 8). A file
302 showing the ORF generated by the different variants of *cox3*, and alignments between them is
303 available on figshare (https://doi.org/10.6084/m9.figshare.4970762.v1).
304 Figure 3 shows the *R. decussatus* mtDNA map (external gray circle), and the BLASTN identity
305 (colored inner circles) with complete mtDNAs of other 10 venerid species (see list in
306 Supplementary Table 3). Figure 4 shows the ML tree obtained with the MitoPhast pipeline; the
307 complete input and output of this analysis is available on figshare
308 (https://doi.org/10.6084/m9.figshare.4970762.v1). Figure 5 shows the variation in gene order

between *R. decussatus* and *P. euglypta* (Figure 5A), *M. lamarckii* F-type (Figure 5B), *R. philippinarum* F-type (Figure 5C), and among all the 4 species (Figure 5D).

Discussion

The size of the fully Sanger-sequenced mitochondrial genome of *R. decussatus* (reference female F4) is of 18,995 bp, and it includes 13 protein-coding genes, 22 tRNAs and 2 rRNAs. Our data support the presence of the *atp8* gene in the mtDNA of *R. decussatus; atp8* has been reported as missing in several bivalve species, however more accurate searches often led to the identification of the gene, so, in most cases, the alleged lack of *atp8* is likely ascribable to annotation inaccuracies due to the extreme variability and the small size of the gene (Breton, Stewart & Hoeh, 2010; Breton et al., 2014).

The mitochondrial genome of *R. decussatus* shows a high content of A-T (63%), a common feature in bivalve mtDNAs; moreover, T is the most present nucleotide at the third codon base (64.6%). The most used codon is UUU (Phe), which is also the most commonly used in bivalves, as well as in other invertebrates (Passamonti et al., 2011).

As shown in Table 4, in 16 cases over 20 the most frequently used codon do not correspond to the anticodon of the relative tRNA produced by mtDNA. In other words, there is not a correspondence between the most representative codons and anticodons of the 22 mitochondrial tRNAs. According to the "wobble hypothesis"—first proposed by Francis Crick (1966)— the conformation of the tRNA anticodon loop enables some flexibility at the first base of the anticodon, so a Watson-Crick type of base pairing in the third position of the codon is not strictly necessary. This allows an amino acid to be correctly incorporated by ribosomes even if the tRNA is not rigidly complementary to the codon; according to Crick, this explains the degeneracy of the genetic code. This feature is particularly interesting in the light of the debate on natural selection acting at synonymous sites: since the early 1980s, evidence about a correlation between synonymous codon usage and tRNA abundances started accumulating. According to several

works, synonymous codon usage is biased to match skews in tRNA abundance, as a result of the selective pressure for a maximized protein synthesis rate (reviewed in Chamary, Parmley & Hurst, 2006). Following this rationale, the results here reported and data from other metazoans (see Passamonti et al., 2011 and references therein) would entail that in some mitochondrial genomes the translation efficiency is not maximized, and this observation deserves further investigation.

The mtDNA of *R. decussatus* has a high proportion of URs mostly depending on the length of the LUR (Table 5); on average, bivalve mtDNAs have 1.7x the amount of URs in respect to other analyzed Metazoa (Ghiselli et al., 2013), and it is still unclear whether there is an accumulation of non-functional sequences in bivalve mtDNA due to genetic drift, or if such URs are maintained by natural selection because they contain—so far unknown—functional elements (see for example Milani et al., 2013, 2014b; Breton et al., 2014; Pozzi et al., 2017). The LUR of *R. decussatus* most likely includes the mitochondrial CR, as indicated by the presence of two motifs (Figure 2C, Supplementary Figures 3 and 4) similar to two regulatory elements identified in the sea urchin CR. These two motifs are the same identified in previous analyses on the clam *R. philippinarum* and the mussel *Musculista senhousia* (Ghiselli et al., 2013; Guerra, Ghiselli & Passamonti, 2014) so they are conserved across distant bivalve taxa, and the GO terms associated with such motifs are related to transcriptional control (Table 6). An interesting feature of *R. decussatus* LUR is its variable length (Table 2), most likely due to different repeat content. As a matter of fact, the very same repeats were present in every sequenced LUR, and our data strongly suggest that LUR length variation is actually due to repeat CNV (see supplementary files on figshare: https://doi.org/10.6084/m9.figshare.4970762.v1), as observed in other bivalve species (see for example Ghiselli et al., 2013; Guerra, Ghiselli & Passamonti, 2014). Tandem repeats

have been also reported in the mitochondrial genomes of the bivalves *Acanthocardia tuberculata (Dreyer & Steiner, 2006)*, *Placopecten magellanicus (La Roche et al., 1990)*, *Moerella iridescens*, *Sanguinolaria olivacea*, *Semele scaba*, *Sinonovacula constricta*, *Solecurtus divaricatus* (Yuan et al., 2012), *Ruditapes philippinarum (Ghiselli et al., 2013)*, and *Musculista senhousia* (Guerra, Ghiselli & Passamonti, 2014). These repeats are believed to arise from duplications caused by replication slippage (Buroker et al., 1990; Hayasaka, Ishida & Horai, 1991; Broughton & Dowling, 1994). The tandem repeats found at the 3' end of *R. decussatus* LUR are predicted to form a secondary structure (see Figure 2B, and supplementary files on figshare) composed by multiple stem-loops, which obviously increase in number with the increment of the number of tandem repeats. The effect, if any, of tandem repeats in mtDNA is unknown: since the repeats are almost always localized in proximity of the CR, they might interact with regulatory elements—or even contain some—influencing replication and/or transcription initiation, and such interactions might also be altered by the formation of secondary structures (Passamonti et al., 2011; Ghiselli et al., 2013; Guerra, Ghiselli & Passamonti, 2014). We assessed the genetic variability of *R. decussatus* mtDNA using two different approaches: by SNP calling in CDS (RNA-Seq data on 12 individuals), and by analysis of the LUR (Sanger sequencing of 14 individuals). The CR and its flanking regions are known to be hypervariable, so they are commonly used to assess polymorphism at low taxonomic levels. Our data strongly support a very low genetic variability: the number of SNPs in CDS is 145, of which 103 are private of a single individual (mRDI01)—thus reducing the number to 42—while the number of variable sites in the analyzed LURs is 98 over 3,095 aligned positions. Considering the known variability of mtDNA in bivalves (Gissi, Iannelli & Pesole, 2008; Ghiselli et al., 2013; Breton et al., 2014; Plazzi, Puccio & Passamonti, 2016), this is a surprising result. Even more if we

compare the results of the present work to a methodologically identical analysis performed on 12 *R. philippinarum* samples from the Pacific coast of USA, performed by Ghiselli et al. (2013): in that work, GATK yielded 194 SNPs in the M-type mtDNA and 293 in the F-type. Strikingly, the 12 *R. philippinarum* samples analyzed were actually two families (6 siblings + 6 siblings). This means that randomly sampled individuals of *R. decussatus* used in this work showed a much lower mtDNA variability than *R. philippinarum* siblings. A previous analysis on the *cox1* gene of *R. decussatus* reported a nucleotide diversity (π) of 0.15 for a population from the Northern Adriatic Sea (Cordero, Peña & Saavedra, 2014). Another analysis on the same gene of *R. philippinarum* from the same range resulted in a π=0.25 (Cordero et al., 2017), so *R. decussatus* has a lower nucleotide diversity at the *cox1* locus. The difference between the variability in mtDNA of *R. decussatus* that we are reporting here and that of *R. philippinarum* reported in Ghiselli et al. (2013) appear to be more marked. It is known that the genetic variability of *R. philippinarum* in the Adriatic Sea is lower than in populations from its native range in Asia (Cordero et al., 2017), probably because of the bottlenecks that this species had to go through during the multiple colonization events. The introduction in North America from Asia happened first (in the 1930s), and from there the Manila clam was introduced in Northern Europe (in the 1970s and 1980s), and lastly in the Adriatic Sea (1983 and 1984), and it is plausible that the genetic diversity decreased at each introduction event. Accordingly, Cordero et al. (2017) observed that *R. philippinarum* genetic variability in Europe is lower compared to that of the Pacific coast of the USA, so the samples analyzed in Ghiselli et al. (2013) could have been more polymorphic than those analyzed in Cordero et al. (2014), thus explaining the more pronounced differences in genetic variability between the Manila clam and the European clam discussed above. In any case, all the available data point to a lower genetic diversity of *R. decussatus*

mtDNA, and it would be interesting to know whether it is a cause or an effect of the ongoing replacement of *R. decussatus* with the invasive *R. philippinarum*. It will also be important to investigate genetic variability of the nuclear genes, especially after Cordero et al. (2014) reported contrasting levels of differentiation between mitochondrial and nuclear markers.

With respect to SNP effects, we found 6 indels in CDS, 2 of which do not cause frameshift, but a simple insertion/deletion of 1 amino acid (SNP_1698, and SNP_17619, see Table 8). Of the remaining 4, SNP_6364 and SNP_10449 consist of a deletion and an insertion of a single T in two homopolymeric sequences (CTTTTTTT and CTTTTTT, respectively), raising the suspect of a sequencing error. In any case, the two SNPs yield a shorter CDS (*cytb* and *nd5*, respectively), and are present at relatively low frequencies in the specimens carrying them, except for SNP_6364 which has a frequency of 80% in fRDI04. The *cox3* gene shows 3 SNPs: the first one, SNP_17619, does not cause frameshift, results in the deletion of 1 alanine residue, and its frequency in mRDI01 is 97%. The second one, SNP_17621, consists of a deletion of a G in respect to the reference sequence, which is the Sanger-sequenced mtDNA of sample F4; all the individuals analyzed with RNA-Seq carry this deletion except for mRDI01 which, at that position, has the same sequence of the reference mtDNA (reference-like allele frequency in mRDI01 = 99%). The third indel, SNP_17624, consists of an insertion of two nucleotides, and its frequency in mRDI01 is 99%. So, basically, for *cox3* we have three types of sequences: *i)* the Sanger-sequenced reference, which yields a 966 bp (321 aa) ORF; *ii)* a sequence found in 11/12 of samples analyzed with RNA-Seq (except mRDI01) that carries a single-nucleotide deletion (SNP_17621), and yields a 963 bp (320 aa) ORF; *iii)* a sequence, private of mRDI01, which is obtained by combining SNP_17624 and SNP_17621 (both 99% of frequency, so most likely co-occurring), which produces a 963 bp (320 aa) ORF. Interestingly, the ORFs obtained from the

sequences described in *ii)* and *iii)*, are almost identical, namely the sequence obtained by RNA-seq in 11/12 samples and the sequence obtained by RNA-Seq in mRDI01 are basically the same, and differ from the Sanger-sequenced reference, yielding an amino acid sequence that differs in the last 35 residues (all data available in supplementary files on figshare: https://doi.org/10.6084/m9.figshare.4970762.v1). Given this consisten difference between the sequence obtained by Sanger-sequencing of DNA, and those obtained by RNA-Seq, it is tempting to speculate that this difference might be caused by RNA editing, a mechanism observed in mtDNA of some animals (Lavrov & Pett, 2016), and recently reported to be widely used in cephalopod (Liscovitch-Brauer et al., 2017). Post-transcriptional modifications (thus including RNA-editing) are still poorly understood mechanisms, but they appear to be responsible for most of the mitochondrial gene expression regulation (Scheibye-Alsing et al., 2007; Scheffler, 2008; Milani et al., 2014a).

Interestingly, in contrast with a low nucleotide variability along the entire mitochondrial genome, we observed a pretty high polymorphism in LUR length due to CNV of tandem repeats, and even a LUR length heteroplasmy: two females yielded two electrophoretic bands each (~2,100 and ~3,500 bp in F3; ~2,500 and ~3,500 bp in F17; see Table 5).

A possible explanation is that the diversity (CNV) detected in the LURs could be recent: the accumulation of nucleotide variation at different sites along the mitochondrial genome needs time, while the kind structural variability we observed can be achieved in few generations (or even one) considering that replication slippage is common in repeat-rich regions.

Despite *R. decussatus* and *R. philippinarum* being morphologically similar and being ascribed to the same genus, the results here reported clearly show that they are quite different both for mtDNA sequence (Figures 3 and 4) and mtDNA gene arrangement (Figure 5). This is an unusual

finding, even among bivalves, which are known to be fast-evolving for this characters. This may point to the fact that these two species are less related than previously thought. Actually, this is not the first clue that *R. decussatus* and *R. philippinarum* are quite different genetically, as allozyme electrophoresis (Passamonti, Mantovani & Scali, 1997, 1999) and satellite DNA content (Passamonti, Mantovani & Scali, 1998) pointed out. More in depth analyses are therefore needed to correctly trace the phylogenetic relationships of these two Ruditapes species, which may eventually end up in two different Genera. As shown in Figures 3, 4 and 5, the Genus Paphia is the most similar to *R. decussatus*. Finally, even using a HTS approach we could not find evidence for sex-specific mtDNAs, so our data appear to confirm that *R. decussatus* does not have DUI. Among Veneridae, only 3 species have been found with DUI, so far: *Cyclina sinensis*, *R. philippinarum*, and *Meretrix lamarckii* (Plazzi, Cassano & Passamonti, 2015; Gusman et al., 2016). The status of Paphia is still unknown, and in future works it would be interesting to investigate this Genus as well as other Heterodonta to understand better the distribution of DUI in this derived group of bivalves.

References

Alikhan NF., Petty NK., Ben Zakour NL., Beatson SA. 2011. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC genomics* 12:402.

Attwood TK., Bradley P., Flower DR., Gaulton A., Maudling N., Mitchell AL., Moulton G., Nordle A., Paine K., Taylor P., Uddin A., Zygouri C. 2003. PRINTS and its automatic supplement, prePRINTS. *Nucleic acids research* 31:400–402.

Bailey TL., Boden M., Buske FA., Frith M., Grant CE., Clementi L., Ren J., Li WW., Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic acids research* 37:W202–8.

Bernt M., Donath A., Jühling F., Externbrink F., Florentz C., Fritzsch G., Pütz J., Middendorf M., Stadler PF. 2013. MITOS: improved de novo metazoan mitochondrial genome annotation. *Molecular phylogenetics and evolution* 69:313–319.

Breton S., Milani L., Ghiselli F., Guerra D., Stewart DT., Passamonti M. 2014. A resourceful genome: updating the functional repertoire and evolutionary role of animal mitochondrial DNAs. *Trends in genetics: TIG* 30:555–564.

Breton S., Stewart DT., Hoeh WR. 2010. Characterization of a mitochondrial ORF from the gender-associated mtDNAs of *Mytilus spp*. (Bivalvia: Mytilidae): identification of the "missing" ATPase 8 gene. *Marine genomics* 3:11–18.

Breton S., Stewart DT., Shepardson S., Trdan RJ., Bogan AE., Chapman EG., Ruminas AJ., Piontkivska H., Hoeh WR. 2011. Novel protein genes in animal mtDNA: a new sex determination system in freshwater mussels (Bivalvia: Unionoida)? *Molecular biology and evolution* 28:1645–1659.

Broughton RE., Dowling TE. 1994. Length variation in mitochondrial DNA of the minnow

493 *Cyprinella spiloptera*. *Genetics* 138:179–190.
494 Buroker NE., Brown JR., Gilbert TA., O'Hara PJ., Beckenbach AT., Thomas WK., Smith MJ.
495 1990. Length heteroplasmy of sturgeon mitochondrial DNA: an illegitimate elongation
496 model. *Genetics* 124:157–163.
497 Buske FA., Bodén M., Bauer DC., Bailey TL. 2010. Assigning roles to DNA regulatory motifs
498 using comparative genomics. *Bioinformatics* 26:860–866.
499 Cao L., Kenchington E., Zouros E., Rodakis GC. 2004. Evidence that the large noncoding
500 sequence is the main control region of maternally and paternally transmitted mitochondrial
501 genomes of the marine mussel (*Mytilus spp*.). *Genetics* 167:835–850.
502 Capella-Gutiérrez S., Silla-Martinez JM., Gabaldón T. 2009. trimAl: a tool for automated
503 alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25:1972–1973.
504 Chamary JV., Parmley JL., Hurst LD. 2006. Hearing silence: non-neutral evolution at
505 synonymous sites in mammals. *Nature reviews. Genetics* 7:98–108.
506 Cordero D., Delgado M., Liu B., Ruesink J., Saavedra C. 2017. Population genetics of the
507 Manila clam (*Ruditapes philippinarum*) introduced in North America and Europe. *Scientific*
508 *reports* 7:39745.
509 Cordero D., Peña JB., Saavedra C. 2014. Phylogeographic analysis of introns and mitochondrial
510 DNA in the clam *Ruditapes decussatus* uncovers the effects of Pleistocene glaciations and
511 endogenous barriers to gene flow. *Molecular phylogenetics and evolution* 71:274–287.
512 Crick FHC. 1966. Codon—anticodon pairing: The wobble hypothesis. *Journal of molecular*
513 *biology* 19:548–555.
514 Darty K., Denise A., Ponty Y. 2009. VARNA: Interactive drawing and editing of the RNA
515 secondary structure. *Bioinformatics* 25:1974–1975.

516 DePristo MA., Banks E., Poplin R., Garimella KV., Maguire JR., Hartl C., Philippakis AA., del
517 Angel G., Rivas MA., Hanna M., McKenna A., Fennell TJ., Kernytsky AM., Sivachenko
518 AY., Cibulskis K., Gabriel SB., Altshuler D., Daly MJ. 2011. A framework for variation
519 discovery and genotyping using next-generation DNA sequencing data. *Nature genetics*
520 43:491–498.
521 Diz AP., Dudley E., Skibinski DOF. 2012. Identification and characterisation of highly
522 expressed proteins in sperm cells of the marine mussel *Mytilus edulis*. *Proteomics*. DOI:
523 10.1002/pmic.201100500.
524 Dreyer H., Steiner G. 2006. The complete sequences and gene organisation of the mitochondrial
525 genomes of the heterodont bivalves *Acanthocardia tuberculata* and *Hiatella arctica*—and
526 the first record for a putative Atpase subunit 8 gene in marine bivalves. *Frontiers in zoology*
527 3:13.
528 Finn RD., Coggill P., Eberhardt RY., Eddy SR., Mistry J., Mitchell AL., Potter SC., Punta M.,
529 Qureshi M., Sangrador-Vegas A., Salazar GA., Tate J., Bateman A. 2016. The Pfam protein
530 families database: towards a more sustainable future. *Nucleic acids research* 44:D279–85.
531 Ghiselli F., Milani L., Chang PL., Hedgecock D., Davis JP., Nuzhdin SV., Passamonti M. 2012.
532 De Novo assembly of the Manila clam *Ruditapes philippinarum* transcriptome provides
533 new insights into expression bias, mitochondrial doubly uniparental inheritance and sex
534 determination. *Molecular biology and evolution* 29:771–786.
535 Ghiselli F., Milani L., Guerra D., Chang PL., Breton S., Nuzhdin SV., Passamonti M. 2013.
536 Structure, transcription, and variability of metazoan mitochondrial genome: perspectives
537 from an unusual mitochondrial inheritance system. *Genome biology and evolution* 5:1535–
538 1554.

539 Ghiselli F., Milani L., Passamonti M. 2011. Strict sex-specific mtDNA segregation in the germ
540 line of the DUI species *Venerupis philippinarum* (Bivalvia: Veneridae). *Molecular biology*
541 *and evolution* 28:949–961.
542 Gissi C., Iannelli F., Pesole G. 2008. Evolution of the mitochondrial genome of Metazoa as
543 exemplified by comparison of congeneric species. *Heredity* 101:301–320.
544 Gosling EM. 2003. *Bivalve molluscs*. Wiley Online Library.
545 Gruber AR., Lorenz R., Bernhart SH., Neuböck R., Hofacker IL. 2008. The Vienna RNA
546 websuite. *Nucleic acids research* 36:W70–4.
547 Guerra D., Ghiselli F., Passamonti M. 2014. The largest unassigned regions of the male- and
548 female-transmitted mitochondrial DNAs in *Musculista senhousia* (Bivalvia Mytilidae).
549 *Gene* 536:316–325.
550 Gusman A., Lecomte S., Stewart DT., Passamonti M., Breton S. 2016. Pursuing the quest for
551 better understanding the taxonomic distribution of the system of doubly uniparental
552 inheritance of mtDNA. *PeerJ* 4:e2760.
553 Hayasaka K., Ishida T., Horai S. 1991. Heteroplasmy and polymorphism in the major noncoding
554 region of mitochondrial DNA in Japanese monkeys: association with tandemly repeated
555 sequences. *Molecular biology and evolution* 8:399–415.
556 He Z., Zhang H., Gao S., Lercher MJ., Chen W-H., Hu S. 2016. Evolview v2: an online
557 visualization and management tool for customized and annotated phylogenetic trees.
558 *Nucleic acids research* 44:W236–41.
559 Huang X., Madan A. 1999. CAP3: A DNA sequence assembly program. *Genome research*
560 9:868–877.
561 La Roche J., Snyder M., Cook DI., Fuller K., Zouros E. 1990. Molecular characterization of a

562 repeat element causing large-scale size variation in the mitochondrial DNA of the sea
563 scallop *Placopecten magellanicus*. *Molecular biology and evolution* 7:45–64.
564 Laslett D., Canback B. 2008. ARWEN: a program to detect tRNA genes in metazoan
565 mitochondrial nucleotide sequences. *Bioinformatics* 24:172–175.
566 Lavrov DV., Pett W. 2016. Animal Mitochondrial DNA as We Do Not Know It: mt-Genome
567 Organization and Evolution in Nonbilaterian Lineages. *Genome biology and evolution*
568 8:2896–2913.
569 Leite RB., Milan M., Coppe A., Bortoluzzi S., dos Anjos A., Reinhardt R., Saavedra C.,
570 Patarnello T., Cancela ML., Bargelloni L. 2013. mRNA-Seq and microarray development
571 for the Grooved Carpet shell clam, *Ruditapes decussatus*: a functional approach to unravel
572 host-parasite interaction. *BMC genomics* 14:741.
573 Liscovitch-Brauer N., Alon S., Porath HT., Elstein B., Unger R., Ziv T., Admon A., Levanon
574 EY., Rosenthal JJC., Eisenberg E. 2017. Trade-off between Transcriptome Plasticity and
575 Genome Evolution in Cephalopods. *Cell* 169:191–202.e11.
576 Lubośny M., Przyłucka A., Sańko TJ., Śmietanka B., Rosenfeld S., Burzyński A. 2017/2. Next
577 generation sequencing of gonadal transcriptome suggests standard maternal inheritance of
578 mitochondrial DNA in *Eurhomalea rufa* (Veneridae). *Marine genomics* 31:21–23.
579 McKenna A., Hanna M., Banks E., Sivachenko A., Cibulskis K., Kernytsky A., Garimella K.,
580 Altshuler D., Gabriel S., Daly M., DePristo MA. 2010. The Genome Analysis Toolkit: a
581 MapReduce framework for analyzing next-generation DNA sequencing data. *Genome*
582 *research* 20:1297–1303.
583 Milani L., Ghiselli F. 2015. Mitochondrial activity in gametes and transmission of viable
584 mtDNA. *Biology direct* 10:22.

585 Milani L., Ghiselli F., Guerra D., Breton S., Passamonti M. 2013. A comparative analysis of
586 mitochondrial ORFans: new clues on their origin and role in species with doubly
587 uniparental inheritance of mitochondria. *Genome biology and evolution* 5:1408–1434.
588 Milani L., Ghiselli F., Iannello M., Passamonti M. 2014a. Evidence for somatic transcription of
589 male-transmitted mitochondrial genome in the DUI species *Ruditapes philippinarum*
590 (Bivalvia: Veneridae). *Current genetics* 60:163–173.
591 Milani L., Ghiselli F., Maurizii MG., Nuzhdin SV., Passamonti M. 2014b. Paternally transmitted
592 mitochondria express a new gene of potential viral origin. *Genome biology and evolution*
593 6:391–405.
594 Milani L., Ghiselli F., Passamonti M. 2016. Mitochondrial selfish elements and the evolution of
595 biological novelties. *Current zoology* 62:687–697.
596 Milbury CA., Lee JC., Cannone JJ., Gaffney PM., Gutell RR. 2010. Fragmentation of the large
597 subunit ribosomal RNA gene in oyster mitochondrial genomes. *BMC genomics* 11:485.
598 Mortazavi A., Williams BA., McCue K., Schaeffer L., Wold B. 2008. Mapping and quantifying
599 mammalian transcriptomes by RNA-Seq. *Nature methods* 5:621–628.
600 Passamonti M., Ghiselli F. 2009. Doubly uniparental inheritance: two mitochondrial genomes,
601 one precious model for organelle DNA inheritance and evolution. *DNA and cell biology*
602 28:79–89.
603 Passamonti M., Mantovani B., Scali V. 1997. Allozymic characterization and genetic
604 relationships among four species of Tapetinae (Bivalvia, Veneridae). *Italian journal of*
605 *zoology* 64:117–124.
606 Passamonti M., Mantovani B., Scali V. 1998. Characterization of a highly repeated DNA family
607 in tapetinae species (mollusca bivalvia: veneridae). *Zoological science* 15:599–605.

608 Passamonti M., Mantovani B., Scali V. 1999. Allozymic analysis of some Mediterranean
609 Veneridae (Mollusca: Bivalvia): preliminary notes on taxonomy and systematics of the
610 family. *Journal of the Marine Biological Association of the United Kingdom. Marine*
611 *Biological Association of the United Kingdom* 79:899–906.
612 Passamonti M., Ricci A., Milani L., Ghiselli F. 2011. Mitochondrial genomes and Doubly
613 Uniparental Inheritance: new insights from *Musculista senhousia* sex-linked mitochondrial
614 DNAs (Bivalvia Mytilidae). *BMC genomics* 12:442.
615 Pesole G., Allen JF., Lane N., Martin W., Rand DM., Schatz G., Saccone C. 2012. The neglected
616 genome. *EMBO reports* 13:473–474.
617 Plazzi F., Cassano A., Passamonti M. 2015. The quest for Doubly Uniparental Inheritance in
618 heterodont bivalves and its detection in *Meretrix lamarckii* (Veneridae: Meretricinae).
619 *Journal of zoological systematics and evolutionary research* 53:87–94.
620 Plazzi F., Puccio G., Passamonti M. 2016. Comparative Large-Scale Mitogenomics Evidences
621 Clade-Specific Evolutionary Trends in Mitochondrial DNAs of Bivalvia. *Genome biology*
622 *and evolution* 8:2544–2564.
623 Pozzi A., Plazzi F., Milani L., Ghiselli F., Passamonti M. 2017. SmithRNAs: could mitochondria
624 "bend" nuclear regulation? *Molecular biology and evolution*. DOI:
625 10.1093/molbev/msx140.
626 Rozen S., Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist
627 programmers. *Methods in molecular biology* 132:365–386.
628 Scheffler IE. 2008. *Mitochondria*. Hoboken, N.J.: Wiley.
629 Scheibye-Alsing K., Cirera S., Gilchrist MJ., Fredholm M., Gorodkin J. 2007. EST analysis on
630 pig mitochondria reveal novel expression differences between developmental and adult

631 tissues. *BMC genomics* 8:367.
632 Serb JM., Lydeard C. 2003. Complete mtDNA sequence of the North American freshwater
633 mussel, *Lampsilis ornata* (Unionidae): an examination of the evolution and phylogenetic
634 utility of mitochondrial genome organization in Bivalvia (Mollusca). *Molecular biology and*
635 *evolution* 20:1854–1866.
636 Sievers F., Wilm A., Dineen D., Gibson TJ., Karplus K., Li W., Lopez R., McWilliam H.,
637 Remmert M., Söding J., Thompson JD., Higgins DG. 2011. Fast, scalable generation of
638 high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems*
639 *biology* 7:539.
640 Skibinski DO., Gallagher C., Beynon CM. 1994a. Mitochondrial DNA inheritance. *Nature*
641 368:817–818.
642 Skibinski DO., Gallagher C., Beynon CM. 1994b. Sex-limited mitochondrial DNA transmission
643 in the marine mussel *Mytilus edulis*. *Genetics* 138:801–809.
644 Smith DR. 2013. RNA-Seq data: a goldmine for organelle research. *Briefings in functional*
645 *genomics* 12:454–456.
646 de Sousa JT., Milan M., Bargelloni L., Pauletto M., Matias D., Joaquim S., Matias AM., Quillien
647 V., Leitão A., Huvet A. 2014. A microarray-based analysis of gametogenesis in two
648 Portuguese populations of the European clam *Ruditapes decussatus*. *PloS one* 9:e92202.
649 Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of
650 large phylogenies. *Bioinformatics* 30:1312–1313.
651 Stothard P. 2000. The sequence manipulation suite: JavaScript programs for analyzing and
652 formatting protein and DNA sequences. *BioTechniques* 28:1102, 1104.
653 Sullivan MJ., Petty NK., Beatson SA. 2011. Easyfig: a genome comparison visualizer.

654 *Bioinformatics* 27:1009–1010.
655 Tamura K., Stecher G., Peterson D., Filipski A., Kumar S. 2013. MEGA6: Molecular
656 Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution* 30:2725–
657 2729.
658 Tan MH., Gan HM., Schultz MB., Austin CM. 2015. MitoPhAST, a new automated
659 mitogenomic phylogeny tool in the post-genomic era with a case study of 89 decapod
660 mitogenomes including eight new freshwater crayfish mitogenomes. *Molecular*
661 *phylogenetics and evolution*. DOI: 10.1016/j.ympev.2015.02.009.
662 Theologidis I., Fodelianakis S., Gaspar MB., Zouros E. 2008. Doubly uniparental inheritance
663 (DUI) of mitochondrial DNA in *Donax trunculus* (Bivalvia: Donacidae) and the problem of
664 its sporadic detection in Bivalvia. *Evolution; international journal of organic evolution*
665 62:959–970.
666 Tritt A., Eisen JA., Facciotti MT., Darling AE. 2012. An integrated pipeline for de novo
667 assembly of microbial genomes. *PloS one* 7:e42304.
668 Wheeler DL., Church DM., Federhen S., Lash AE., Madden TL., Pontius JU., Schuler GD.,
669 Schriml LM., Sequeira E., Tatusova TA., Wagner L. 2005. Database resources of the
670 National Center for Biotechnology. *Nucleic Acid Research* 33:D39–D45.
671 Yuan Y., Li Q., Yu H., Kong L. 2012. The complete mitochondrial genomes of six heterodont
672 bivalves (Tellinoidea and Solenoidea): variable gene arrangements and phylogenetic
673 implications. *PloS one* 7:e32353.
674 Yuan S., Xia Y., Zheng Y., Zeng X. 2016. Next-generation sequencing of mixed genomic DNA
675 allows efficient assembly of rearranged mitochondrial genomes in *Amolops chunganensis*
676 and *Quasipaa boulengeri*. *PeerJ* 4:e2786.

677 Zouros E. 2013. Biparental Inheritance Through Uniparental Transmission: The Doubly
678 Uniparental Inheritance (DUI) of Mitochondrial DNA. *Evolutionary biology* 40:1–31.
679 Zouros E., Ball AO., Saavedra C., Freeman KR. 1994a. Mitochondrial DNA inheritance. *Nature*
680 368:818.
681 Zouros E., Oberhauser Ball A., Saavedra C., Freeman KR. 1994b. An unusual type of
682 mitochondrial DNA inheritance in the blue mussel Mytilus. *Proceedings of the National*
683 *Academy of Sciences of the United States of America* 91:7463–7467.

Figure 1(on next page)

R. decussatus mtDNA gene arrangement

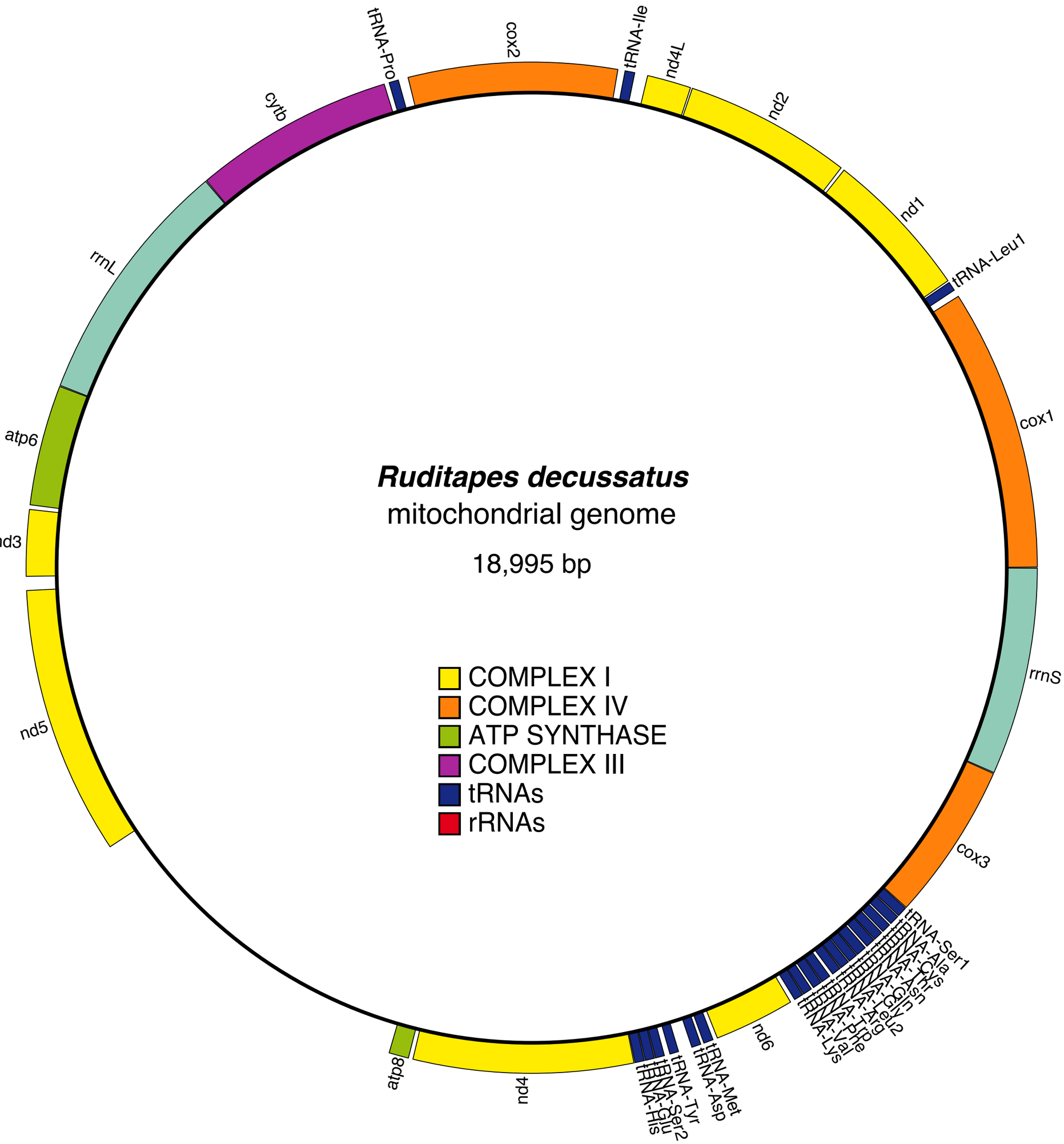

Ruditapes decussatus
mitochondrial genome
18,995 bp
COMPLEX I
COMPLEX IV
ATP SYNTHASE
COMPLEX III
tRNAs
rRNAs
tRNA-Pro
cox2
tRNA-Ile
nd4L
nd2
nd1
tRNA-Leu1
cox1
rrnS
cox3
tRNA-Ser1
tRNA-Ala
tRNA-Cys
tRNA-Trp
tRNA-Asn
tRNA-Gly
tRNA-Leu2
tRNA-Arg
tRNA-Phe
tRNA-Val
tRNA-Lys
nd6
tRNA-Met
tRNA-Asp
tRNA-Tyr
tRNA-Ser2
tRNA-Glu
tRNA-His
nd4
atp8
nd5
nd3
atp6
rrnL
cytb

Figure 2(on next page)

Principal features of the Largest Unassigned Region (LUR)

A: map of the lUR; B: DNA secondary structure predicted in the repeat region (boxed in A); C: Logos of the two DNA motifs found in the LUR.

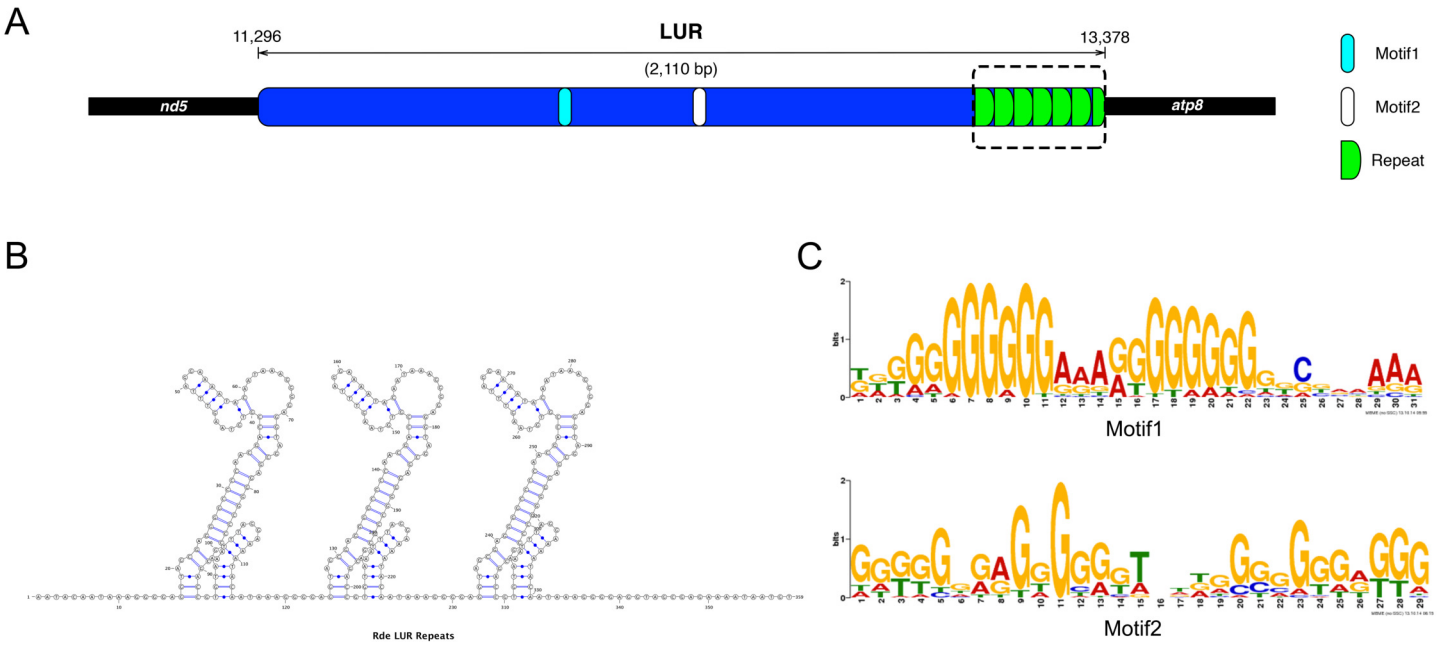
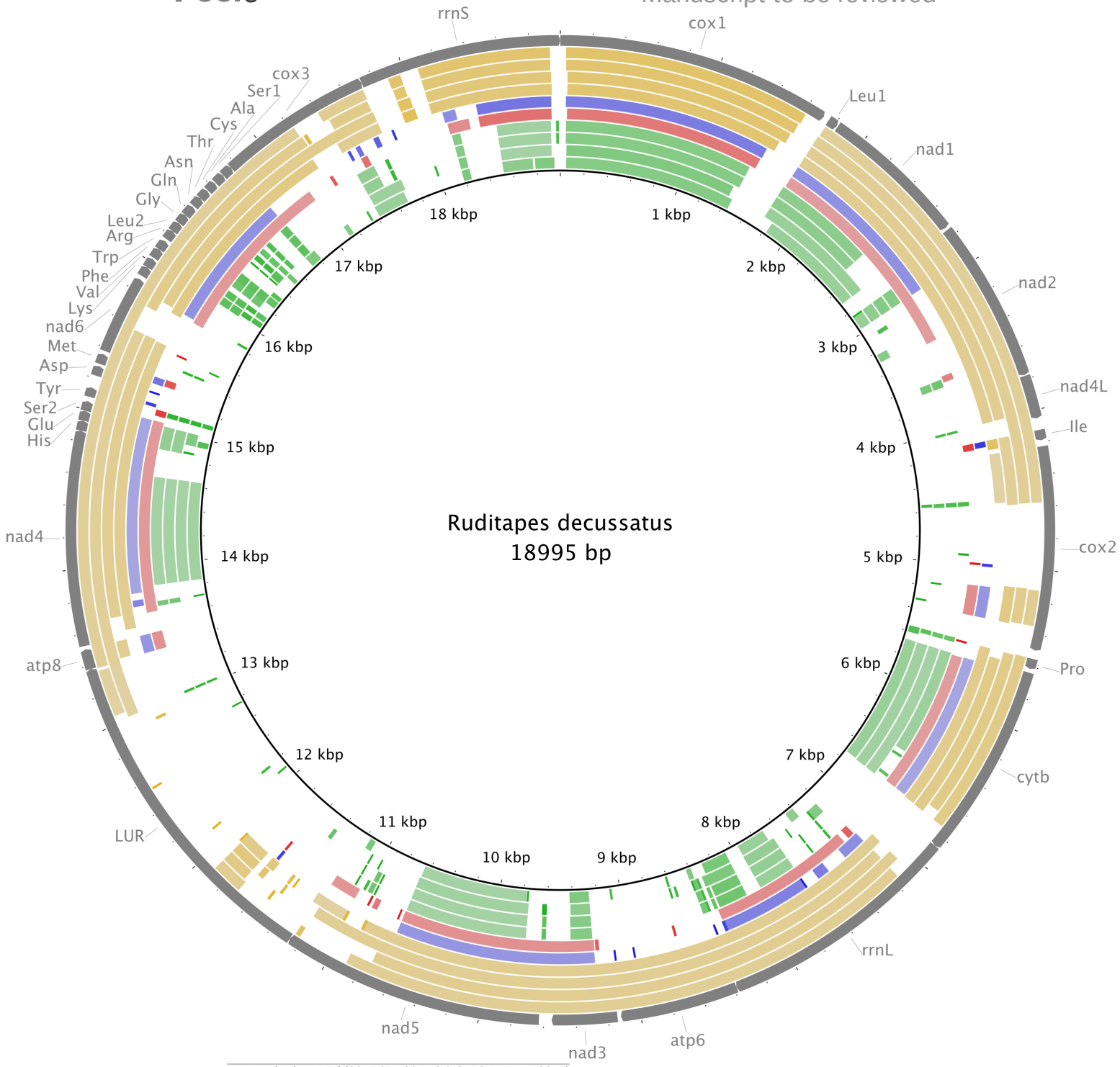

A
11,296
LUR
13,378
(2,110 bp)
nd5
atp8
Motif1
Motif2
Repeat
B
Rde LUR Repeats
C
Motif1
Motif2

Figure 3(on next page)

BLASTN comparison of *R. decussatus* and other Veneridae

R. decussatus mtDNA map (external gray circle), and BLASTN identity (colored inner circles) with complete mtDNAs of other 10 venerid species (see list in Supplementary Table 3).



38 of the 38 labels are shown.

Figure 4(on next page)

Maximum Likelihood (ML) tree of Veneridae obtained with all mitochondrial coding genes.

ML tree obtained with the MitoPhast pipeline; the complete input and output of this analysis is available on figshare (https://doi.org/10.6084/m9.figshare.4970762.v1).

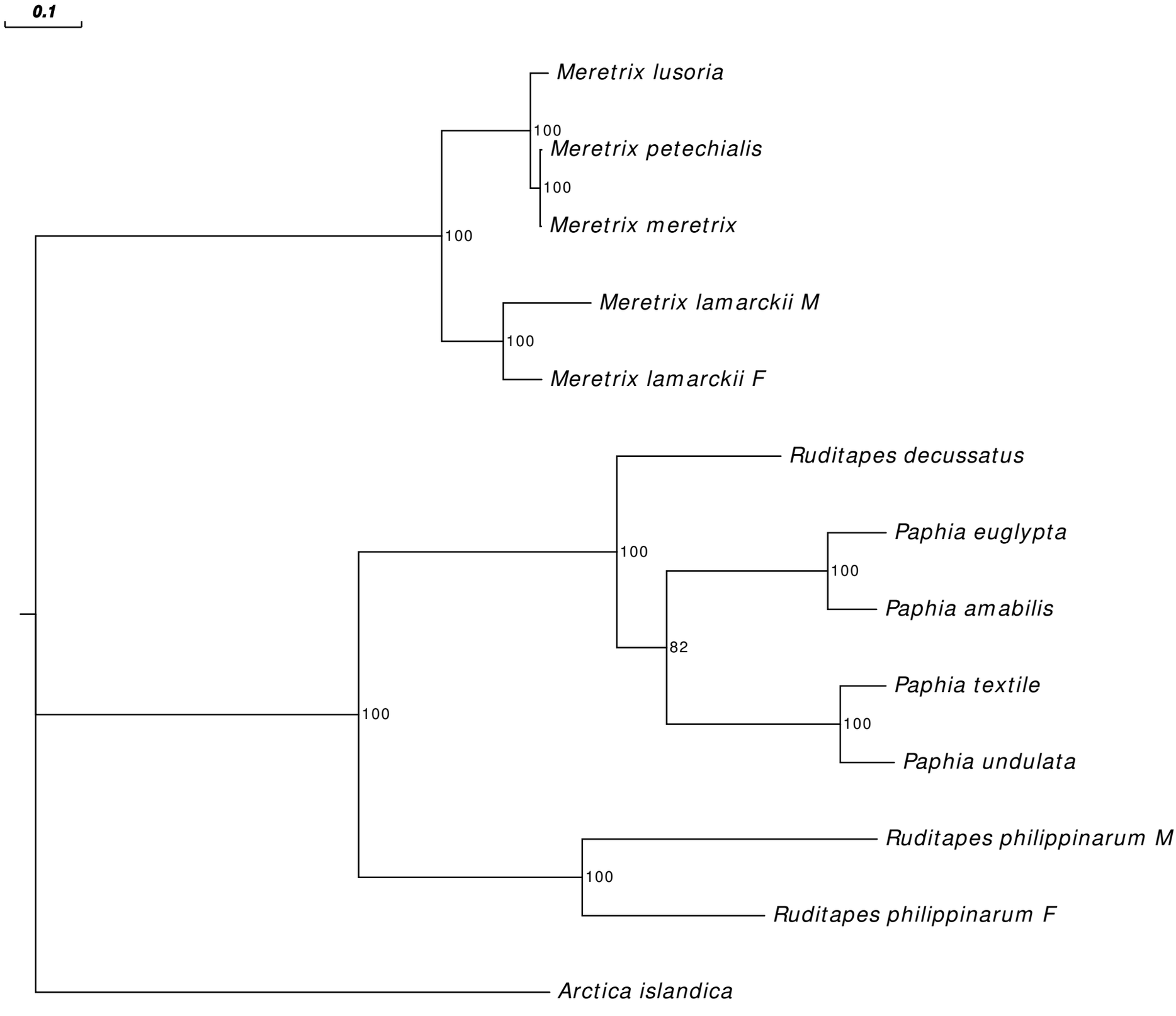

0.1
Meretrix lusoria
100
Meretrix petechialis
100
Meretrix meretrix
100
Meretrix lamarckii M
100
Meretrix lamarckii F
Ruditapes decussatus
100
Paphia euglypta
100
Paphia amabilis
82
Paphia textile
100
Paphia undulata
100
Ruditapes philippinarum M
100
Ruditapes philippinarum F
Arctica islandica

Figure 5(on next page)

Comparison of gene order in venerid mtDNAs

Variation in gene order between *R. decussatus* and *P. euglypta* (Figure 5A), *M. lamarckii* F-type (Figure 5B), *R. philippinarum* F-type (Figure 5C), and among all the 4 species (Figure 5D).

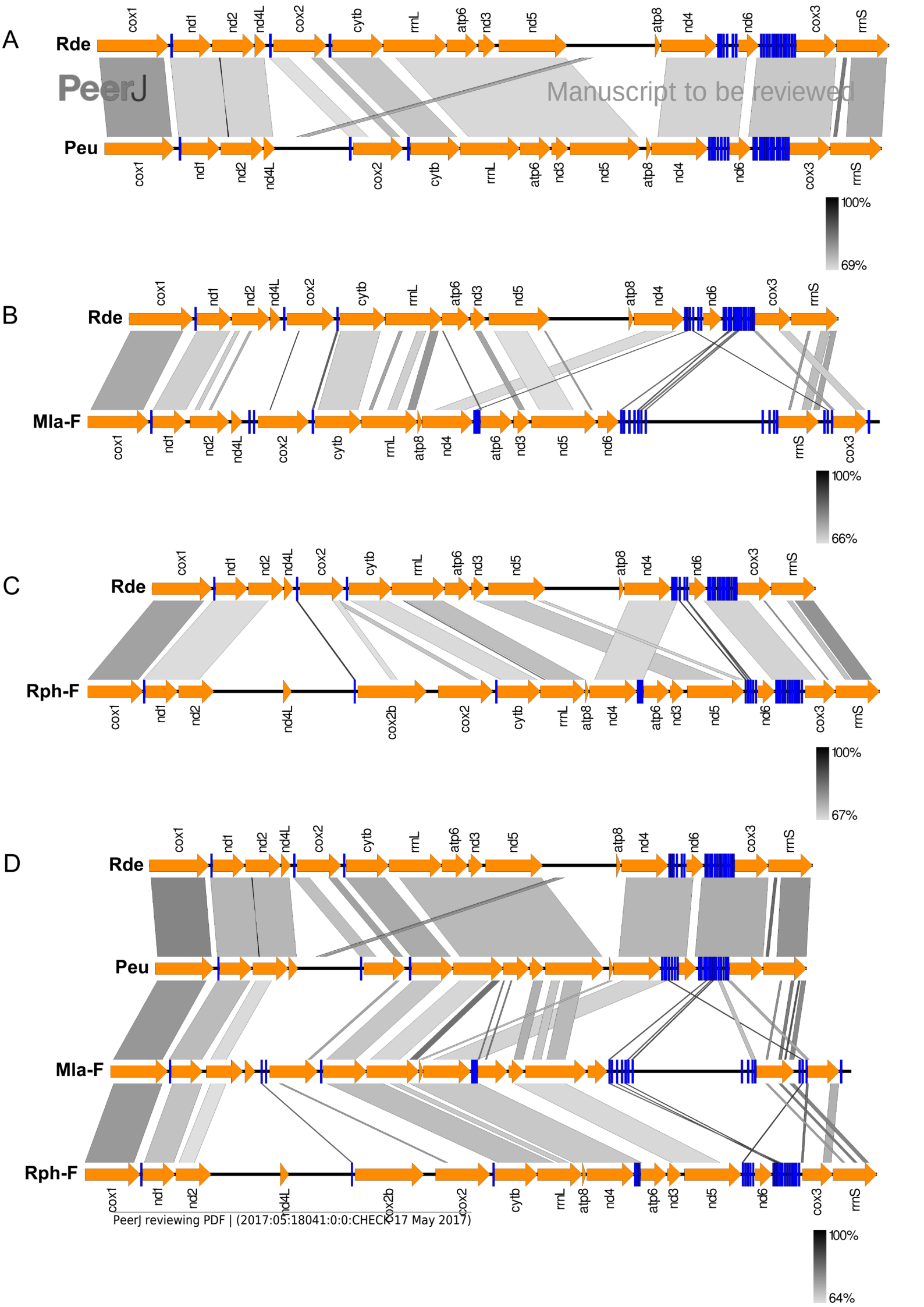


Table 1(on next page)

MtDNA gene arrangement of *R. decussatus*.

The anticodon of tRNAs are reported in the 5’-3’ direction.

Table 1 MtDNA gene arrangement of *R. decussatus*. The anticodon of tRNAs are reported in the 5'-3' direction.

Name	Type	Start	Stop	Length (bp)	Start	Stop	Anticodon
cox1	Coding	1	1,716	1,716	ATA	TAG	
tRNA-Leu1	tRNA	1,754	1,815	62			TAG
nd1	Coding	1,822	2,739	918	ATA	TAA	
nd2	Coding	2,755	3,774	1,020	ATG	TAG	
nd4l	Coding	3,780	4,052	273	ATA	TAG	
tRNA-Ile	tRNA	4,125	4,190	66			GAT
cox2	Coding	4,228	5,499	1,272	ATA	TAG	
tRNA-Pro	tRNA	5,553	5,616	64			TGG
cytb	Coding	5,641	6,864	1,224	ATA	TAG	
rrnL	rRNA	6,865	8,385	1,521			
atp6	Coding	8,386	9,123	738	ATG	TAA	
nd3	Coding	9,145	9,552	408	ATG	TAA	
nd5	Coding	9,631	11,268	1,638	ATG	TAG	
atp8	Coding	13,379	13,504	126	ATA	TAA	
nd4	Coding	13,526	14,865	1,340	ATA	TA*	
tRNA-His	tRNA	14,866	14,928	63			GTG
tRNA-Glu	tRNA	14,929	14,990	62			TTC
tRNA-Ser2	tRNA	14,991	15,052	62			TGA
tRNA-Tyr	tRNA	15,081	15,140	60			GTA
tRNA-Asp	tRNA	15,218	15,280	63			GTC
tRNA-Met	tRNA	15,294	15,358	65			CAT
nd6	Coding	15,380	15,874	495	ATG	TAA	
tRNA-Lys	tRNA	15,897	15,959	63			TTT
tRNA-Val	tRNA	15,960	16,021	62			TAC
tRNA-Phe	tRNA	16,030	16,092	63			GAA
tRNA-Trp	tRNA	16,093	16,155	63			TCA
tRNA-Arg	tRNA	16,171	16,232	62			TCG
tRNA-Leu2	tRNA	16,233	16,295	63			TAA
tRNA-Gly	tRNA	16,297	16,358	62			TCC
tRNA-Gln	tRNA	16,359	16,427	69			TTG
tRNA-Asn	tRNA	16,435	16,497	63			GTT
tRNA-Thr	tRNA	16,498	16,560	63			TGT
tRNA-Cys	tRNA	16,565	16,626	62			GCA
tRNA-Ala	tRNA	16,632	16,696	65			TGC
tRNA-Ser1	tRNA	16,698	16,764	67			TCT
cox3	Coding	16,765	17,730	966	ATG	TAA	
rrnS	rRNA	17,731	18,995	1,265			

13
14
15
16
17

Table 2(on next page)

Unassigned Regions (URs)

Table 2 Unassigned Regions (URs)

UR name	Start	Stop	Length (bp)
UR1	1,717	1,753	37
UR2	1,816	1,821	6
UR3	2,740	2,754	15
UR4	3,775	3,779	5
UR5	4,053	4,124	72
UR6	4,191	4,227	37
UR7	5,500	5,552	53
UR8	5,617	5,640	24
UR9	9,124	9,144	21
UR10	9,553	9,630	78
UR11 (LUR)	11,269	13,378	2,110
UR12	13,505	13,525	21
UR13	15,053	15,080	28
UR14	15,141	15,217	77
UR15	15,281	15,293	13
UR16	15,359	15,379	21
UR17	15,875	15,896	22
UR18	16,022	16,029	8
UR19	16,156	16,170	15
UR20	16,296	16,296	1
UR21	16,428	16,434	7
UR22	16,561	16,564	4
UR23	16,627	16,631	5
UR24	16,697	16,697	1

Table 3(on next page)

Nucleotide composition.

URs = Unassigned Regions.

Table 3 Nucleotide composition. URs = Unassigned Regions.

Name	Length (bp)	T %	C %	A %	G %	A+T %	T3 %	C3 %	A3 %	G3 %	A3+T3 %
cox1	1,716	35.8	15.5	25.8	22.9	61.6	39	12.1	28.0	21.3	67.0
nd1	918	38.7	12.5	24.0	24.8	62.7	38	10.1	30.7	21.2	68.7
nd2	1,020	38.3	11.0	24.8	25.9	63.1	35	11.5	29.4	24.4	64.4
nd4l	273	39.9	12.8	25.3	22.0	65.2	34	14.3	30.8	20.9	64.8
cox2	1,272	29.7	14.8	29.1	26.4	58.8	30	15.3	27.4	27.6	57.4
cob	1,224	37.4	17.2	22.7	22.6	60.1	41	14.7	21.8	22.1	62.8
rrnL	1,749	33.2	11.5	32.6	22.6	65.8	33	10.6	33.4	23.0	66.4
atp6	510	42.0	15.7	20.8	21.6	62.8	45	13.5	21.8	20.0	66.8
nd3	408	39.5	11.0	24.8	24.8	64.3	33	11.0	30.1	25.7	63.1
nd5	1,638	37.6	11.7	27.7	23.0	65.3	35	11.0	34.2	19.8	69.2
atp8	126	44.4	11.9	19.0	24.6	63.4	45	4.8	23.8	26.2	68.8
nd4	1,340	38.9	12.9	22.1	26.1	61.0	41	10.8	24.9	23.5	65.9
nd6	495	39.2	12.1	23.0	25.7	62.2	38	13.9	27.9	20.0	65.9
cox3	966	36.9	12.7	24.8	25.6	61.7	39	9.6	28.6	23.0	67.6
rrnS	1,265	32.7	12.3	32.9	22.1	65.6	35	13.5	31.6	19.5	66.6
All coding	14,920	36.3	13.2	26.5	24.0	63.0	37	12.0	28.9	22.4	65.7
All *rRNAs*	3,014	32.9	23.8	32.7	22.3	65.7					
All *tRNAs*	1,394	35.4	12.8	30.2	21.7	65.6					
All URs	2,681	28.2	14.1	34.1	23.6	62.3					
All genic DNA	16,314	36.2	13.2	26.8	23.8	63.0					
All DNA	18,995	35.1	13.3	27.9	23.7	63.0					

Table 4(on next page)

Codon usage.

The codons corresponding to a tRNA present in the mitochondrial genome are underlined and in bold. The highest frequency among synonymous codons is also underlined and in bold. # = number of codons; Frequency = frequency of each codon among synonymous codons; %TOT =frequency of each codon among all the codons.

Table 4 Codon usage. The codons corresponding to a tRNA present in the mitochondrial genome are underlined and in bold. The highest frequency among synonymous codons is also underlined and in bold. # = number of codons; Frequency = frequency of each codon among synonymous codons; %TOT =frequency of each codon among all the codons.

Amino Acid	Codon	#	Frequency	%TOT	Amino Acid	Codon	#	Frequency	%TOT
Ala	GCG	29	0.15	0.73	Pro	CCG	16	0.12	0.40
	GCA	44	0.23	1.11		**CCA**	36	0.27	0.91
	GCT	85	**0.45**	2.14		CCT	58	**0.43**	1.46
	GCC	30	0.16	0.76		CCC	24	0.18	0.61
Cys	TGT	94	**0.76**	2.37	Gln	CAG	25	0.44	0.63
	TGC	30	0.24	0.76		**CAA**	32	**0.56**	0.81
Asp	GAT	54	**0.66**	1.36	Arg	CGG	23	0.31	0.58
	GAC	28	0.34	0.71		**CGA**	21	0.28	0.53
Glu	GAG	87	**0.6**	2.19		CGT	25	**0.33**	0.63
	GAA	58	0.4	1.46		CGC	6	0.08	0.15
Phe	TTT	269	**0.78**	6.78	Ser	AGG	69	0.19	1.74
	TTC	78	0.22	1.97		**AGA**	69	0.19	1.74
Gly	GGG	131	**0.4**	3.30		AGT	55	0.15	1.39
	GGA	61	0.19	1.54		AGC	23	0.06	0.58
	GGT	98	0.3	2.47		TCG	18	0.05	0.45
	GGC	36	0.11	0.91		**TCA**	33	0.09	0.83
His	CAT	37	**0.62**	0.93		TCT	76	**0.21**	1.92
	CAC	23	0.38	0.58		TCC	22	0.06	0.55
Ile	ATT	165	**0.8**	4.16	Thr	ACG	21	0.17	0.53
	ATC	40	0.2	1.01		**ACA**	30	0.24	0.76
Lys	AAG	61	0.41	1.54		ACT	57	**0.46**	1.44
	AAA	87	**0.59**	2.19		ACC	16	0.13	0.40
Leu	TTG	122	0.23	3.08	Val	GTG	113	0.3	2.85
	TTA	210	**0.39**	5.29		**GTA**	121	**0.32**	3.05
	CTG	43	0.08	1.08		GTT	119	0.32	3.00
	CTA	70	0.13	1.76		GTC	23	0.06	0.58
	CTT	75	0.14	1.89	Trp	TGG	58	**0.54**	1.46
	CTC	20	0.04	0.50		**TGA**	49	0.46	1.24
Met	**ATG**	86	0.36	2.17	Tyr	TAT	103	**0.69**	2.60
	ATA	155	**0.64**	3.91		**TAC**	47	0.31	1.18
Asn	AAT	76	**0.66**	1.92	STOP	TAG	34	0.58	0.86
	AAC	39	0.34	0.98		TAA	25	0.42	0.63

7
8
9
10

Table 5(on next page)

LUR length and number of repeats in the 13 female samples analyzed.

Table 5 LUR length and number of repeats in the 13 female samples analyzed. F3 and F17 are heteroplasmic with LURs of different length.

Specimen	Length (bp)	Number of Repeats	GenBank Acc. No.
F3	2,100 - 3,500	6.5 - 25	MF055702
F5	5,000	45	MF055703
F7	3,500	25	MF055704
F9	3,500	25	MF055705
F10	3,000	20	MF055706
F11	3,000	20	MF055707
F13	3,500	25	MF055708
F15	3,000	20	MF055709
F16	3,500	25	MF055710
F17	2,500 - 3,500	8 - 25	MF055711
F19	3,500	25	MF055712
F20	2,500	8	MF055713
F21	2,100	6.5	MF055714

Table 6(on next page)

Most significant GO terms associated with the two DNA motifs found in the LUR.

BP = Biological Process; CC = Cellular Component; MF = Molecular Function.

Table 6 Most significant GO terms associated with the two DNA motifs found in the LUR. BP = Biological Process; CC = Cellular Component; MF = Molecular Function.

Motif 1	Motif 2
Positive regulation of transcription from RNA polymerase II promoter (BP)	Transcription (BP)
Transcription (BP)	Negative regulation of transcription from RNA polymerase II promoter (BP)
Negative regulation of transcription from RNA Polymerase II promoter (BP)	-
Transcription factor complex (CC)	-
Transcription activator activity (MF)	-

Table 7 (on next page)

SNP features

CDS = coding sequences.

Table 7 SNP features. CDS = coding sequences.

FEATURE	VALUE	MIN	MEDIAN	MEAN	MAX
Depth (all SNPs)	-	6	1,357	1,521	3,880
Phred Score (all SNPs)	-	3.30E+01	5.76E+03	4.18E+07	2.15E+09
Depth (SNPs in CDS)	-	222	2,038	2,150	3,880
Phred Score (SNPs in CDS)	-	1.18E+02	1.01E+04	4.45E+07	2.15E+09
Total number of SNPs	257	-	-	-	-
Number of SNPs in CDS	145 (56.4% of the Total)	-	-	-	-
Number of mRDI01 private SNPs	103 (71% of the SNPs in CDS)	-	-	-	-
Number of SNPs in CDS (excluding mRDI01)	42	-	-	-	-
Frequency of SNPs in CDS	0.0097 (~ 1 every 103bp)	-	-	-	-
Frequency of SNPSs in CDS (excluding mRDI01)	0.0028 (~1 every 355bp)	-	-	-	-
Total number of indels	18	-	-	-	-
Number of indels in CDS	6	-	-	-	-
Number of indels causing frameshift	4	-	-	-	-

Table 8(on next page)

Indels located in coding sequences

DEPTH = sequencing depth; QUAL = quality of the called SNP expressed in Phred score; ALLELE FREQUENCY = frequency of the alternative allele in each sample indicated in the "SAMPLE" column.

Table 8 Indels located in coding sequences. DEPTH = sequencing depth; QUAL = quality of the called SNP expressed in Phred score; ALLELE FREQUENCY = frequency of the alternative allele in each sample indicated in the "SAMPLE" column.

POSITION	DEPTH	QUAL	GENE	SNP	FRAMESHIFT	SAMPLE	ALLELE FREQUENCY	NOTES
1,698	3,732	1.38E+04	*cox1*	C/CAAA	No	mRDI02, mRDI03	0.089, 0.85	Insertion of 1 Lysine
6,364	1,929	2.15E+09	*cytb*	CT/C	Yes	fRDI04, mRDI05	0.80, 0.81	Yields a shorter Cytb. Possible sequencing error due to the homopolymer CTTTTTTT
10,449	1,780	2.15E+09	*nd5*	C/CT	Yes	fRDI01, fRDI04, fRDI05	0.11, 0.10, 0.11	Yields a nd5 gene divided in 2 ORFs. Possible sequencing error due to the homopolymer CTTTTTT
17,619	2,272	5.98E+03	*cox3*	AGCG/A	No	mRDI01	0.97	Deletion of 1 Alanine
17,621	2,188	9.99E+04	*cox3*	CG/C	Yes	mRDI01	0.99	Always combined with SNP_17624. Together change the last 35 amino acids.
17,624	2,287	5.98E+03	*cox3*	C/CAT	Yes	mRDI01	0.99	Always combined with SNP_17621. Together change the last 35 amino acids.