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Did doubly uniparental inheritance (DUI) of mtDNA originate as a cytoplasmic male sterility (CMS) system?

Sophie Breton[1], **Donald T. Stewart**[2], **Julie Brémaud**[1], **Justin C. Havird**[3], **Chase H. Smith**[3], **Walter R. Hoeh**[4]

[1]Département des sciences biologiques, Université de Montréal, Montréal, Québec, Canada

[2]Department of Biology, Acadia University, Wolfville, Nova Scotia, Canada

[3]Department of Integrative Biology, The University of Texas at Austin, Austin, Texas, USA

[4]Department of Biological Sciences, Kent State University, Kent, Ohio, USA

Abstract

Animal and plant species exhibit an astonishing diversity of sexual systems, including environmental and genetic determinants of sex, with the latter including genetic material in the mitochondrial genome. In several hermaphroditic plants for example, sex is determined by an interaction between mitochondrial cytoplasmic male sterility (CMS) genes and nuclear restorer genes. Specifically, CMS involves aberrant mitochondrial genes that prevent pollen development and specific nuclear genes that restore it, leading to a mixture of female (male-sterile) and hermaphroditic individuals in the population (gynodioecy). Such a mitochondrial-nuclear sex determination system is thought to be rare outside plants. Here, we present one possible case of CMS in animals. We hypothesize that the only exception to the strict maternal mtDNA inheritance in animals, the doubly uniparental inheritance (DUI) system in bivalves, might have originated as a mitochondrial-nuclear sex-determination system. We document and explore similarities that exist between DUI and CMS, and we propose various ways to test our hypothesis.

Keywords

bivalves; mitochondria; mitonuclear interactions; plants; sex determination

INTRODUCTION

Although a rich diversity of sexual systems exists in animals and plants, two are most common and evolutionary stable. The first is dioecy or gonochorism, a system in which individuals reproduce in only one sexual role during their lifetime with males producing

Correspondence: Sophie Breton, Département des sciences biologiques, Université de Montréal, Montréal, QC H2V0B3, Canada. s.breton@umontreal.ca.

CONFLICT OF INTEREST

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sperm and females producing eggs. The second is simultaneous hermaphroditism with out-crossing, in which individuals can reproduce through both sperm and eggs in a single breeding season, and mate with other individuals in both the male and female sexual roles.[1] The other, less evolutionary stable sexual systems include environmental sex determination, exclusively selfing hermaphroditism, sequential hermaphroditism, trioecy, gynodioecy and androdioecy[1] (Table 1). Sexual systems can be conserved across a given taxonomic level, but some lineages exhibit a wide diversity among species, or even among populations within the same species.[2] Indeed, it remains enigmatic why some taxa quickly evolve diversity in sexual systems while others remain unchanged over hundreds of millions of years.

Multiple transitions from simultaneous hermaphroditism to dioecy (or the reverse) have been studied in depth in plants,[3,4] but fewer examples are known in animals.[1,5] For angiosperms, two main paths to dioecy from hermaphroditism have been proposed: one is via monoecy, which involves hermaphrodites with separate male and female flowers as opposed to “perfect” hermaphrodites with flowers bearing both male and female parts.[6,7] In this scenario, a hermaphroditic population gradually increases sexual specialization because of disruptive selection (in which extreme values for a trait are favored over intermediate values), such that some individuals produce more male flowers over female ones and vice versa.[7] In these situations, individuals that specialize in being either male or female are more fit than those with both flower types. The second path to dioecy implicates a gynodioecious intermediate.[3,6–8] The underlying genetic transitions have been modeled for this scenario,[3,6–8] in which a male-sterility mutation first occurs in a hermaphroditic population (Figure 1). Once gynodioecy has evolved, hermaphrodites will experience greater selective pressure to produce more pollen and specialize in being male, eventually leading to dioecy if selection ultimately favors those individuals that only produce pollen (Figure 1). For the reverse path, that is, dioecy to simultaneous hermaphroditism, it is assumed that females able to produce small amounts of pollen for self-fertilization might be favored when population density drops and mates are difficult to find,[1,8,9] thus leading to androdioecy (Figure 1). Once androdioecy has evolved, males will experience a relative disadvantage as fewer ova become available for them to fertilize. Selection for more male sex allocation in hermaphrodites can ultimately lead to stochastic loss of males and a simultaneous hermaphroditic sexual system[1,8] (Figure 1).

In the majority of gynodioecious plant species, sex is determined by an interaction between cytoplasmic male-sterility (CMS) genes encoded in the mitochondrial genome that disrupt viable pollen production (leading to female individuals), and nuclear restorer-of-fertility alleles (restorer genes or *Rf* genes) that counteract the CMS factor when they occur in the same individual (leading to hermaphroditic individuals).[7,10,11] CMS genes are often chimeric and can contain pieces of multiple mitochondrial genes fused together. They are thought to be created by recombination and rearrangement of mtDNA, which is rampant in plants[10,12] (see also next section). Compared to plant mtDNAs (200–2000 kb),[13] the extremely compact nature of bilaterian animal mtDNAs (typically ~16–17 kb) and putatively limited role for mtDNA recombination in animals suggests that there is little room for evolutionary novelties such as the emergence of chimeric or novel genes. This, together with the rarity of gynodioecious animals,[5] suggests that CMS might be a rather uncommon phenomenon outside plants.

However, CMS in bivalve mollusks might be possible. Bivalves display relatively large variation in the size of their mitochondrial genomes (< 14 to > 67 kb), their gene order, and even gene content.[14,15] Moreover, gynodioecy, androdioecy and trioecy have all been reported in bivalves[16–19]; indeed, bivalves, and molluscs more generally, exhibit diverse sexual systems.[20] Mitochondrial genes have also been hypothesized to influence sex-determination pathways in bivalves with doubly uniparental inheritance (DUI) of mtDNA[21,22] (see also the DUI section below). DUI is a bivalve-specific mitochondrial transmission system discovered in the early 1990s[23–26] that involves two divergent sex-linked mtDNAs that are transmitted through eggs or sperm.[27–30] The association between a male-transmitted genome and maleness, which has been observed in more than a hundred species of freshwater mussels, marine mussels and marine clams representing five bivalve orders,[31–33] has led some to hypothesize a role for mitochondria and mtDNA in sex-determination mechanisms in bivalves.[21,22,27,29,34–36] DUI bivalves also contain novel, mitochondrial protein-coding genes that are specific to either the male-transmitted or the female-transmitted genomes in addition to the typical set of 13 oxidative phosphorylation (OXPHOS) genes found across animals.[21,37–41]

Although many parallels exist between DUI and CMS, previous studies have only cursorily explored the hypothesis that DUI had its origins as a cytoplasmic sex-determination system.[14,21,42,43] In this paper, we document and further explore similarities that exist between DUI and CMS. We hypothesize that DUI originated as a nuclear-cytoplasmic sex determining system and that mitonuclear sex determining mechanisms might either still be operational in extant species or be resurrected in certain contexts in contemporary bivalves. We also propose various ways to test this hypothesis.

CMS: SELFISH MITOCHONDRIA, THEIR NUCLEAR EQUALIZERS, AND SEX DETERMINATION

As mentioned above, mitochondrial-encoded genes in plants can cause CMS.[44–46] Because mtDNA is primarily maternally inherited, any mitochondrial mutations that increase female fitness might spread, even if they decrease male function.[47–50] In other words, mutations that negatively affect only males might accumulate in populations because deleterious male-specific fitness effects are “invisible” to natural selection of the maternally transmitted mitochondria. This process has been termed the “mother’s curse”.[49] CMS is a classic example of such selfish mitonuclear conflict: mtDNA variants that cause male sterility can also cause increased female fitness through reallocation of resources to female reproduction or increased offspring fitness through forced outcrossing.[51–53]

The genetic bases of CMS systems are beginning to be described in detail. The key mitochondrial genes responsible for CMS have been identified in several lineages and show remarkable variability. Two main types of CMS genes have been documented: (i) chimeric genes that contain parts of typical mitochondrial genes (e.g., OXPHOS genes), and (ii) novel genes which do not have known homology with other species (ORFans).[44,46,54] CMS genes have arisen independently many times and likely relatively quickly. For example, in different populations of *Lobelia siphilitica*, several different mitotypes (mitochondrial

or mtDNA haplotypes) were found to be segregating at different frequencies, likely representing many novel CMS types.[55] Because plant mtDNA undergoes intragenomic recombination regularly, structural variation can be rapidly generated in plant mitochondrial genomes,[56] which in combination with gene duplication can provide the raw genomic fuel to generate new CMS genes.[57] Also, novel mtDNA can often be acquired in plant mitogenomes via horizontal gene transfer from other lineages.[58,59] Plant mtDNA is therefore a hotspot for generating the chimeric genes and ORFans underlying CMS.

When new CMS genes arise, there should be strong selection on nuclear-encoded genes to counteract them and restore fertility, as nuclear encoded genes benefit from male transmission. Such nuclear restorer of fertility (*Rf*) genes have been documented in many species and are predominantly members of the pentatricopeptide repeat (PPR) family of genes.[46,57,60–63] PPR genes are numerous in plant nuclear genomes (about ~450 *Arabidopsis* proteins contain PPR motifs[64]), are usually targeted to the mitochondria or other organelles, and often function in RNA editing or other posttranscriptional processing. As expected, putative *Rf* genes are under strong positive selection in populations with CMS genes,[61,65,66] consistent with an evolutionary arms race between mitochondrial CMS genes and nuclear *Rf* genes. The result is that many plant populations show no outward signs of CMS due to suppression by locally adapted *Rf* genes. However, during crosses or migration between populations or species, CMS genes are left unchecked when they are placed against a naïve nuclear background and male sterile individuals (i.e., females) are produced.[67]

While the genetic controls of CMS have received much attention, less is known regarding the physiological mechanisms of male sterility and fertility restoration. The “ATP hypothesis” posits that ATP production is compromised by CMS genes (e.g., because CMS proteins might disturb the formation or the conformation of OXPHOS complexes[68]). Although CMS genes are expressed in all tissues, only developing pollen grains are affected because pollen development is especially energetically expensive.[69,70] This hypothesis would explain how many diverse CMS genes all cause similar aberrant pollen formation phenotypes. However, this assumption has been questioned, as *Arabidopsis* with knocked down ATP production still produce functional pollen.[71] Another set of hypotheses, collectively termed the “pollen hypothesis”, suggests there is a pollen-specific substance that interacts with mitochondria in anthers that ensure pollen development, and this interaction is altered in CMS lines.[70,72] This hypothesis has received some empirical support, as CMS proteins appear to preferentially accumulate in anthers,[73] but are degraded by a protease in vegetative tissue.[74] Transcriptomic and proteomic CMS studies have identified putative nuclear interactors in pollen grains,[75,76] but these remain to be confirmed. CMS lines also show increased ROS production and altered patterns of programmed cell death in tapetal cells, which are critical to pollen development.[73,77–80] Presumably, *Rf* genes mainly counteract CMS by post-transcriptional silencing of CMS genes, although CMS restoration at the genomic level and through translational and post-translational mechanisms has also been reported.[54,63,81,82] The exact mechanisms by which CMS genes cause male sterility and how much variation in these processes exist across plants remain important questions.

DUI: SEX-SPECIFIC MITOCHONDRIAL LINEAGES AND SEX-SPECIFIC MITOCHONDRIAL GENES

As in plants, mtDNA is primarily maternally inherited in animals.[83,84] The DUI system in bivalves is one exception, where both parents transmit their mitochondria and mtDNA to their offspring: the female-type (referred to as F-mtDNA) is transmitted by the mother to both daughters and sons, and the male-type (referred to as M-mtDNA) is transmitted by the father to only sons.[25,26] Females are mostly homoplasmic and only possess the F-mtDNA, whereas males are globally heteroplasmic and possess the maternally inherited F-mtDNA in their somatic tissues (with M-mtDNA sometimes present in variable amounts) and exclusively paternally inherited M-mtDNA in their sperm.[29,30,85–88]

The F- and M-mtDNA can be highly divergent, reaching more than 50% divergence in amino acid sequences in some freshwater mussels (e.g., *Quadrula, Echyridella menziesi*; order Unionoida) and marine clams (e.g., *Scrobicularia plana*; Cardioida),[32,89,90] and 10%–35% in marine mussels (e.g., *Mytilus* spp., *Geukensia demissa*; *Modiolus*; Mytiloida) and marine clams (e.g., *Ruditapes philippinarum*; Veneroida).[29,30,91] There is still an ongoing debate regarding DUI origin, that is, a single origin of DUI in an ancestral bivalve lineage, with multiple losses during bivalve phylogenesis, or multiple independent origins of DUI.[29,30] Nevertheless, the existence of a radically different mitochondrial inheritance system raises the questions of how and why DUI has been maintained within bivalves.

The molecular basis of DUI has been investigated mainly through transcriptomic studies,[92–94] and there seems to be a consensus for a role of ubiquitination, a process that is also involved in the strict maternal inheritance (SMI) of mitochondria in other animal species.[84] The question of why DUI has appeared and been maintained in bivalves has also received much attention. One hypothesis is that in the absence of heteromorphic sex chromosomes, sex-specific mtDNAs could be involved in sex determination.[21,22,27,34] This hypothesis has gained strong support with the discovery of mitochondrial sex-specific genes in DUI bivalves with still unknown functions, that is, the F-*orf* gene (F-specific functional Open Reading Frame) in the F-mtDNA and the M-*orf* gene (M-specific functional Open Reading Frame) in the M-mtDNA.[21,32,37,39,41,42] Breton and colleagues[21] hypothesized that DUI would be responsible for the maintenance of dioecy in unionoids based on their demonstration that sex-specific mt genomes follow a traditional XY system of sex determination, and closely-related hermaphroditic species do not possess M-type mtDNA. In addition, the remaining maternally-transmitted genome in hermaphroditic species experiences highly divergent evolution in its F-*orf* gene. Specifically, hermaphrodites possess a highly modified F-*orf* sequence, called H-*orf*, relative to their dioecious relatives, and this situation has evolved independently at least four times in freshwater mussels.[21,40,42] However, the link among DUI, sex-specific mt genes, and the maintenance of dioecy remains uncertain.

Finally, DUI species possess other uncommon features regarding mitochondrial genes, particularly the cytochrome *c* oxidase subunit II gene in M-mtDNA (M*cox2*). In most DUI species, including all DUI unionoids and cardioids, and some veneroids and mytiloids, M*cox2* shows a unique 3′-extension or a large in-frame insertion, extending the COX2

protein, which is usually ~250 amino acids long, by ~100 up to 1892 additional amino acids.[32,95–99] In freshwater mussels, this 3′-M*cox2*-extention is transcribed, translated and localized in both inner and outer membranes of sperm mitochondria, suggesting that the exposed C-terminus tail of MCOX2 at the mitochondrial surface could act as a specific tag determining the fate of sperm mitochondria in embryos.[95,96] As noted by Stewart and colleagues,[30] gaining novel and/or chimeric genes in the mitogenomes of DUI species might generate new gene products with functions beyond metabolic roles. As explored below, among these putative roles is the possible involvement of the DUI system in sex determination in bivalves, as is the case for the CMS system in plants.

SIMILARITIES BETWEEN CMS AND DUI

Cyto-nuclear sex determination and gynodioecy

The CMS-*Rf* system is the prevalent sex determination system found in natural gynodioecious plant species,[11] and such nuclear-cytoplasmic sex determination is theoretically expected to occur in the majority of natural gynodioecious species.[100] Interestingly, Bivalvia is one of the rare animal groups in which gynodioecy and trioecy, a system often associated with evolutionary transitioning from gynodioecy to dioecy (or reverse), have been functionally identified, and to our knowledge, mainly involves species with DUI or closely-related species.[16–19,101–103]

In gynodioecious plants, theory predicts that female frequency might be highly variable among populations when sex is determined by interactions between several nuclear and cytoplasmic genetic factors, some of which may not be present in all populations.[104] For example, if a female parent gives rise to all-female progeny when crossed with different pollen-parents, one might suspect that the female carried a type of CMS factor for which nuclear *Rf* alleles were relatively rare or absent in the population.[104] In other words, within and among CMS plant populations, hermaphrodite individuals may have a male-fertile cytoplasm, or a male-sterile cytoplasm interacting with a nuclear restorer allele, and when the correct complement of nuclear *Rf* allele(s) is absent, some parental crosses may produce 100% female progeny.[100,104] Interestingly, the sex ratio resulting from parental crosses in several marine and freshwater DUI species can vary from one extreme (100% female progeny) to the other (100% male progeny) depending on the impact of the mother only, suggesting the presence of a key maternal factor in eggs involved in sex determination.[105–109] In *Mytilus* spp., the proportion of female parents that give rise to all-female progeny can be high [e.g., 25% (6/24) in wild-caught *M. galloprovincialis*,[105] 29% (14/49) in a pedigreed experiment in *M. edulis*[106] and 32% (10/31) in wild-caught *M. edulis*,[35,107]] whereas female parents that give rise to all-male progeny are relatively rare (i.e., < 1%).[35,105,106] In other words, a pattern of producing purely female offspring, as predicted with mitonuclear mismatches and CMS, is common in the DUI-possessing genus *Mytilus*.

Plant-like mtDNAs in bivalves

CMS has been linked to the recombinogenic and repetitive nature of plant mitogenomes, two characteristics that may cause genomic rearrangements that are a source of novel ORFs (ORFans that do not match a known coding DNA sequence in other species) or chimeric

genes, which are key drivers in the evolution of CMS.[10,110–112] Genomic rearrangements are thus frequently observed within a species (also among closely-related species) for CMS-inducing genomes versus "fertile" ones. These characteristics, that is, recombination, repeats and rearrangements, have also been reported in bivalve mtDNAs,[113,114] including in DUI species.[15] Furthermore, genomic rearrangements with novel ORFs or chimeric genes have been observed among closely-related DUI and non-DUI species,[21,40,115] as well as within DUI species (e.g., *Mytilus*).[116–118] For example, the F-*orf* and M-*orf* genes both occur in DNA regions corresponding to gene order rearrangements observed among F or M mtDNAs or between F and M mtDNAs in freshwater mussels.[21,42] In particular, the *atp8-nad4L* region, which contains the M-*orf* gene in the M mtDNA of freshwater mussels, is known as a hotspot for significant rearrangements, gene duplications and "gene chimerization"[40,90] (see also below). Another observation congruent with plants is recombination events between the F and M mtDNAs in marine mussels *Mytilus*.[29] These events appear to primarily occur in the control regions (CRs), in which sex specific F- and M-*orfs* are housed,[38,39] and often result in the introduction of the M-*orf* gene (i.e., M-type CR portions containing M-*orf*) in an otherwise F-type mtDNA. To our knowledge, the reverse (i.e., the introduction of F-type CR portions and F-*orf* in an M-type mtDNA) has yet to be reported. Interestingly, the acquisition of the M-*orf* gene by an F mtDNA also often results in the "masculinization" of the F mtDNA, which is subsequently transmitted through sperm[29,117,119,120] (see Box 1). It thus seems that the acquisition of an M-*orf* is a prerequisite for a mtDNA that functioned as an F-type genome to become a functional M-type genome, although this has not been fully demonstrated.[29,121]

ORFans and chimeric ORFs in CMS and DUI systems

As seen above, CMS is associated with ORFans or chimeric genes that contain parts of typical mitochondrial genes. In other words, CMS genes in plant mitochondria are often composed of pieces of duplicated OXPHOS genes. Evidence suggests a similar situation in some DUI bivalves. Sex-specific F-*orfs* and M-*orfs* in DUI bivalves are speculated to have originated from endogenization of viral genes[39,42] or mitochondrial gene duplication.[42] In freshwater mussels in particular, *in silico* analyses suggested that the F-*orf* might have originated from a *nad2* mitochondrial gene duplication, and the M-*orf* from an *atp8* mitochondrial gene duplication, with the M-*orf* clearly containing segments of *atp8* in some species.[40,42,90]

CMS genes are usually known to encode small transmembrane proteins (10-35 kDa) that sometimes show specific spatiotemporal accumulation in male parts of the flower such as anthers and microspores to interfere with pollen development.[82] Moreover, mitochondrially-encoded CMS proteins may sometimes localize outside of mitochondria and exert their function via interactions with cellular components other than the mitochondrion.[122] In DUI species, F-*orf* and M-*orf* genes (and H-*orf* genes in hermaphroditic unionoids) are also known to encode transmembrane proteins that localize inside but also outside mitochondria.[21,39,41,42,123,124] While spatiotemporal expression has not been studied for H-ORF proteins in hermaphroditic freshwater mussels, the available data for F-ORF proteins in dioecious freshwater mussels indicate a presence in mitochondria and nuclei of eggs,[21] as well as sperm mitochondria.[123] In the marine mussel *M.*

edulis, the F-ORF protein seems to accumulate preferentially in male gonads and sperm mitochondria.[41] Because gametes are usually homoplasmic for their sex-specific mtDNA in DUI bivalves,[86] this suggests that the mtDNA-encoded F-ORF protein is exported from F-type mitochondria and imported into sperm mitochondria.[41] The data available for M-ORF proteins indicate a presence in male gonads in freshwater mussels,[37] and a progressive accumulation in mitochondria and nuclei of spermatogenic cells (and an absence in eggs) in the marine clam *Ruditapes philippinarum*.[124]

To date, the functions of F-ORF, M-ORF, and H-ORF proteins are unknown. However, it has been hypothesized that the M-ORF protein could be a masculinizing factor whereas the F-ORF protein could be a feminizing factor.[39,42] In freshwater DUI mussels in particular, hermaphroditism has evolved independently multiple times and in each of the hermaphroditic lineages, the M genome is apparently lost, and the F-*orf* of the F genome experiences highly divergent evolution and becomes an H-*orf*.[21,40,42,90] Therefore, it has been suggested that the F-ORF protein could inhibit testicular development in embryos that will become females in dioecious species, while the extreme modifications seen in the H-ORF protein (or its absence) could explain why the development of some testicular tissue is not completely inhibited in closely-related hermaphroditic species.[30,42] This hypothesis is also supported by the observation in marine mussels *Mytilus* that recombinant mtDNAs that have transitioned from F- to M-types (see point 3 above and Box 1), always contain truncated F-*orfs* or no F-*orf* in addition to having acquired an M-*orf* gene.[38,121] This suggests that either a complete, intact F-ORF protein might be necessary to avoid producing males and/or a complete, intact M-ORF protein might be necessary to produce sperm.

Heteroplasmy and substochiometric shifting

Mitochondria in plants with CMS are frequently heteroplasmic, often containing substoichiometric mtDNA molecules at low copy number in addition to an abundant primary mitogenome.[125] Sometimes, these coexisting substoichiometric mtDNAs are amplified and take on the role of the primary molecule, thus being responsible for "substoichiometric shifting" or rapid and dramatic changes in relative copy number of substoichiometric molecules within a generation.[125,126] Genomic shifting usually involves a single substoichiometric molecule, often containing recombination-derived chimeric sequences, and can alter plant phenotype by activating or silencing mtDNA sequences located on the shifted molecule. Thus, if the mitochondrial population consists of both CMS and "male-fertile" mtDNAs, substoichiometric shifting might be responsible for transition between the CMS and hermaphroditic condition and back again.[125,126] This heteroplasmy may result from recombination and/or mutations but also from paternal leakage of mtDNA.[125,127] Also, intra-individual segregation of different mitotypes may result in plants with mosaic phenotypes. For example, the segregation of one mitotype conditioning development of the male flower function and another inhibiting it was proposed to explain the spatial distribution of female and hermaphrodite flowers found on the same Silene vulgaris plants.[128] It was also proposed that the proportion of the mitotypes could change progressively during plant growth, leading to different sexual phenotypes in different parts of the plant.[125] These phenomena could result from an interaction between particular nuclear genes and

mitotypes that are under selection to guard specific mtDNA configurations and eliminate or reduce the copy number of others.[125]

Interestingly, recent investigations on the DUI bivalve *R. philippinarum* revealed the presence of heteroplasmy (i.e., presence of both F- and M-mtDNAs) at the organelle level in undifferentiated germ cells of both sexes, whereas homoplasmy was observed for the F mtDNA in eggs and for the M mtDNA in sperm.[86] It is tempting to speculate that a mechanism exists in DUI species that is similar to what has been proposed in plants, where changes in the proportion of the mitotypes in undifferentiated germ cells change during development leads to different sexual phenotypes. The recent report of a hermaphroditic species with DUI (*Semimytilus algosus*), which possesses a sperm producing gonad (that contains M-mtDNA) located on one half of the body and an egg producing gonad (that contains F-mtDNA) located on the opposite side of the body,[103] supports this. In this species, cell-specific segregation and/or preferential replication of mitotypes could lead to different sexual phenotypes in different parts of the animal, similar to the spatial distribution of hermaphrodite and female flowers in some plants.

A HYPOTHESIS FOR DUI ORIGINATING AS A NUCLEAR-CYTOPLASMIC SEX DETERMINING SYSTEM

It has been hypothesized that DUI first emerged in an ancestral hermaphroditic species, in which both eggs and sperm were produced in an ovotestis.[21,27,124,129] Our hypothetical scenario for the origin of DUI as a nuclear-cytoplasmic sex determining system would start with the origin of a CMS-causing gene in this ancestral hermaphroditic lineage, that is, with the origin of the“feminizing” F-*orf* gene[42] (Figure 2). The presence of this CMS factor causing gynodioecy and female-biased sex ratios then selected for paternal transmission (leakage) of the male-fertile mitochondrial genome (i.e., without the F-*orf*). This idea of selection for paternal mitochondrial leakage has been proposed by Burt and Trivers[130] and McCauley[53] for the following reason: in order to transmit genes via sperm, it is evolutionary advantageous for a nuclear genome to be paired with a male-fertile mitotype. In a gynodioecious system, paternal mitochondrial leakage enhances the probability of producing a male-fertile phenotype since a sperm donor must be a hermaphrodite that is more likely to carry a fertile phenotype, while a sperm recipient could be either hermaphroditic or female. As suggested by McCauley,[53] leakage of male-fertile mtDNAs through sperm produced by hermaphrodites can eventually offset the added productivity often associated with females carrying CMS. In early bivalves, this situation might have set the stage for the evolution of pure paternal inheritance or DUI (with further genes, likely nuclear, involved in the process), together with the invasion of gynodioecious populations by female-sterility mutation(s) or factors (i.e., masculinizing factors). If these mutations/factors increase male function sufficiently to compensate for the loss of female function, then “pure males” will successfully establish and lead to subdioecy or dioecy (Figure 2). We propose that such female-sterile or “masculinizing” factors could be the chimeric M-*orf* gene, and/or the elongated *cox2* gene in the paternally-transmitted M mtDNAs of DUI bivalves. This is in line with the recent demonstration that sperm and their M-type mitochondria express a “M-specific phenotype” in DUI species, which is characterized by low OXPHOS rates

and an almost null spare capacity of the cytochrome *c* oxidase complex compared with F-type mitochondria, a phenotype that could potentially be detrimental in somatic tissues or eggs.[131,132]

Our hypothetical scenario is somewhat different than the one proposed by Milani and colleagues[124] to describe how DUI and dioecy might have evolved together in *Ruditapes philippinarum*. Based on in silico evidence that the M-*orf* might be of viral origin in some bivalve species,[39] Milani and colleagues[124] proposed that a viral infection of some mitochondria would have conferred upon them the ability to avoid degradation in embryos, and to be preferentially transmitted through generations (i.e., segregation distortion). Specifically, in a population of hermaphroditic *R. philippinarum*, infected mitochondria would have spread more efficiently through sperm, allowing paternal inheritance of mitochondria, selection for mtDNA mutations that increase male fitness and emergence of males in the population. This transition from hermaphroditism to androdioecy would have set up a condition for selective pressure favoring egg production in hermaphrodites, eventually leading to the evolution of dioecy (with DUI) from androdioecy.[124] While gynodioecy, not androdioecy, precedes dioecy in our hypothesis, viral infections could have led to the establishment of M-*orf* genes in our hypothesis, and the two hypotheses might have complementary components. At this moment, the two hypothetical scenarios need further evaluation.

EVALUATING THE CMS/DUI HYPOTHESIS

As stated above, the unique mode of inheritance and sex-biased mt architecture in DUI bivalves has led to the hypothesis that DUI is a critical component of sex determination.[21,22,29] Herein, we outline some methods to test our hypothesis that the DUI system in bivalves might have originated as a CMS system, in which mitochondrial factors interact with nuclear factors to determine the production of hermaphrodites, females, or males. While we acknowledge that this original system might have been replaced during bivalve evolution, we argue that mitonuclear sex determining mechanisms might still operate in multiple bivalve lineages or might be able to be resurrected in contemporary bivalves.

One way to determine if there are mitochondrial CMS genes in a species of interest is by comparing offspring sex ratios from reciprocal crosses, where pairs of parents are used to produce two sets of offspring, one set with one parent being the “egg parent” and the other the sperm donor, and the second set where their roles are reversed.[100] The two sets of offspring will have the same distribution of nuclear genes but will differ for cytoplasmic genes that are primarily maternally inherited, which will have a major effect on sex ratios in the presence (or absence) of CMS.[100] For DUI bivalves however, this strategy is somewhat problematic because the paternal mtDNA is also transmitted. Moreover, DUI bivalve species that are currently gynodioecious or dioecious might have evolved from populations with mitochondrial sex determining genes but for which sex determination has now come under complete nuclear control. For example, recent studies suggest extremely low within-species variability for the F-*orf* gene in bivalves.[133,134] That said, freshwater mussels might offer an opportunity to investigate the genetic basis of sex determination through reciprocal crosses because of the presence of gynodioecious species with SMI of mtDNA that are

closely related to dioecious DUI species.[16,17,135] Specifically, a cross using eggs from a dioecious (DUI) species and sperm from a closely related hermaphrodite species could be performed,[135,136] which in theory, should lead to all female progeny if the F-*orf* is acting as a CMS gene. Reciprocal crosses could also be done using pairs of SMI hermaphrodites, and if the offspring sex ratios differ, sex determination in the species would be under nuclear-cytoplasmic control.[100] Crossing distant DUI populations or even closely-related DUI species with divergent F-*orf* genes could also be considered. For example, we could expect that a female would produce male embryos when crossed with males from the same population but only (or predominantly) female embryos when crossed with males from distant populations (i.e., if the F-*orf* acts as a CMS gene and restorers are absent in the distant population).

Another approach would be to silence the F-*orf* gene (e.g., RNA interference or RNAi) and see if this results in hermaphroditic or male individuals, or if it causes downstream dysregulation of specific genes (e.g., genes in sex determination/differentiation pathways). An RNAi-like mechanism has been proven to operate in the mitochondria in an Ago2-dependent manner.[137] However, the technique is relatively untested with respect to the study of mitochondrial biology and has limitations of its own, for example, transfected small interference RNAs (siRNAs) are able to enter the matrix of mitochondria and affect mRNA levels, but their translational effect seems to be only recordable on relatively unstable proteins.[137] That said, RNAi has been widely used in bivalves,[138–142] although not at the mitochondrial level. An alternative approach would be to overexpress the F-*orf* or M-*orf* gene. In this case, we would expect sperm sterility with an overexpression of F-*orf* in male individuals (and female sterility with overexpression of M-*orf*), and potentially also cytotoxicity following heterologous gene expression, an effect that has been reported several times for CMS genes introduced in *Escherichia coli* or yeast cells.[110,111,143] Although the above-mentioned approaches might never convincingly confirm the hypothesis that DUI originally arose via CMS, they could provide a framework for testing the hypothesis that mitonuclear sex determining mechanisms still operate or might be reactivated in DUI bivalves. It is worth mentioning that the origin of DUI via CMS and mitochondrial sex determination in extant bivalve species could be two different, non-mutually exclusive hypotheses. In either case, this would indicate, for the first time, that mitochondrial genetic elements might be involved in sex determination in animals. Many of the above-mentioned studies are currently underway.

CONCLUSION

There are many intriguing parallels between bivalves with DUI and CMS in plants. Based on these similarities, we present a novel and testable hypothesis, namely that the DUI system in bivalves might have originated as a CMS system, in which mitochondrial factors interact with nuclear factors to determine sex, and that mitonuclear sex determining mechanisms could still perform their roles or might be reactivated in extant bivalve species. It is conceivable that nuclear-cytoplasmic sex determination might be more common than previously thought in animal species.[144,145] In order to further uncover and fully appreciate the implications of mitochondria in sex determination (and sex-ratio distortion), we must expand taxonomic sampling in a comprehensive manner, for example starting with animal

species with atypical sexual systems. Such studies will significantly contribute to a better understanding of fundamental evolutionary processes, such as the role of intergenomic conflict in sex determination.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Abbreviations:

CMS	cytoplasmic male sterility
CR	control region
DUI	doubly uniparental inheritance
ORF	open reading frame
OXPHOS	oxidative phosphorylation
PPR	pentatricopeptide repeat family of genes
Rf	restorer-of-fertility genes
RNAi	RNA interference
SMI	strict maternal inheritance
siRNA	small interference RNA

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Box 1:

Role reversal of the F mitogenome

Given that F and M mtDNAs regularly coexist in male embryos of DUI species, intermolecular mitochondrial recombination between both genomes has been frequently reported or inferred, but only fully described and characterized in *Mytilus* mussels.[29] Intermolecular recombination in *Mytilus* spp. often results in a "role-reversal" event where a F mtDNA invades the male route of inheritance and becomes transmitted through sperm, effectively reversing its sex-specific role.[29,119–121] This phenomenon has been highlighted following the discovery of genomes recovered from male gonads but with coding sequences almost identical to the F mtDNA.[29] Specifically, role-reversed mtDNAs are mostly F-type genomes but with mosaic control regions (CRs) consisting of M- and F-type segments.[29,119–121] It thus seems that the switch from F to M function is mediated through intermolecular recombination introducing M-like CRs (and M-*orfs*) in an otherwise F-type mtDNA, although this hypothesis still need to be clearly demonstrated.[29,121] Because they reset M-mtDNAs to be nearly identical to F-mtDNAs (except for the control region), role-reversal events have further complicated DUI origination hypotheses.[27,29,30,146,147]

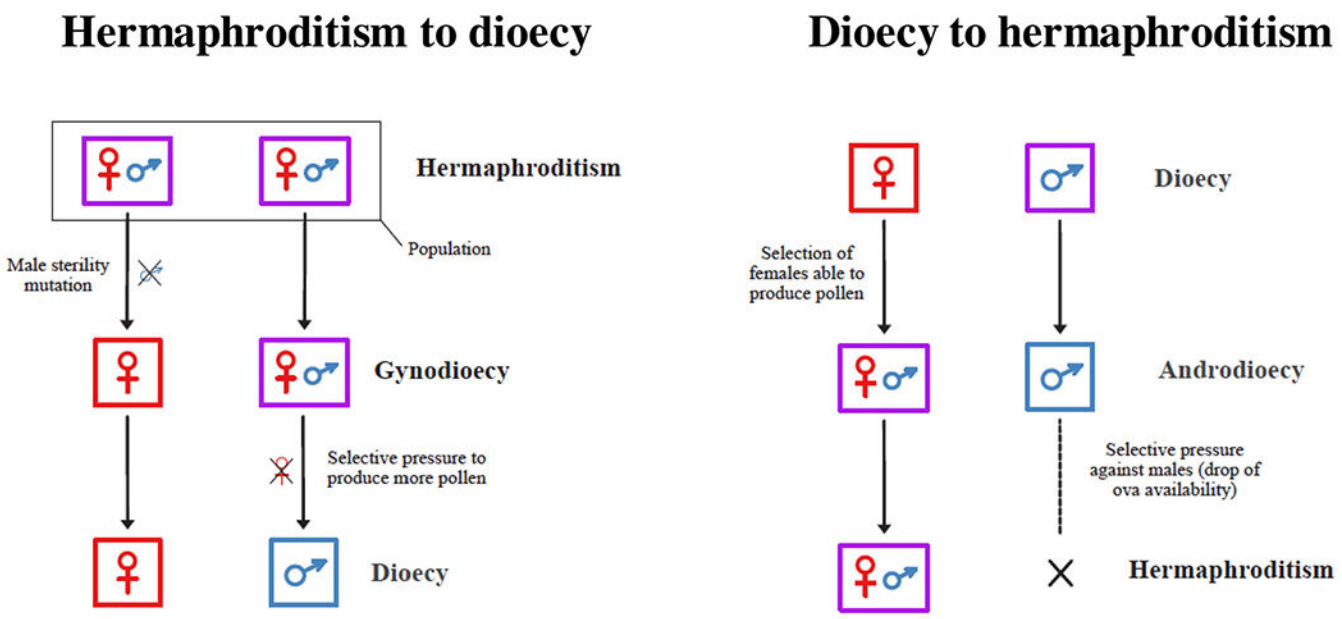

FIGURE 1.

Simultaneous hermaphroditism to dioecy (with a gynodioecious intermediate) and dioecy to simultaneous hermaphroditism (with an androdioecious intermediate)

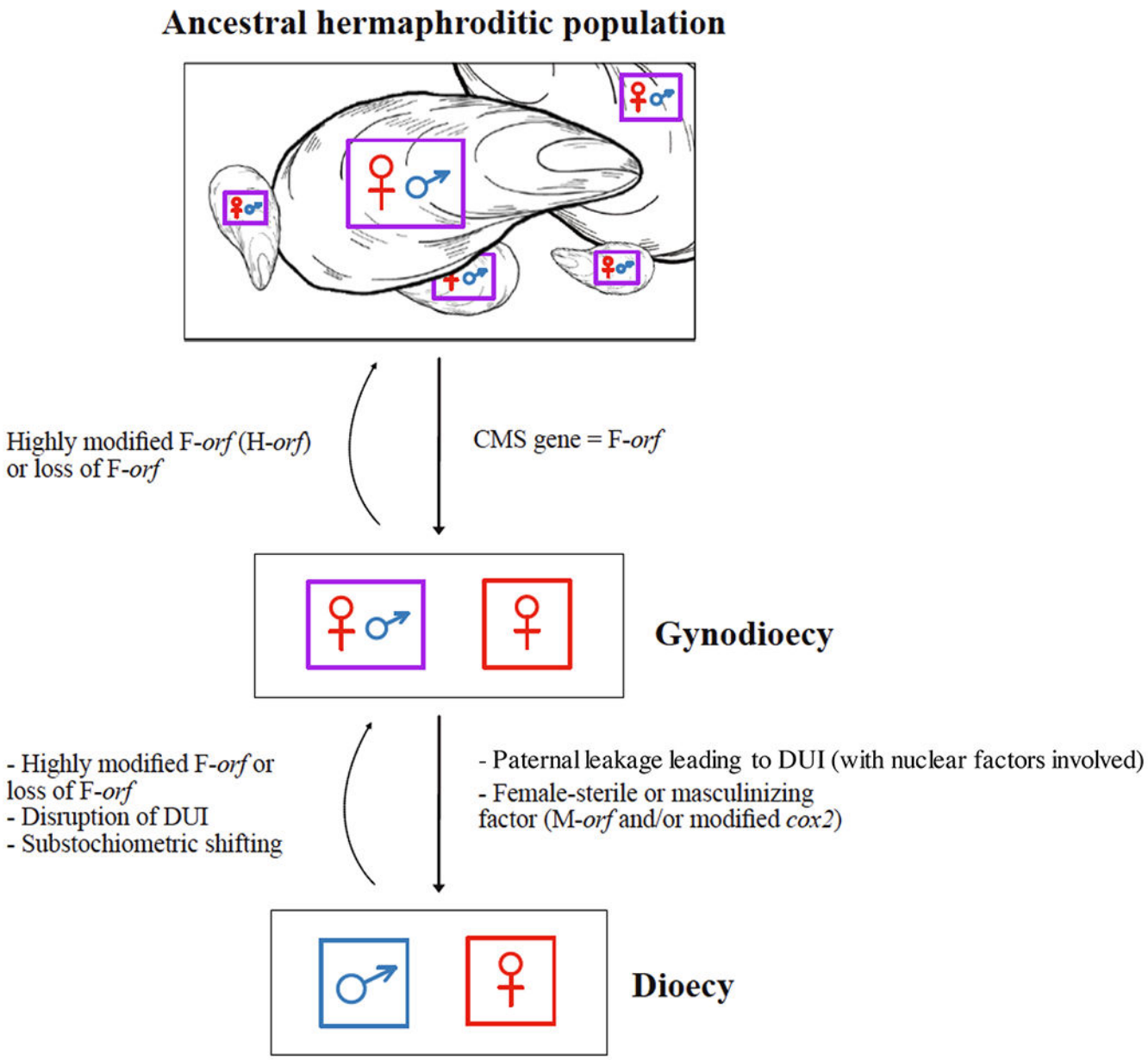

FIGURE 2.
Hypothetical scenario for the origin of DUI as a nuclear-cytoplasmic sex determining system

TABLE 1

Sexual systems in plants and animals

Sexual system	Definition
Dioecy or gonochorism	A sexual system in which individuals reproduce in only one sexual role during their lifetime with males producing sperm and females producing eggs
Hermaphroditism	A sexual system in which individuals can reproduce through both sperm and eggs in a single breeding season, and mate with other individuals in both the male and female sexual roles
Androdioecy	A sexual system which consists of populations composed of simultaneous hermaphrodites and males
Gynodioecy	A sexual system which consists of populations composed of simultaneous hermaphrodites and females
Trioecy	A sexual system which consists of populations composed of simultaneous hermaphrodites, females, and males
Environmental sex determination	A sexual system in which sex is established by non-genetic cues
Exclusively selfing hermaphroditism	A sexual system in which individuals reproduce through self-fertilization exclusively
Sequential hermaphroditism	A sexual system in which individuals reproduce through eggs during one part of their life and through sperm during another