Mitochondrial selfish elements and the evolution of biological novelties

Liliana Milani*,§, Fabrizio Ghiselli§, and Marco Passamonti

Department of Biological, Geological and Environmental Sciences, University of Bologna, Via Selmi 3, 40126, Bologna, Italy

*Address correspondence to Liliana Milani. E-mail: liliana.milani@unibo.it.

§These authors contributed equally to this work.

Received on 31 October 2015; accepted on 18 March 2016

Abstract

We report the present knowledge about RPHM21, a novel male-specific mitochondrial protein with a putative role in the paternal inheritance of sperm mitochondria in the Manila clam Ruditapes philippinarum, a species with doubly uniparental inheritance of mitochondria (DUI). We review all the available data on rphm21 transcription and translation, analyze in detail its female counterpart, RPHF22, discuss the homology with RPHM21, the putative function and origin, and analyze their polymorphism. The available evidence is compatible with a viral origin of RPHM21 and supports its activity during spermatogenesis. RPHM21 is progressively accumulated in mitochondria and nuclei of spermatogenic cells, and we hypothesize it can influence mitochondrial inheritance and sexual differentiation. We propose a testable model that describes how the acquisition of selfish features by a mitochondrial lineage might have been responsible for the emergence of DUI, and for the evolution of separate sexes (gonochorism) from hermaphroditism. The appearance of DUI most likely entailed the invasion of at least 1 selfish element, and the extant DUI systems can be seen as resolved conflicts. It was proposed that hermaphroditism was the ancestral condition of bivalves, and a correlation between DUI and gonochorism was documented. We hypothesize that DUI might have driven the shift from hermaphroditism to gonochorism, and androdioecy as transition state. The invasion of sex-ratio distorters and the evolution of suppressors can prompt rapid changes among sex-determination mechanisms, and DUI might have been responsible for one of such changes in some bivalve species. If true, DUI would represent the first animal sex-determination system involving mtDNA-encoded proteins.

Key words: bivalve mollusc, doubly uniparental inheritance (DUI), endogenized virus, germ line, gonochorism, hermaphroditism.
control, chromatin remodeling, and transcriptional control (Kurz et al. 2002; Ronkina et al. 2007, 2008; Milani et al. 2013a, 2014a). Following these observations, Milani et al. (2014a) proposed that: (1) M-specific elements may have a role in preventing the recognition of male-transmitted mitochondria by the degradation machinery, thus allowing their survival in male embryos, and their preferential transmission to the progeny; (2) these elements might also function as transcriptional regulators.

The aim of this article is to report the present knowledge about RPHM21, proposed to be one of the factors responsible for paternal inheritance of sperm mitochondria in *R. philippinarum*. The evidence collected so far strongly supports the activity of this protein during spermatogenesis, and we hypothesize that the viral sequence provided infected mitochondria with the ability to escape the degradation process in early embryos, invading the germ line, and being preferentially transmitted to the progeny. In the present work we: (1) review all the available data on *rpm21* transcription and translation, providing new in situ hybridization and confocal data; (2) analyze in detail the F-type specific mtORFan RPHF22, discussing its homology with RPHM21, its putative function, and its origin; (3) analyze the M-type and F-type polymorphism; (4) propose a testable model that describes how the acquisition of selfish features by a mitochondrial lineage (through endogenesis of a viral sequence in its mtDNA) might have been responsible for the emergence of an aberrant mitochondrial inheritance system, and for the evolution of separate sexes (condition usually referred to as gonochorism in animals and dioecy in plants) from hermaphroditism.

Materials and Methods

Real-Time qPCR data

A comparative analysis of *rpm21* transcript amount obtained with Real-Time qPCR on young and adult male specimens was performed to highlight possible differences between tissues and developmental stages. The target (129 bp sequence) was quantified using the primers Rph_SYBR_orf21_f, forward TCTGTGAAAGAAA CCCATGTGAG and Rph_SYBR_orf21_r, reverse ACTAATATATA TGGAGCGCGAATAACTTGT (Milani et al. 2014b, 2015). In total, data from 112 male samples were used: 28 juveniles (whole bodies), 16 gonads from gametogenic adults (adults during gonad maturation), 20 gonads of ripe adults (adults with mature gametes), 24 adductor muscles, and 24 mantles. Details about experimental design and quantification method are available in Milani et al. (2014b, 2015). Statistical analyses and plots were performed with R 3.1.1.

Microscopy

Mature adult specimens (gonads and surrounding tissues of 5 individuals, 3 males, and 2 females) were used for in situ hybridization with *rpm21* riboprobes and immunological localization of RPHM21 in tissues to document the presence and localization of transcript and protein in tissues. A digoxigenin (DIG)-labeled riboprobe antisense to *rpm21* transcript was obtained using the Roche in vitro transcription labeling protocol (Roche DIG RNA labeling kit). Cryosections of gonads of 10–20 μm were used for in situ hybridization, following the method in Milani et al. (2013b, 2014a). Samples were observed with a Nikon Eclipse 80i microscope and images were captured using NIS-Elements D3.2 software.

An anti-RPHM21 antibody was used to immunolocalize the male-specific mitochondrial protein, following the method in Milani et al. 2013a. That said, the most noteworthy feature of bivalve mitochondrial biology is the doubly uniparental inheritance (DUI) of mitochondria (Skibinski et al. 1994a, 1994b; Zouros et al. 1994a, 1994b) reported, so far, in about 50 species. In animals, DUI is the only known evolutionarily stable exception to the common strictly maternal inheritance (SMI) of mitochondria. Under DUI, mitochondria follow two distinct inheritance paths: 1 type of mitochondria is inherited through females (F type), the other through males (M type). The zygote receives both the mtDNA types then, if the embryo develops into a female, the M type is lost (degraded or diluted), and the adult will be homoplasmic for the F type. Conversely, if the embryo develops into a male, the M type is retained, it becomes the predominant mtDNA type in the gonad, and sperm will be homoplasmic for the M type. The somatic tissues of males show variable proportions of M and F type, depending on tissue and species (Ghiselli et al. 2011). It has to be noted that DUI is not a case of biparental inheritance, since each mtDNA lineage experiences a uniparental transmission. F and M mtDNAs are highly differentiated, with an amino acid divergence up to 50% (see Zouros 2013 for a thorough review of DUI). DUI represents an exceptional model for studying multiple aspects of mitochondrial biology (Passamonti and Ghiselli 2009; Milani et al. 2011; Breton et al. 2014), and since its discovery, the main focus has been to unveil the origin and the molecular mechanism behind it. The data gathered so far are consistent with DUI being originated from SMI by modification of the molecular machinery of SMI (Breton et al. 2007; Zouros 2013; Milani and Ghiselli 2015), but the underlying factors are still unknown. A fair amount of research was done on the mitochondrial genomes of DUI species to identify elements that could be responsible for, or involved in, the modification of the inheritance mechanism, and some good candidates have been spotted. Lineage specific mtORFans were identified in all the analyzed DUI species: fORFans specific to the F mtDNA, and mORFans specific to the M mtDNA have been found in the families Unionidae (Breton et al. 2009; Breton et al. 2011a), Mytilidae (Breton et al. 2011b), and Veneridae (Ghiselli et al. 2013; Milani et al. 2013a). Given the absence of identifiable homologs in databases, the function of these mtORFans has to be inferred by the presence of short regions showing similarity with known structural motifs. An *in silico* meta-analysis (Milani et al. 2013a) has highlighted some interesting patterns: features shared by MORFs (we refer to the translations of fORFs and mORFs as FORF and MORF, respectively) point to a role in the targeting of M-type mitochondria to germ line precursor blastomeres through interactions with the cytoskeleton. Moreover, both FORF and MORF features point to a role in nucleic acid binding and transcription regulation, and, most strikingly, all the analyzed mtORFans showed signatures that are consistent with a viral origin. Up to now, evidence for a protein product has been provided for only 3 mtORFans: RPHM21, specific to the M mtDNA of the Manila clam *Ruditapes philippinarum*, and the F and M mtORFans (here reported as Vel-FORB and Vel-MORB) specific to the freshwater mussel *Venericardia aequalis* (Breton et al. 2009, 2011a). Immunoelectron microscopy and confocal microscopy showed that Vel-FORB and RPHM21 are localized in both mitochondria and nucleus (Breton et al. 2011a; Milani et al. 2014a). Milani et al. (2014a) found similarities between RPHM21 and MK3, a member of modulators of immune recognition (MIRs), which are viral proteins involved in immune recognition functioning as ubiquitin ligases (Coscoy and Ganem 2003). RPHM21 and MK3 show structural similarities, are both localized in mitochondria and nucleus, and share putative functions such as reorganization of cytoskeleton, cell migration, cell cycle...
et al. (2014a). Nuclei were stained with 1 mM TO-PRO3 (Molecular Probes). Some sections were incubated with a monoclonal anti-alpha-tubulin (clone DM1A; Sigma) (method as in Milani et al. 2011) to stain microtubules and to better show the structure of both male and female acinus (i.e., the structural unit of bivalve gonad; a gonad is formed by thousands of acini). Imaging was recorded by a confocal laser scanning microscope (Leica confocal SP2 microscope), using Leica software.

In silico analyses on RPHM21 and RPHF22

We performed in silico analyses to better characterize the novel mitochondrial elements RPHM21 and RPHF22 because they were reported not to show a clear sequence similarity with known proteins. The tertiary structure of RPHM21 and RPHF22 was predicted using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER; Zhang 2008). The best model in pdb format was used as input for the Chimera 1.8.1 software (Pettersen et al. 2004) to obtain the 3D structure.

Alignments between RPHM21 and RPHM22 were performed with TM-COFFEE (http://tcoffee.crg.cat/apps/tcoffee/do:tmcoffee; Chang et al. 2012), which is specifically designed to align transmembrane proteins, and with HMMER 3.1b2 (Finn et al. 2011). HMMER uses profile hidden Markov models (profile HMMs; Eddy 1998) to perform alignments. Profile HMM converts an alignment into a position-specific scoring system that is particularly efficient in searches for remote homology. To obtain specific-profile HMMs for RPHM21 and RPHF22, we obtained an amino acid sequence alignment for each protein using Clustal Omega (Sievers et al. 2011), and then we used the hmmbuild tool of the HMMER 3.1b2 package. We then aligned the 2 proteins using hmalign bidirectionally (RPHM21 profile HMM vs. RPHF22 and vice versa).

Interproscan5 (http://www.ebi.ac.uk/Tools/ftp/interproscan5; Jones et al. 2014) was used to search InterPro signatures in RPHM21 and RPHF22.

Polymorphism analysis was performed on F and M mitochondrial genes for which the number of sequences available was ≥10, namely: atp6, cox3, nd3, nd4, nd5, nad (F type: n = 12; M type n = 11), rphm21 (n = 16), rphf22 (n = 20). We combined data obtained by Sanger sequencing (F type: AB065375, AF484332-36, KC243324-31; M type: AB065374, AF484337-40, KC243347-53) with data obtained from a single nucleotide polymorphism (SNP) analysis performed on RNA-Seq data (BioProject PRJNA68513, Ghiselli et al. 2012). SNPs were called from gonad transcriptomes of 6 males and 6 females. Details about SNP calling procedure are in Ghiselli et al. (2013). To obtain alignable FASTA sequences from the VCF file containing the SNP informations, we first prepared the reference FASTA by building an index with the Samtools faidx tool (Li et al. 2009), and creating a sequence dictionary with the CreateSequenceDictionary program from PicardTools (http://broad institute.github.io/picard/). Then we used the FastAAlternateReferenceMaker command from the program GenomeAnalysisTK of the GATK distribution 3.4-46 (McKenna et al. 2010). Later, we used ClustalW to align the FASTA files containing the alternative alleles obtained from the SNP calling with the Sanger sequences. Nucleotide and amino acid p-distances, number of synonymous substitutions for synonymous sites (synonymous polymorphism, pS), and number of nonsynonymous substitutions per nonsynonymous sites (nonsynonymous polymorphism, pN) were calculated using MEGA 5 (Tamura et al. 2011) with the following settings: 1,000 bootstrap replicates, transitions+transversions, complete deletion, Kumar method.

Results

Real-Time qPCR

This analysis was performed in male individuals only; sex in juveniles was assigned by the presence of the M-type genome, while in adults by microscope examination of gonadic tissue. Real-time qPCR data indicate that rphm21 transcript abundance increases from the time of gonad formation (juveniles) to the fully mature gonad of ripe adults (Figure 1). rphm21 transcript amount is lower and uniform in the 2 somatic tissues analyzed (Figure 1).

In situ hybridization

Figure 2 shows the morphology of male and female acini. Consistently with what was previously described (Milani et al. 2014a), rphm21 transcripts are localized in male gonads (Figure 3). Many more spermatozoa are produced in male acini compared to eggs in female acini (50:1, spermatozoa:eggs per z-section at the confocal microscope; Figure 2). In male acini, the transcript is present in early spermatogenic cells along the acinus wall (Figure 3A,B), then, during spermiogenesis, it accumulates in spermatozoa that fill the acinus lumen when gonads are mature. The transcript is not detected in female acini (Figure 3C).

Immunohistochemistry

In the male gonad, anti-RPHM21 labels a clear spot at one side of the nucleus of spermatocytes and spermatids (Figure 3D). Anti-RPHM21 staining is also present in spermatozoa that fill the acinus (Figure 3D), showing the staining both in the sperm mitochondrial midpiece and in the nucleus (Figure 3D, box). The nuclei of spermatogenic cells appear with different color, depending on the different proportion of colocalizing fluorescence signals (TO-PRO3, in green,
and anti-RPHM21, in red). RPHM21 protein is not detected in female acini and eggs (Figure 3E).

In silico analyses
I-TASSER proposed 5 models for RPHM21 and RPHF22. The C-score for the inferred proteins was in the range $[-4.31, -4.96]$ and $[-1.96, -5.00]$, respectively. The 10 threading templates used by I-TASSER to model RPHF22 and RPHM21 are reported in Table 1. The results of InterProScan 5, TM-COFFEE, and HMMER are shown in Figure 4A–D. 3D models of RPHM21 and RPHF22 obtained using structures predicted by I-TASSER are shown in Figure 4E,F.

Polymorphism analysis (Table 2) shows that both rphm21 and rphf22 are quite conserved, and that, compared with other mitochondrial genes, their synonymous and nonsynonymous polymorphism is low. rphm21 has the lowest nonsynonymous polymorphism of all (both F and M mtDNAs) the analyzed genes ($p_N = 0.003, 95\% CI [0.000, 0.007]$).

Discussion
Lineage-specific mitochondrial elements and DUI
In the absence of proper functional studies, the role of RPHM21 and of other DUI mtORFans can be inferred only through in silico predictions and bioinformatics analyses. In the last years, several works have provided new insights about these novel mitochondrial elements (Breton et al. 2011a, 2011b, 2014; Ghiselli et al. 2013; Milani et al. 2013a, 2014a, 2015), but clear evolutionary and functional patterns have not emerged yet. The difference among these elements might be the result of divergent evolution after a common origin, or it might be indicative of an independent origin; in the latter case the genes involved in DUI would be taxon-specific. Accordingly, and alternatively to what hypothesized earlier (see Zouros 2013), it was recently proposed that DUI might have evolved multiple times through the endogenization of different viral elements in the mitochondrial genome of some bivalve species (Milani et al. 2013a), thus explaining the scattered distribution of DUI in the class Bivalvia (Zouros 2013) and the higher difference in amino acid sequence among the above-mentioned lineage-specific mitochondrial elements when compared to standard mitochondrial proteins (see Figure 5 in Milani et al. 2013a). If so, DUI in different species could be achieved by different modifications of SMI, and the genes involved could be nonhomologous.

M-type specific RPHM21
The sequence of rphm21 was reported to be quite conserved (Ghiselli et al. 2013), and the new analysis performed on additional sequences (both from Sanger sequencing and RNA-Seq SNP calling) confirms this observation. Both the nucleotide and amino acid p-distances are among the lowest of all the analyzed mitochondrial genes (see Table 1) and the synonymous-nonsynonymous polymorphism indicates negative selection ($p_S = 0.011, 95\% CI [0.001, 0.021]; p_N = 0.003, 95\% CI [0.000, 0.007]$). All the data collected so far show that rphm21 transcript is more abundant in gonads of ripe adults, when compared with juveniles and gametogenic adults (Figure 1), and that rphm21 transcript is localized in gametes, as shown by the riboprobe labeling the acinus lumen (Figure 3A–C). The protein appears to be produced and stored in the cytoplasm portion in which the mitochondrial midpiece forms, as shown by the red spot at one
Figure 3. Localization of *rphm21* products. (A–C) *rphm21* transcript localization with in situ hybridization in male (A, B) and female (C) gonadic tissue of the Manila clam *Ruditapes philippinarum*. (A) Male immature acinus in which *rphm21* riboprobe labels spermatogenic cells along the acinus wall (positive signal in black). (B) Male mature acinus in which spermatozoa stored in the acinus lumen are deeply stained with *rphm21* riboprobe. (A, B scale bars = 100 μm). (C) No staining is present in eggs (C scale bar = 50 μm). *n*—egg nucleus; *cyt*—egg cytoplasm; *ct*—connective tissue. (D, E) RPHM21 protein localization in male and female gonadic tissue, respectively. (D) In the male gonad, anti-RPHM21 (in red) labeled a clear spot at 1 side of the nucleus of spermatocytes and spermatids (arrow and arrowhead, respectively). Anti-RPHM21 staining is strong in mature spermatozoa in the acinus lumen in both mitochondrial midpiece and nucleus (yellow due to the colocalization of the nuclear dye, in green, and the antibody, in red). Initially, in spermatocytes the nuclei are visible in green, and no RPHM21 appears to be stored in the nucleus; in spermatozoa the nuclei are brownish as indicating RPHM21 storing (clearly seen in the bottom of the image going from the right to the left). (D scale bar = 15.77 μm). (E) Eggs do not show anti-RPHM21 detectable staining. The smaller nuclei of somatic cells surrounding acini are also visible. (E scale bars = 75 μm). RPHM21 immunolabeling in red; TO-PRO3 nuclear dye in green.

A–C: optical microscope. D, E: confocal microscope.
The presence of RPHF22 and RPHM21 proteins in male and female germ cells suggests that these proteins may play a role in the process of spermatozoa elimination/maintenance, as well as in the targeting of proteins to mitochondria (Howard et al. 2004; Bouaouina et al. 2012). Interestingly, the sequence similarity of RPHF22 and RPHM21 proteins is higher in the predicted transmembrane region (Figure 4) and lower in the cytoplasmic region. This suggests that RPHF22 and RPHM21 may have distinct functions in male and female gametes.

A model for the evolution of DUI and gonochorism

In the present article, we focused on the characterization, origin, and possible function of RPHM21 and RPHM22 and their role in the evolution of DUI and gonochorism. The presence of RPHF22 and RPHM21 proteins in male and female germ cells suggests that these proteins may have sex-specific functions.
Figure 4. Structural analysis of RPHM21 and RPHF22. (A, B) Protein domains detected with InterProScan 5: RPHM21 (A) and RPHF22 (B) both show transmembrane domains in their N-terminus, whereas the C-terminus is cytoplasmic. (C) Similarities in domain localization detected with TM-COFFEE alignment. (D) HMMER detected good alignment of profile HMMs in correspondence of the transmembrane domains. (E, F) 3D models of RPHM21 (E) and RPHF22 (F) obtained using structures predicted by I-TASSER.
likely derivation from the same selfish element. Starting from the assumption that the 2 elements have the same origin, we formulate a model on how selection on selfish mitochondria may have resulted in the appearance of DUI.

There is a strict correlation between the presence of DUI and gonochorism, indeed, hermaphrodites closely related to DUI species do not have DUI and show extensive mutations in the mitochondrial genome. Only genes with specific mtORFs in the maintenance of gonochorism was hypothesized as a consequence of this observation, a role of DUI lineage-specific mtORFs in the maintenance of gonochorism was hypothesized by Breton et al. (2011a). However, we still do not know if the strict correlation between mitochondrial inheritance and sex is actually causal or coincidental. Here we propose a model that describes a scenario under which DUI and gonochorism might have evolved together in some bivalve species.

**Step 1**
In a population of hermaphroditic bivalves, a virus infects some mitochondria and confers them the ability to avoid degradation in embryos, and to be preferentially transmitted through generations (segregation distortion).

**Step 2**
Infected mitochondria end up both in male and female germ lines, but we could expect that they spread more efficiently through sperm for the following reasons:

a. males produce more gametes than females. Actually, in *R. philippinarum*, as visible in cross sections of male and female acini, many more spermatozoa are produced in male acini compared to eggs in female acini (spermatogenesis:eggs ratio indicatively 50:1; Figure 2). According to some authors, the majority of retroviral insertions are acquired by the male germ line due to the relatively high number of cell divisions involved in the production of sperm in comparison to eggs (Katzourakis et al. 2007; Mishra and Chan 2014).

b. even though eggs contain more mitochondria than sperm, the mtDNA copy number in *R. philippinarum* gonads is much higher in males than in females (at least 1 order of magnitude, see Ghiselli et al. 2011); the integration of viral elements is easier in presence of a high replication rate, a condition that well matches mitochondrial proliferation during spermatogenesis. In fact, proliferating cells usually experience higher mitochondrial replication, with the only notable exception of the early stages of embryo development (Yamano and Youle 2011; Milani et al. 2012; Mishra and Chan 2014). This mitochondrial proliferation may be even stronger for germ cells, when a proper distribution and quality control of mitochondria is needed more;

c. the high incidence of mitochondrial fusion and fission during cell replication can be an additional mechanism allowing the element to spread in the mitochondrial population (Mitra 2013; Mishra and Chan 2014).

All that considered, given its specific feature, the spermatozoon appears to be more suited to incorporate and transmit mtDNA carrying the integrated element.

**Step 3**
The frequency of infected sperm increases in the population. If we assume that the viral sequence allows mitochondria containing it to survive and to be preferentially transported to the germ line (a behavior clearly visible for sperm mitochondria in *R. philippinarum*, Milani et al. 2012, and in other DUI species, Zouros 2013) and transmitted to the progeny, in the subsequent generations the viral element can widely spread in the population.

**Step 4**
The infection allowed both maternal and paternal inheritance of mitochondria. In this condition, mitochondria are under selection...
also for male functions, such as spermatogenesis and—most importantly for a broadcast spawning species—sperm swimming. So now mutations that increase male fitness have a high selection coefficient and can spread quickly in the population.

Step 5
Emergence of males and transition from hermaphroditism to androdioecy. Gonochorism can evolve when a unisexual mutant (i.e., carrying a female- or male-sterility mutation) invades the population and the remaining hermaphrodites specialize in the complementary unisexual type (Charlesworth and Charlesworth 1978). Theoretical models show that this transition can occur through 2 intermediate transitory stages: gynodioecy (females + hermaphrodites), and androdioecy (males + hermaphrodites). In animals, the transition from hermaphroditism to androdioecy is much more common (Weeks 2012). Unisexual mutants can be maintained in hermaphrodite populations if they gain a strong fitness advantage over the hermaphrodites (Charlesworth and Charlesworth 1978, Equation 10), and this can be achieved either by avoiding self-fertilization (thus inbreeding depression), or through a more efficient resource allo- location: spermatozoa are cheaper to produce, so the reproductive success of males is size-independent, namely given the same energy allocated in reproduction, male relative fitness is higher (Charlesworth and Charlesworth 1978; Weeks et al. 2006; Weeks 2012).

Going back to our model, we think that in R. philippinarum 2 main factors contributed to the increase in male fitness that allowed the establishment of androdioecy: (1) bivalves show the kind of resource allocation discussed above, for example, in most hermaphrodite species, younger/smaller individuals are males, and they switch to females when they grow older/bigger (Wright 1998); (2) considering what we mentioned in Step 2, we expect a longer permanence of the selfish element in male germ cells than in female germ cells, so infected mtDNA will coevolve mostly with male-specific alleles and male-biased genes. This suggests that males with infected mitochondria (now under selection for male functions, see Step 4) may have an even higher fitness in respect to other males. That said, the evolution of androdioecy and eventually separate sexes can occur even in the absence of resource reallocation, if segregation distortion is present (Billiard et al. 2015).

Step 6
Evolution of gonochorism from androdioecy. The proportion of males increases in the population, until their initial fitness advantage is neutralized by the disadvantage of a male-biased population. In this condition, the selective pressure on egg production would be strong, and male-sterility mutation in hermaphrodites highly favored.

Step 7
Evolution of one or more restorer genes that counteract the action of RPHM21. The coevolutionary interactions of the nuclear genome with cytoplasmic sex-ratio distorters can lead to the evolution of compensatory genes, to restore a balanced sex ratio (Hurst 1992). Such genes might induce monogeny, which is a condition where all offspring of each individual female are either exclusively male or exclusively female (Werren and Beukeboom 1998). Interestingly, DUI species show sex-biased lineages (Kenchington et al. 2002; Ghiselli et al. 2012; Machordom et al. 2015): independent of the sire, there are females producing exclusively or almost exclusively female offspring, females producing almost exclusively male offspring, and females producing intermediate proportions of the 2 sexes. In the literature about genomic conflicts, the restorer genes are usually nuclear, and that might be the case also for R. philippinarum, but then this would not explain the persistence of 2 lineage-specific elements (RPHM21 and RPHF22). Indeed, if RPHM21 was the segregation distorter, and a restorer system evolved from nuclear genes, most likely RPHF22 would have pseudogenized, remaining a genic relict. This is not what we observe, since RPHF22 sequence is well conserved, as suggested by polymorphism data ($p_S = 0.020, 95\% CI [0.002, 0.038]$; $p_N = 0.007, 95\% CI [0.001, 0.013]$, see also Table 2). RPHF22 might have become part of the restorer mechanism, interacting with nuclear elements to counteract RPHM21.

We propose 3 different scenarios.

a. The viral element also kept infecting mtDNA transmitted through eggs, but the slower replication in the female germ line led to a less efficient insertion ratio and a consequently slower diffusion in the population, so the process of differentiation from the original sequence would have been slowed down as well. Interestingly, I-TASSER generated a better model for RPHF22 compared with RPHM21 (−1.96 versus less than −4, respectively, see ‘Results’ section), because of a higher structural similarity found with known protein sequences.

b. The viral element was first endogenized in sperm-transmitted mtDNA, starting DUI, and then, by recombination (an event that was documented multiple times in DUI species, see Zouros 2013), it invaded the original mitochondrial lineage that was still transmitted maternally. At this point the 2 elements diverged by evolving sex-specific features.

c. RPHF22 originated from a different viral element, but with similar features.

Given the fast-evolving nature of viral sequences, the fast-evolving nature of mtDNA in bivalves, and also given the long divergence time between F and M mtDNAs in R. philippinarum, it will be very difficult (if not impossible) to resolve this issue.

How to test the model
In follow-up work, we will test our working hypotheses using mathematical models and simulations. This approach will show which biological and evolutionary parameters are compatible with our model (e.g., number of male and female gametes, number of cell divisions in gametogenesis, mtDNA copy number, selection coefficient on infected mitochondria, relative fitness, etc.), and will greatly help us to understand if it is realistic and consistent with the available data. The ongoing increase of bivalve genomic resources will also help to better understand the mechanisms of germ line establishment, gametogenesis and mitochondrial inheritance, thus providing fundamental knowledge to test our suppositions. Another approach that would answer the question of the role of RPHM21 and RPHF22 would be the manipulation of their transcription/ expression (e.g., over/under expression, knock-out, RNAi), but this is a medium-term goal, since such techniques still need to be developed for bivalve molluscs. Last, to answer more general questions about the evolution of DUI, a more comparative approach will be necessary, but this will require the overcoming of the intrinsic challenges of comparative analyses (see, for example, Dunn et al. 2013; Roux et al. 2015).
Conclusions

The origin of DUI most likely entailed the invasion of at least one selfish genetic element, and the extant DUI systems can be seen as resolved conflicts (Passamonti and Ghiselli 2009). Specifically, we think that the acquisition of selfish features by a mitochondrial lineage could have started the genetic conflict that originated DUI, and that this process could have happened in independent events for single bivalve species, as described by the model in R. philippinarum. Under this light it is interesting to point out the similarities between the DUI system and one of the most studied cases of genomic conflicts, the cytoplasmic male sterility (CMS) in plants (reviewed in Chase 2007). CMS is responsible for gynodioecy, it consists of a condition under which a plant is unable to produce functional pollen, and it is determined by mitochondrial ORFs derived either from mitochondrial gene-coding and gene-flanking sequences, or from sequences of unknown origin. The 2 most striking CMS features that could be applied also to the model for DUI origin proposed here are the selfish nature of mitochondria, and the influence of a mitochondrial ORF on germ line development. Also, CMS is a clear example of nuclear-cytoplasmic sex determination, a scenario hypothesized also for DUI (Breton et al. 2007; Passamonti and Ghiselli 2009; Zouros 2000; Yusa et al. 2013). It was proposed that hermaphroditism was the ancestral condition of bivalves (Davison 2006), and a correlation between DUI and gonochorism was documented (Breton et al. 2011a). The invasion of sex-ratio distorters and the evolution of suppressors can prompt rapid transitions among sex-determination mechanisms (Bachtrog et al. 2014), and DUI might have been responsible for the shift from hermaphroditism to gonochorism in some bivalve species. If true, DUI would represent the first animal sex-determination system involving mtDNA-encoded proteins, paralleling CMS in plants.

But why do only bivalves have DUI? Is bivalve mitochondrial genome more prone to foreign sequence insertion/endogenization? Or is the nuclear genome more permissive to such kind of biological novelties? First of all, it is possible that modifications of SMI are present in other organisms, but they have not been discovered yet. That said, we think that bivalve molluscs have several features that could favor the emergence of a system like DUI. First, it is known that bivalve mitochondria can function in absence of oxygen, by switching to an anaerobic energy metabolism. This “anaerobically functioning mitochondria” appear to be widespread in species that often have to face hypoxic or anoxic conditions (Mueller et al. 2012). Functioning mitochondria appear to be widespread in species that could favor the emergence of a system like DUI. First, it is known that this process could have happened in independent events for single bivalve species, as described by the model in R. philippinarum. Under this light it is interesting to point out the similarities between the DUI system and one of the most studied cases of genomic conflicts, the cytoplasmic male sterility (CMS) in plants (reviewed in Chase 2007). CMS is responsible for gynodioecy, it consists of a condition under which a plant is unable to produce functional pollen, and it is determined by mitochondrial ORFs derived either from mitochondrial gene-coding and gene-flanking sequences, or from sequences of unknown origin. The 2 most striking CMS features that could be applied also to the model for DUI origin proposed here are the selfish nature of mitochondria, and the influence of a mitochondrial ORF on germ line development. Also, CMS is a clear example of nuclear-cytoplasmic sex determination, a scenario hypothesized also for DUI (Breton et al. 2007; Passamonti and Ghiselli 2009; Zouros 2000; Yusa et al. 2013). It was proposed that hermaphroditism was the ancestral condition of bivalves (Davison 2006), and a correlation between DUI and gonochorism was documented (Breton et al. 2011a). The invasion of sex-ratio distorters and the evolution of suppressors can prompt rapid transitions among sex-determination mechanisms (Bachtrog et al. 2014), and DUI might have been responsible for the shift from hermaphroditism to gonochorism in some bivalve species. If true, DUI would represent the first animal sex-determination system involving mtDNA-encoded proteins, paralleling CMS in plants.

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At the moment it is not known whether in DUI species the presence of lineage-specific mitochondrial proteins (or RNAs) in germ cells can drive the gonad development toward 1 specific sex, or if their segregation into male (female) germ cells is simply a consequence of being a male (female). If the scenario proposed here will be proved correct, DUI will become another example of how a selfish element (in this particular case a segregation distorter of viral origin) can profoundly affect the biology of an organism, and eventually its evolution.

Funding

This study was supported by the Italian Ministry of Education, University and Research MIUR - SIR Programme no. RBSS14G0F5 funded to LM, by the Italian Ministry of Education, University and Research MIUR - FIR Programme no. RBFR13T97A funded to FG, and by the Canziani bequest funded to MP.

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