Unusual Patterns of Mitochondrial Inheritance in the Brown Alga *Ectocarpus*

Laure Mignerot,[1] Chikako Nagasato,[2] Akira F. Peters,[3] Marie-Mathilde Perrineau,[1,4] Delphine Scornet,[1] Florian Pontheaux,[1] Walid Djema,[5] Yacine Badis,[1,4] Taizo Motomura,[2] Susana M. Coelho,[1] and J. Mark Cock*[,1]

[1]Sorbonne Université, CNRS, Algal Genetics Group, UMR 8227 Integrative Biology of Marine Models (LBI2M), Station Biologique de Roscoff (SBR), Roscoff, France

[2]Muroran Marine Station, Hokkaido University, Japan

[3]Bezhin Rosko, Santec, France

[4]The Scottish Association for Marine Science, Scottish Marine Institute, Oban, Argyll, Scotland

[5]Inria Sophia-Antipolis, Côte d'Azur University, Bicore and McTAO Teams, France

***Corresponding author:** E-mail: cock@sb-roscoff.fr.

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All the sequence data used in this study have been submitted to public databases and can be recovered using the accession numbers provided in supplementary table S1, Supplementary Material online.

Abstract

Most eukaryotes inherit their mitochondria from only one of their parents. When there are different sexes, it is almost always the maternal mitochondria that are transmitted. Indeed, maternal uniparental inheritance has been reported for the brown alga *Ectocarpus* but we show in this study that different strains of *Ectocarpus* can exhibit different patterns of inheritance: *Ectocarpus siliculosus* strains showed maternal uniparental inheritance, as expected, but crosses using different *Ectocarpus* species 7 strains exhibited either paternal uniparental inheritance or an unusual pattern of transmission where progeny inherited either maternal or paternal mitochondria, but not both. A possible correlation between the pattern of mitochondrial inheritance and male gamete parthenogenesis was investigated. Moreover, in contrast to observations in the green lineage, we did not detect any change in the pattern of mitochondrial inheritance in mutant strains affected in life cycle progression. Finally, an analysis of field-isolated strains provided evidence of mitochondrial genome recombination in both *Ectocarpus* species.

Key words: **brown algae, *Ectocarpus*, life cycle, uniparental inheritance, mitochondria, parthenogenesis, recombination.**

Article

Introduction

The sexual progeny of most eukaryotes inherit mitochondria from only one of their two parents (Birky 2001; Breton and Stewart 2015). This uniparental pattern of inheritance is thought to exist to control the spread of selfish genetic elements that may arise in the mitochondrial genome and to limit conflicts between mitochondrial and nuclear genomes (Sato and Sato 2013; Breton and Stewart 2015; Greiner et al. 2015). In organisms with different sexes, it is usually the mitochondria of the female parent (i.e., the partner with the largest gametes) that are transmitted to the progeny. One possible reason for this is that male gametes are usually more metabolically active, for example, because they are motile, and this may increase the risk of oxidative damage to the paternal mitochondrial genomes (Allen 1996; Lynch 1996; Roze et al. 2005; Greiner et al. 2015). In addition, in many species, the production of male gametes involves more cell divisions than the production of female gametes and this also increases the risk of mitochondrial genome mutation (Crow 2000; Greiner et al. 2015). Note that maternal mitochondrial inheritance may therefore be conducive to the production of large amounts of sperm, which will tend to improve fitness under conditions of broadcast dispersal or when sperm competition is high.

In oogamous species, where the large female gamete (the egg cell) contributes more mitochondria to the zygote than the small male gamete (sperm cell), a bottleneck phenomenon (Breton and Stewart 2015) could explain the disappearance of the paternal mitochondria. However, uniparental mitochondrial inheritance is also observed in isogamous species where the two gametes carry similar numbers of mitochondria, implying the existence of specific mechanisms that eliminate the mitochondria of one parent. For example, in the unicellular green alga *Chlamydomonas reinhardtii*, the mitochondrial genome contributed by the plus mating type parent is specifically eliminated during zygote maturation (Nakamura 2010) and this appears to be under genetic control (Nishimura et al. 2012). Specific mechanisms also exist to promote uniparental mitochondrial inheritance in oogamous species (Mishra and Chan 2014; Greiner

et al. 2015). These mechanisms are highly diverse and can act either before or after zygote formation. Prezygotic mechanisms include the elimination of mitochondria from male gametes, degradation of male gamete mitochondria before fertilization and prevention of male mitochondria from entering the egg cell during fertilization. Alternatively, selective degradation of the mitochondria or mitochondrial DNA of one parent can occur after formation of the zygote and again this can occur via several different mechanisms involving, for example, the ubiquitin-proteasome system or autophagy (Sato and Sato 2013).

There is accumulating evidence that many mitochondrial inheritance systems that have been classed as uniparental actually exhibit some level of heteroplasmy (i.e., transmission of both parental mitochondrial genomes to the offspring) or paternal leakage (Breton and Stewart 2015; Greiner et al. 2015). Strict uniparental inheritance of mitochondria is expected to lead to the accumulation of deleterious mutations in the mitochondrial genome due to the action of Muller's ratchet. It has been proposed that the mechanisms that promote uniparental inheritance are periodically relaxed over evolutionary time to allow mitochondrial genomes to recombine and thereby eliminate deleterious mutations (Takano et al. 2010; Greiner et al. 2015). "Leakage" of paternal mitochondria through to the progeny is also expected to limit the effects of Muller's ratchet but it is not clear whether leakage alone is sufficient. The broad diversity of the mechanisms by which paternal mitochondria are eliminated (see above) is consistent with periodical relaxation of uniparental inheritance in the sense that these mechanisms would need to re-evolve after each period of relaxed inheritance.

A number of organisms exhibit patterns of mitochondrial inheritance that deviate from the usual situation of uniparental maternal inheritance. These variations are of considerable interest because they can provide insights into the evolutionary and molecular mechanisms underlying mitochondrial inheritance. Examples include strict paternal inheritance in some plants including the sequoia tree (Neale et al. 1989), banana (Fauré et al. 1994), and cucumber (Havey et al. 2004). In some organisms, more than one mitochondrial genome may be transmitted to the offspring. For example, stable inheritance of maternal heteroplasmy has been described for terrestrial isopod crustaceans (Doublet et al. 2012). Biparental mitochondrial transmission has been reported for several species but there do not appear to be any cases where both maternal and paternal mitochondria are systematically transmitted to the zygote and then stably inherited throughout development (Breton and Stewart 2015). Therefore, even when inheritance is biparental, there are usually mechanisms that limit heteroplasmy, usually by ensuring that individual offspring carry either the maternal or the paternal mitochondria, but not both. For example, in the fungus *Coprinopsis cinerea*, progeny can inherit mitochondria from either one or the other parent (Wilson and Xu 2012). This pattern of inheritance occurs because heterokaryon formation involves an exchange of parental nuclei, but not mitochondria, between mating partners. In this case, therefore, there is no stage where mitochondria from both parents are mixed in a cell fusion product and therefore no need for selective elimination of mitochondria derived from one of the parents. In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, fusion of isogametes results in heteroplasmy but partitioning of mitochondria during budding actively promotes the formation of homoplasmic daughter cells (Birky 2001). Several bivalve species exhibit doubly uniparental inheritance, with mitochondrial being transmitted in a sex-specific manner (Zouros 2013). The female (F) mitochondrial genome is transmitted to females and males but the males do not transmit the F genome to their progeny, rather they transmit the male (M) genome, which is transmitted uniquely through the male line. Finally, novel patterns of mitochondrial inheritance have often been reported following interspecific crosses in a broad range of eukaryotic taxa (Hoarau et al. 2009; Breton and Stewart 2015; Montecinos et al. 2017). Under these conditions, mitochondrial inheritance systems may exhibit breakdown due to genome incompatibilities and therefore it is possible that the patterns observed are dysfunctional.

Studies of mitochondrial inheritance in the brown algae have reported maternal inheritance (Motomura et al. 2010). In oogamous species, male mitochondria are digested by lysosomes in the zygote (Motomura 1990), whereas in the anisogamous species, *Scytosiphon lomentaria* male mitochondria persist until the four-cell stage of sporophyte development (Motomura et al. 2010).

Ectocarpus is an emerging model species for the brown algae (Peters, Marie, et al. 2004; Cock et al. 2011). An earlier study indicated that mitochondrial inheritance is strictly maternal in *Ectocarpus* (Peters, Scornet, et al. 2004). However, a recent analysis of the species structure of the genus *Ectocarpus* (Montecinos et al. 2017) has indicated that the strains used in the 2004 study belonged to distinct cryptic species and therefore that the crosses were interspecific. Here, we analyzed mitochondrial inheritance in intraspecific crosses using pairs of strains from two of the recently defined *Ectocarpus* species (Montecinos et al. 2017). For one species (*Ectocarpus siliculosus*), we observed strict maternal inheritance, as reported previously, but, surprisingly, crosses between different strains of a second species (*Ectocarpus* species 7) produced progeny that exhibited either paternal uniparental inheritance or an unusual pattern of transmission where progeny inherited either maternal or paternal mitochondria, but not both.

The haploid–diploid, sexual life cycle of *Ectocarpus* involves alternation between the sporophyte generation and male and female individuals (dioicy) of the gametophyte generation (Müller 1967). Facultative variations on the sexual cycle, observed in laboratory cultures, include the capacity of gametes that do not fuse with a gamete of the opposite sex to develop parthenogenetically to produce a partheno-sporophyte (Müller 1967). Parthenogenetic capacity appears to be a ubiquitous feature of female gametes but, in some strains, the male gametes are also capable of parthenogenetic development (Mignerot et al. 2019). Based on an earlier suggestion that the mechanism that regulates mitochondrial inheritance in brown algae might influence male gamete parthenogenetic capacity (Han et al. 2014), we investigated a possible

correlation between the unusual patterns of inheritance observed in *Ectocarpus* and the parthenogenetic capacity of male gametes.

In addition, based on an earlier observation that a mutation affecting the *C. reinhardtii* gene *GSP1*, which is required for deployment of the diploid program in this green alga, exhibited aberrant mitochondrial DNA inheritance (Nishimura et al. 2012), we investigated patterns of mitochondrial inheritance in equivalent life cycle mutants in *Ectocarpus*. Finally, field-isolated *Ectocarpus* strains were analyzed for evidence of mitochondrial genome recombination.

Results

Development of Markers to Follow Mitochondrial Inheritance in Intraspecific Crosses

A recent analysis by Montecinos et al. (2017) identified the presence of at least 15 cryptic species within the genus *Ectocarpus* and indicated that an earlier study of mitochondrial inheritance in *Ectocarpus* (Peters, Scornet, et al. 2004) was based on interspecific crosses. To determine whether the conclusions of the earlier study held for intraspecific crosses, we developed molecular markers to distinguish between polymorphic forms of the mitochondrial genome in two of the *Ectocarpus* species defined by Montecinos et al. (2017): *E. siliculosus* sensu stricto (hereafter *E. siliculosus*) and *Ectocarpus* species 7. Note that *Ectocarpus* species 7, which corresponds to the reference genome species (Cock et al. 2010; Cormier et al. 2017), was earlier referred to as *E. siliculosus* under the older classification system but this nomenclature needs to be revised.

The mitochondrial genome sequence of the male *Ectocarpus* species 7 strain Ec32, which had been initially assembled using Sanger sequence data (deposited as *E. siliculosus* with Genbank accession number FP885846.1), was re-evaluated using high-coverage Illumina shotgun sequence data and two sequencing errors were corrected. The corrected *Ectocarpus* species 7 strain Ec32 mitochondrial genome is available through the accession number FP885846.2 (fig. 1A). The mitochondrial genome of the female *E. siliculosus* strain EA1 was assembled using whole genome shotgun sequence data (supplementary table S1, Supplementary Material online) and the Ec32 genome as a guide (fig. 1A). The EA1 mitochondrial genome is available through the accession number MK045263. Whole genome sequence data were then generated for independently isolated strains of both species (RB1 and EcNAP-12-24 for *E. siliculosus* and Ec568 for *Ectocarpus* species 7) and each data set was mapped onto the corresponding, conspecific mitochondrial genome to identify intraspecific polymorphisms. This analysis identified 28 and 6 intraspecific SNPs for the *E. siliculosus* and *Ectocarpus* species 7 mitochondrial genomes, respectively (fig. 1A and supplementary table S2, Supplementary Material online). Two additional mitochondrial SNPs, between *Ectocarpus* species 7 male strain Ec246 and female strain Ec856 (a sister of Ec568) were detected by Sanger sequencing of a region of the mitochondrial genome amplified by PCR from strains Ec246 and Ec856. These latter SNPs correspond to A to G transitions at positions 31684 and 31744 (fig. 1A and supplementary table S2, Supplementary Material online).

Based on the above SNPs, two and five dCAPS markers (Neff et al. 1998) were developed for *E. siliculosus* and *Ectocarpus* species 7, respectively (fig. 1A and supplementary table S3, Supplementary Material online). The sensitivity of the dCAPS markers was tested by carrying out amplifications from samples in which parental DNA had been mixed in different proportions (50:50, 20:80, 10:90; 5:95). This analysis showed that the dCAPS assays distinguished between male and female alleles and were able to detect the presence of mixtures of mitochondrial DNA from the two parents (equivalent to biparental inheritance) provided they were in approximately equal proportions (fig. 1B).

Mitochondria DNA Inheritance in *E. siliculosus* and *Ectocarpus* Species 7

To analyze mitochondrial inheritance, intraspecific crosses were carried out between various male and female strains of *E. siliculosus* and *Ectocarpus* species 7. The heterozygous sporophytes derived from the crosses were isolated and PCR amplifications were carried out to verify that they carried both the female (U) and the male (V) sex chromosome. This step allowed the elimination of any haploid individuals that had arisen via gamete parthenogenesis rather than gamete fusion and zygote formation (supplementary table S1, Supplementary Material online).

dCAPS analysis of 20 sporophytes derived from a cross between the *E. siliculosus* strains EcNAP12-24 and Ec236-191 (supplementary fig. S1, Supplementary Material online) indicated that they had all inherited their mitochondrial genomes from the mother (fig. 2 upper panel and supplementary table S1, Supplementary Material online). This result was consistent with the maternal uniparental inheritance pattern observed by Peters, Scornet, et al. (2004) following interspecific crosses. Analysis of 13 sporophytes derived from crosses between the *Ectocarpus* species 7 female strains Ec721-18-9 and Ec721-18-10 (both derived from the sporophyte Ec721, supplementary fig. S1 and table S1, Supplementary Material online) and the male strain Ec246 also indicated uniparental inheritance but, surprisingly, all of the progeny had inherited their mitochondrial DNA from the father (fig. 2 upper panel and supplementary table S1, Supplementary Material online). Uniparental inheritance was also observed in a second series of crosses between female strains derived from the sporophyte Ec721 (sisters Ec568, Ec855, and Ec343) and an independently isolated male strain, Ec32, but in this case a very unusual pattern of inheritance was observed, with about half the progeny inheriting their mitochondria from the mother (8/15) but the others from the father (fig. 2 upper panel and supplementary table S1, Supplementary Material online). In *Ectocarpus* species 7, therefore, inheritance appears to be uniparental but two unusual patterns of inheritance were observed, depending on the male parent used: either paternal uniparental inheritance or a situation in which either the maternal or

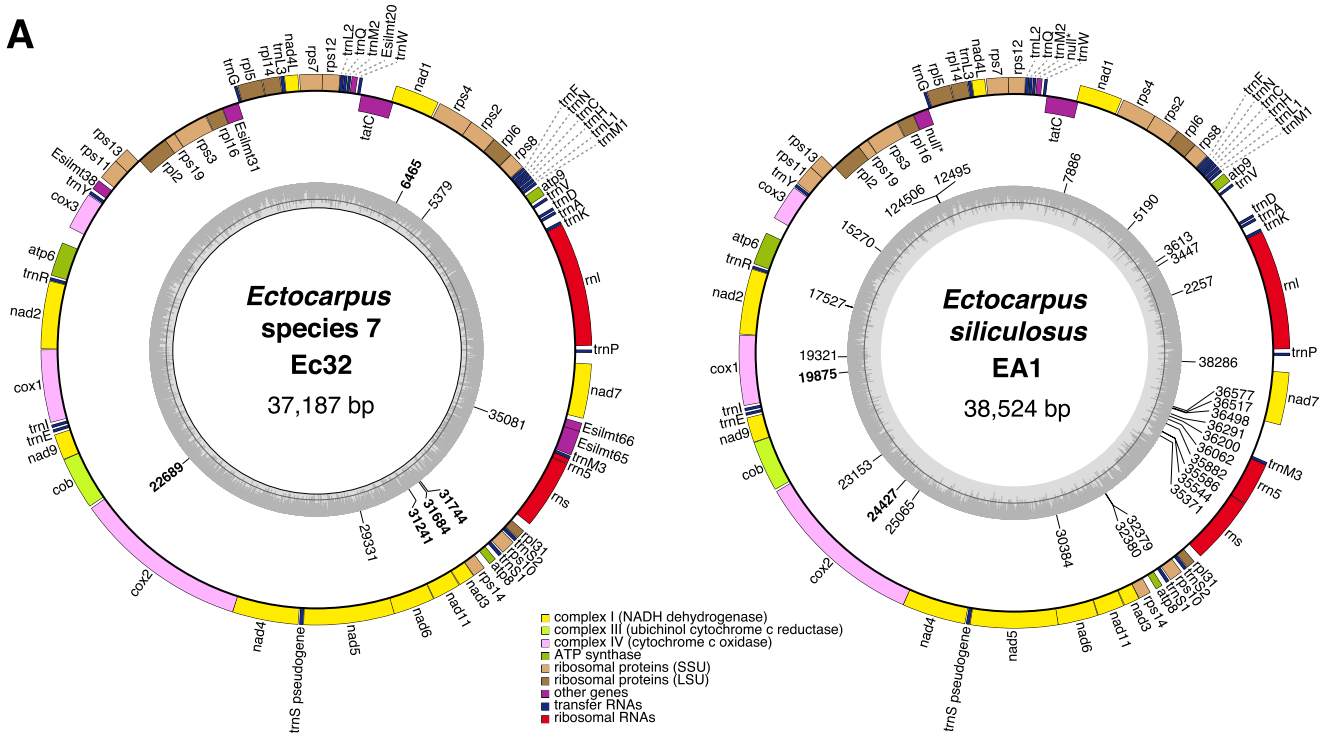

Fig. 1. Mitochondrial genome analysis and development of dCAPS markers. (*A*) Mitochondrial genomes of *Ectocarpus* species 7 strain Ec32 and *Ectocarpus siliculosus* strain EA1. The inner circles show GC content and the positions of the intraspecific polymorphisms detected by this study. Polymorphisms indicated in bold were used to develop dCAPS markers. (*B*) In vitro tests of dCAPS markers. PCR amplifications were carried out using genomic DNA from the two parental strains mixed in different proportions. f, female; m, male; bp, base pairs.

paternal mitochondrial genome was retained, apparently at random.

Droplet Digital PCR Analysis Indicates Uniparental Inheritance of Mitochondria in *Ectocarpus*

In all the above assays, the results of the dCAPS marker analyses were consistent with uniparental inheritance of mitochondrial DNA but test assays using mixes of male and female DNA (fig. 1*B*) had indicated that these markers were not sensitive enough to rule out some level of biparental inheritance. A more sensitive assay method, droplet digital PCR (ddPCR), was therefore used to detect mitochondrial inheritance patterns in which one of the parental genomes represented a minor component of the mitochondrial DNA inherited by an individual. The analysis was carried out on three classes of genetic cross that were representative of the three patterns of mitochondrial inheritance observed: maternal uniparental inheritance, paternal uniparental inheritance, and random maternal or paternal uniparental inheritance. Analyses of series of samples where parental DNAs had been mixed in different proportions indicated that the ddPCR assays were sensitive enough to detect minority mitochondrial DNA species, even if these genomes constituted of only 1% of an individual's total mitochondrial DNA pool (fig. 3). The results of analyses of three independent progeny

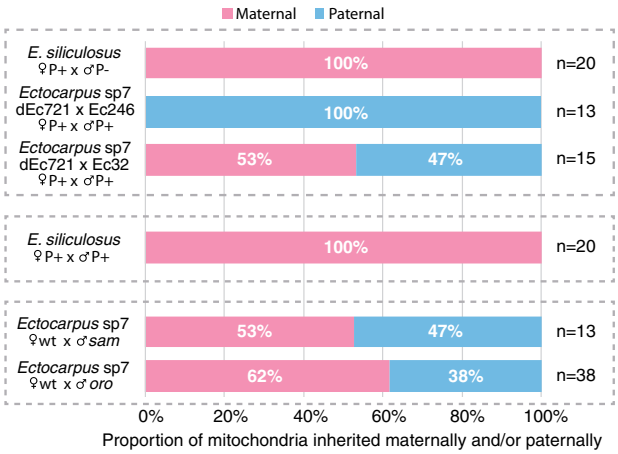

Fig. 2. Inheritance of mitochondrial genomes following different intraspecific crosses. Percentage of sporophyte progeny carrying maternal (pink) or paternal (blue) mitochondrial genomes. *Ectocarpus* sp7, *Ectocarpus* species 7; dEc721, daughters of the sporophyte Ec721; Ec32 and Ec246, male gametophytes corresponding to independently isolated strains; P+, parthenogenetic; P−, nonparthenogenetic; wt, wild type; *sam*, *samsara* mutants; *oro*, *ouroboros* mutant. See supplementary table S1, Supplementary Material online, for further details about the genetic crosses.

for each of the three observed patterns of inheritance (maternal, paternal, and randomly maternal or paternal) were consistent with uniparental mitochondrial DNA transmission in all cases (fig. 3).

Does Male Parthenogenetic Capacity Affect Mitochondrial Inheritance?

One difference between the gametes of male *Ectocarpus* species 7 strains Ec246 and Ec32 and those of the male *E. siliculosus* strains RB1, Ec236-154 and Ec236-191 is that the former possess parthenogenetic capacity (i.e., they can develop parthenogenetically if they fail to fuse with a female gamete) whereas the latter do not (note that the gametes of all the female strains used, EA1, EcNAP12-24 and all female strains derived from Ec721, are parthenogenetic; supplementary table S1, Supplementary Material online). It is possible that the requirement for functional mitochondria during parthenogenesis leads to the attenuation of mechanisms that would normally prepare male mitochondria for destruction following zygote formation, resulting in a higher probability of the male mitochondria being transmitted to heterozygous sporophyte offspring (Han et al. 2014). This is because both zygote development and gamete parthenogenesis involve deployment of the sporophyte developmental program. Consequently, if male mitochondria are marked in some way for destruction during the development of the sporophyte, this process would have to be modified during parthenogenesis because male-gamete-derived partheno-sporophytes possess only male mitochondrial. We hypothesized that the emergence of mechanisms that promote the maintenance of mitochondria in male-gamete-derived partheno-sporophytes might also have led to increased transmission of male mitochondria following sexual crosses. In other words, paternal transmission of mitochondria during sexual crosses may occur as a result of selection to maintain male mitochondria in male-gamete-derived partheno-sporophytes. To investigate this hypothesis, we compared the number of mitochondria in female and male gametes for both *Ectocarpus* species 7, using the parthenogenetic male strain Ec32, and for *E. siliculosus*, using the nonparthenogenetic male strains EcNAP12-83, Ec236-191, EcNAP12-80, and Ec236-87 (supplementary table S1, Supplementary Material online and fig. 4). This analysis showed that, although female gametes (which are slightly larger than male gametes; Lipinska et al. 2015) from both species contained more mitochondria than male gametes on average (fig. 4), this difference was significant for *E. siliculosus* (Kruskal–Wallis test, P value $< 2.2\times10^{-16}$; then Dun's post hoc test, P value $= 0$) but not for *Ectocarpus* species 7 (Dun's post hoc test, P value $= 0.052$). This observation suggested a possible link between the number of mitochondria carried by the male gamete and transmission of the male mitochondrial genome.

Parthenogenetic Male *E. siliculosus* Strains Exhibit Maternal Uniparental Inheritance

A recent study has shown that about a third of the male gametophytes derived from the cross between *E. siliculosus* strains EA1 and RB1 (parthenogenetic female x nonparthenogenetic male) produce parthenogenetic gametes (Mignerot et al. 2019). Counts of mitochondria in *E. siliculosus* gametes indicated that parthenogenetic male gametes had significantly more mitochondria than nonparthenogenetic male gametes, but less than female gametes (fig. 4). This difference suggests that parthenogenetic capacity may influence the number of mitochondria carried by a gamete, with possible consequences for the transmission of mitochondria to the next generation. However, when one of the parthenogenetic *E. siliculosus* males (Esil236-154) was crossed with the female strain EcNAP12-24, mitochondrial inheritance was 100% maternal (fig. 2 middle panel and supplementary table S1, Supplementary Material online). When these observations are taken together with the analyses described in the previous section, they suggest a broad correlation between the number of mitochondria carried by a gamete and parthenogenetic capacity but there does not appear to be a simple relationship between parthenogenetic capacity and the pattern of inheritance of mitochondria.

Life Cycle Mutants Do Not Exhibit Altered Patterns of Mitochondrial Inheritance

In the unicellular green alga *C. reinhardtii*, the deployment of the diploid program following gamete fusion is under the control of two genes called *GSP1* and *GSM1*, which encode three amino acid loop extension homeodomain transcription factors (TALE HD TFs) (Lee et al. 2008). A *C. reinhardtii* mutant in which a region of the genome including the gene *GSP1* was deleted exhibited aberrant biparental inheritance of mitochondrial DNA rather than the usual uniparental inheritance (Nishimura et al. 2012). There is accumulating evidence that *GSP1* and *GSM1* are derived from an ancient regulatory

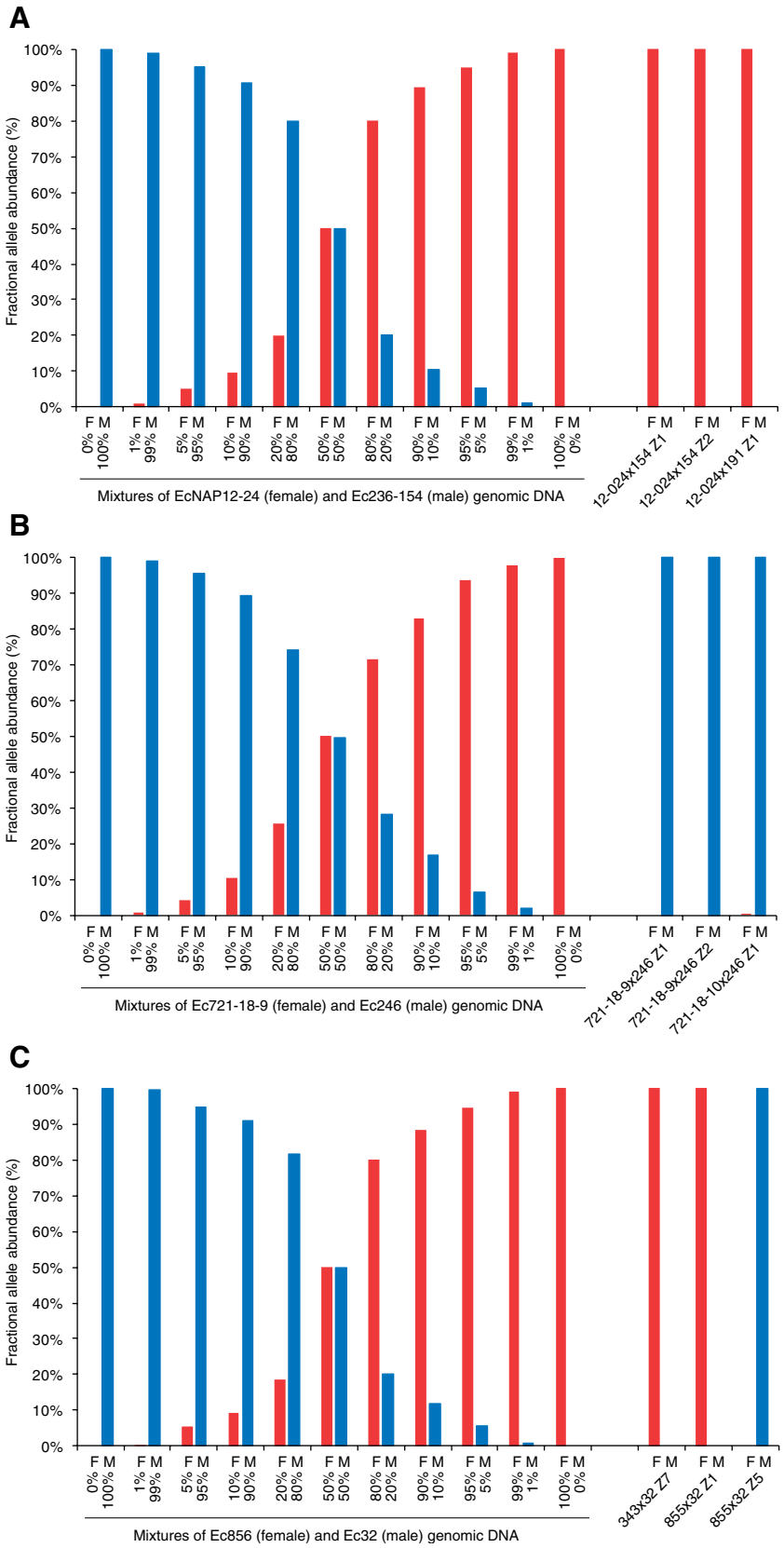

Fig. 3. Droplet digital PCR assays indicate uniparental inheritance of mitochondrial DNA. Each panel includes an in vitro test using genomic DNA from two parental strains mixed in different proportions on the left and analyses of genomic DNA from three hybrid progeny on the right. (*A*) *Ectocarpus siliculosus* crosses showing maternal mitochondrial DNA inheritance. (*B*) *Ectocarpus* species 7 crosses showing paternal mitochondrial DNA inheritance (Ec246 male strain). (*C*) *Ectocarpus* species 7 crosses showing random maternal or paternal mitochondrial DNA inheritance (Ec32 male strain). See supplementary table S1, Supplementary Material online, for information about the strains. F, allele from the female parent; M, allele from the male parent.

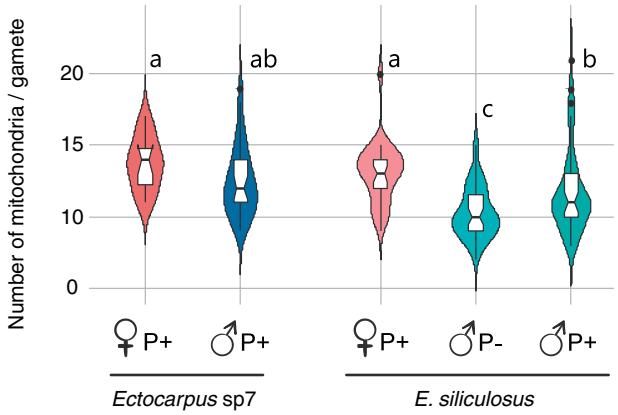

FIG. 4. Number of mitochondria in gametes of different *Ectocarpus* strains. Letters indicate significant differences (Kruskal–Wallis test followed by Dun's post hoc test). *Ectocarpus* sp7, *Ectocarpus* species 7; P+, parthenogenetic; P−, nonparthenogenetic. The strains analyzed for each class of gamete are indicated in supplementary table S1, Supplementary Material online.

module and that related regulatory systems play key roles following zygote formation in diverse eukaryotic lineages. For example, genes related to *GSP1* and *GSM1* have been shown to be necessary for the deployment of the diploid sporophyte generation following gamete fusion in the moss *Physcomitrella patens* (Sakakibara et al. 2013; Horst et al. 2016) and a recent study has uncovered a similar role for two TALE HD TFs named OUROBOROS (ORO) and SAMSARA (SAM) in *Ectocarpus* (Arun et al. 2019). Based on these observations, we were interested to determine whether strains carrying mutations in the *ORO* or *SAM* genes exhibited modified patterns of mitochondrial inheritance, in addition to their life cycle phenotypes. For this, male strains carrying either *oro* or *sam* mutations were crossed with wild type *Ectocarpus* species 7 female strains and the inheritance of mitochondrial DNA followed in the derived sporophyte generation. These experiments indicated that the presence of the *oro* or *sam* mutations did not significantly modify the pattern of mitochondrial inheritance. In both cases, mitochondrial inheritance was uniparental with approximately half of the sporophyte progeny inheriting the maternal mitochondrial genome and half inheriting the paternal mitochondrial genome (fig. 2 lower panel).

Recombination between Mitochondrial Genomes

Motomura et al. (2010) showed that male mitochondria persist until the four-cell stage of sporophyte development in the Ectocarpales species *S. lomentaria*. If male mitochondria also persist during early development of *Ectocarpus* sporophytes, this could potentially allow recombination between mitochondrial genomes to occur provided that the two genomes come into contact, for example, due to male mitochondria fusing with female mitochondria in the same cell. To search for potential recombination events, mitochondrial DNA from 93 independent field isolates of *E. siliculosus* and 40 independent field isolates of *Ectocarpus* species 7 was genotyped with two and four different mitochondrial markers, respectively (supplementary table S4, Supplementary Material online and fig. 5). This analysis indicated that the majority of *E. siliculosus* isolates carried a hybrid genome with allelic variants corresponding to both of the parental genomes that had been used for the crossing experiments. The majority of *Ectocarpus* species 7 isolates carried mitochondrial genomes that had the same genotype as one or the other of the parental genomes that had been used for the crossing experiments for this species but 35% of the isolates had recombinant genomes. Moreover, because four markers were used for this species, we were able to show that the field isolates possessed at least three different types of recombinant mitochondrial genome, indicating at least three independent recombination events. We were not able to determine whether different types of recombinant mitochondrial genome were present in the *E. siliculosus* isolates because only two markers were available for this species. In conclusion, these analyses provided evidence of mitochondrial recombination in both *E. siliculosus* and *Ectocarpus* species 7. Note however that the prevalence of recombinant mitochondrial genomes in field isolates does not allow any inference about the frequency of recombination because prevalence may be influenced by other factors such as the fitness of individuals that possess particular mitochondrial genotypes.

Discussion

Recent work on the species structure of the genus *Ectocarpus* has provided evidence that the crosses carried out by Peters, Scornet, et al. (2004), which indicated strict uniparental maternal inheritance of mitochondria, were between strains that belonged to different cryptic species. Interspecific crosses can lead to aberrant patterns of organelle inheritance due to genome incompatibilities. We therefore sought to repeat these experiments using intraspecific crosses. We used complete assemblies of the mitochondrial genomes of *E. siliculosus* and *Ectocarpus* species 7, together with whole genome shotgun sequence for several *E. siliculosus* and *Ectocarpus* species 7 strains, to identify intraspecific mitochondrial DNA polymorphisms. Intraspecific crosses and genetic markers based on the intraspecific polymorphisms were then used to analyze mitochondrial inheritance in the two species. These analyses detected strict maternal inheritance of mitochondria in *E. siliculosus*, as previously reported. Mitochondrial inheritance was also uniparental in *Ectocarpus* species 7 but two different patterns were observed when crosses were carried out with two independently isolated male strains. Progeny derived from crosses with male strain Ec246 exhibited paternal inheritance whereas progeny derived from crosses with male strain Ec32 exhibited either maternal or paternal inheritance, depending on the gamete fusion event.

The random inheritance of either maternal or paternal mitochondria observed following crosses with strain Ec32 is particularly interesting. It seems unlikely that a mechanism that differentially marks mitochondria from the two parents to allow selective destruction could act randomly to signal destruction of either the maternal or the paternal mitochondria, depending on the diploid individual (and note that

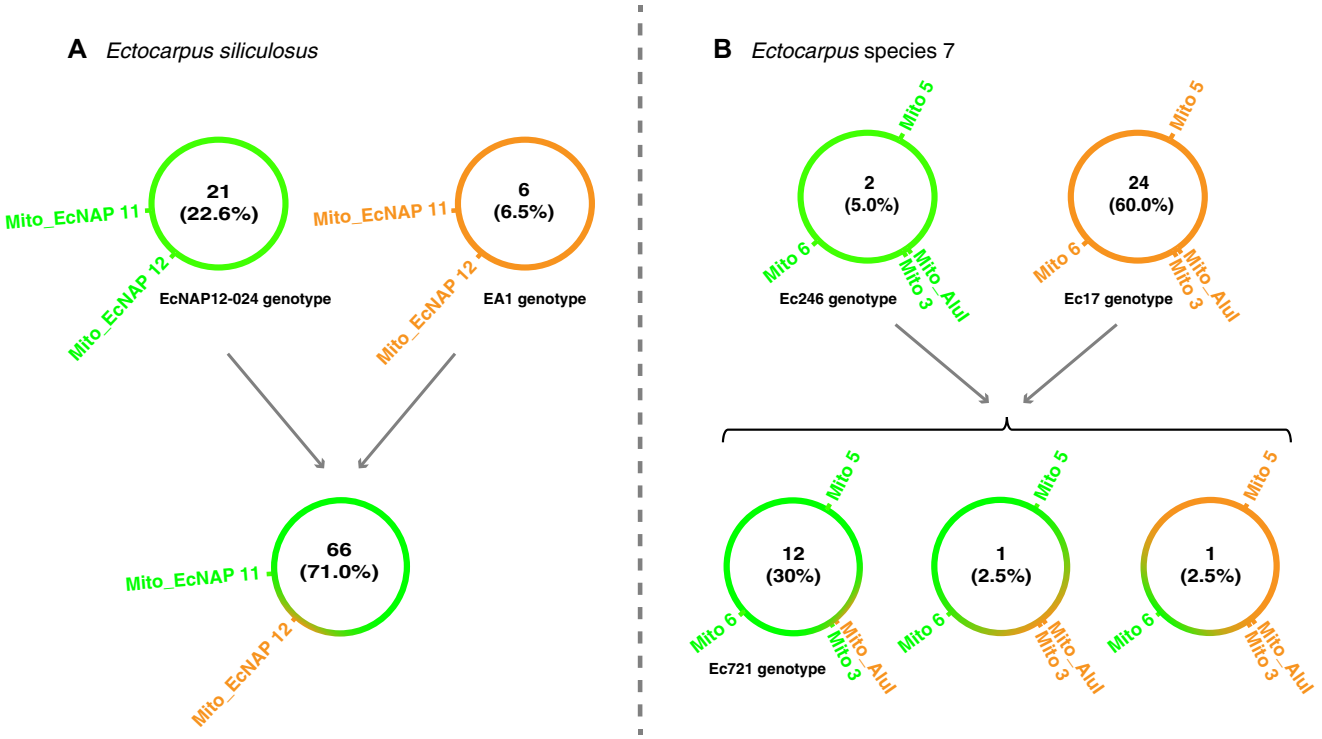

Fig. 5. Recombinant mitochondrial genomes detected in field isolates of (*A*) *Ectocarpus siliculosus* and (*B*) *Ectocarpus* species 7. Colors are used to indicate allelic variants corresponding to one or the other parental genome. Numbers and percentages of each mitochondrial genotype are indicated, as are the genotypes corresponding to strains used for the crossing experiments. See supplementary table S4, Supplementary Material online, for information about the field isolates.

Kimura et al. 2010 did not detect any evidence for differential methylation of mitochondrial DNA in female and male gametes of the related brown alga *S. lomentaria*). A more likely explanation for the observed pattern of mitochondrial inheritance in *Ectocarpus* would be the existence of a mechanism that tends to promote homoplasmy (i.e., retention of only one mitochondrial genotype to avoid intergenomic conflict) but which does not distinguish between maternal and paternal mitochondria. The origin of the mitochondria that are eliminated (i.e., whether they are maternal or paternal) might then simply be determined stochastically or might be influenced by factors such as the relative numbers of mitochondria delivered by each of the two fusing gametes into the zygote or the manner in which mitochondria segregate to daughter cells at each cell division during the growth of the thallus. Note that the analyses carried out here did not provide information about mitochondrial content during the early stages of development of the sporophytes because DNA extractions were carried out after 2–3 months in culture.

One marked difference between the *E. siliculosus* and *Ectocarpus* species 7 strains that were used for these crosses was that male gametes of the latter had been shown to be capable of parthenogenesis. We hypothesized that the mechanisms that allow male gametes to retain functional mitochondria for parthenogenesis may result in an increased likelihood of the male mitochondrial being transmitted through sexual crosses. However, the identification of male *E. siliculosus* strains whose gametes were capable of parthenogenesis and yet which exhibited a strict maternal pattern of mitochondrial inheritance argued against this hypothesis. Nonetheless, we believe that this hypothesis would merit further investigation as it is possible that the situation regarding male gamete parthenogenesis is not the same in the two species, in the sense that parthenogenetic males appear to be rare in *E. siliculosus* but preliminary analyses indicates that they may be a more common, and perhaps universal, phenomenon in *Ectocarpus* species 7. In other words, an effect on mitochondrial inheritance may only be seen when male parthenogenesis become a fixed characteristic within a species.

We also investigated mitochondrial inheritance in two mutant strains affected in life cycle progression, *oro* and *sam*. We did not observe any effect on mitochondrial inheritance in these mutants. This observation suggests that the pleiotropic effect of the *C. reinhardtii GSP1* life cycle mutant on mitochondrial inheritance may represent a secondary function of this gene, acquired in addition to its life cycle function by a green lineage ancestor.

Finally, the intraspecific mitochondrial markers generated during this study were used to search for evidence of recombination between mitochondrial genomes using collections of field isolates. This analysis detected recombinant mitochondrial genomes at high frequencies in natural populations suggesting that recombination may not be a rare event under field conditions. Note that the ddPCR analyses indicated that the unusual patterns of mitochondrial inheritance observed

in this study (paternal or randomly paternal or maternal) all involve uniparental inheritance and, therefore, these inheritance patterns would not necessarily be expected to influence the frequency of mitochondrial genome recombination.

In conclusion, we show here that patterns of mitochondrial inheritance vary across different *Ectocarpus* isolates, with the commonly observed strict maternal inheritance pattern being observed in *E. siliculosus* strains but unusual pattern of inheritance being observed in *Ectocarpus* species 7 strains. These observations indicate that mitochondrial inheritance patterns can vary across related species within the same genus and argue for broader analyses of inheritance using multiple strains. We would also like to underline the importance of using intraspecific mitochondrial DNA polymorphisms, which allows the analysis of intraspecific crosses and reduces the risk of observing aberrant inheritance patterns due to genome incompatibilities, as may often be the case with interspecific crosses. The pattern of randomly maternal or paternal uniparental inheritance observed in *Ectocarpus* species 7 indicates that it is not essential that mitochondria always be inherited from the same parent (i.e., only maternally or only paternally in all crosses) provided that only one parent provides the mitochondria in each particular cross. Consequently, it seems likely that the prevalence of strictly maternal and, more rarely, strictly paternal mitochondrial inheritance across the eukaryotic tree may be the result of it being easier to evolve systems that consistently target the mitochondria from the same parent rather than any fundamental requirement that mitochondrial inheritance be limited to one specific sex or mating type.

Materials and Methods

Ectocarpus Strains, Culture Conditions, and Crosses

The list of strains used in this study, together with their characteristics and genetic history, is shown in supplementary table S1, Supplementary Material online. Pedigrees are shown in supplementary figure S1, Supplementary Material online. The strains corresponded to two *Ectocarpus* species, *E. siliculosus* sensu stricto (referred to herein as *E. siliculosus*) and *Ectocarpus* species 7. Note that *Ectocarpus* species 7 was previously referred to as *E. siliculosus* but actually corresponds to a distinct species (Montecinos et al. 2017). The species classification used by Montecinos et al. (2017) used mitochondrial cytochrome oxidase subunit 1 (COI-5P) and nuclear ribosomal DNA internal transcribed spacer 1 (ITS1) marker data from 729 sampled individuals and was based on two phylogenetic reconstruction methods, maximum likelihood and Bayesian inference, and two species delimitation methods, General Mixed Yule Coalescence (GMYC) and Automatic Barcode Gap Discovery (ABGD). The classification of *E. siliculosus* and *Ectocarpus* species 7 as distinct species is also supported by the observation that hybrid sporophytes derived from crosses between individuals of these two species have been shown to be viable but incapable of meiosis (Peters, Marie, et al. 2004). Moreover, flow cytometry experiments indicate that individuals of the two species have different total genome sizes (Peters, Marie, et al. 2004).

Ectocarpus strains were cultured in autoclaved natural sea water supplemented with half strength Provasoli solution (Starr and Zeikus 1993) at 13 °C, with a light:dark cycle of 12 h:12 h (20 µmol photons m^{-2} s^{-1}) using daylight-type fluorescent tubes (Coelho et al. 2012b). All manipulations were carried out under sterile conditions in a laminar flow hood. Crosses (listed in supplementary table S1, Supplementary Material online) were carried out using the protocol described by Coelho et al. (2012a). Sporophytes derived from crosses were cultivated for 2–3 months before excision of material for DNA extraction. Genomic sequence data accession numbers for strains Ec32, Ec568, EA1, RB1, and EcNAP-12-24 are provided in supplementary table S1, Supplementary Material online.

Extraction of Genomic DNA and Identification of Heterozygous, Diploid Sporophytes

Sporophyte tissue (10–20 mg wet weight) was frozen in liquid nitrogen and DNA was extracted using the NucleoSpin Plant II kit (Macherey Nagel) according to the manufacturer's instructions. As some *Ectocarpus* gametes are able to undergo parthenogenesis to form haploid partheno-sporophytes, the ploidy of the sporophytes derived from each cross was assessed using sex markers (supplementary table S3, Supplementary Material online). Diploid sporophytes are expected to carry both the female (U) and the male (V) sex chromosome whereas partheno-sporophytes carry only one sex chromosome (U or V; supplementary fig. S2, Supplementary Material online). Touchdown PCR reactions, which consisted of 2 ng of DNA, 80 nM of primer mix, 0.2 mM dNTP, 2 µl of 5× Go Taq green buffer (Promega), 2 mM $MgCl_2$, 2 mg/ml of BSA, and 0.05 µl (0.25 units) of Taq polymerase (Promega), were carried out in an Applied Biosystems thermocycler under the following conditions: 3 min at 95 °C, then 10 touchdown cycles of 30 s 95 °C; 30 s at 65 °C (−1 °C/cycle); and 30 s at 72 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C and a final incubation at 72 °C for 5 min before storage at 4 °C. After amplification, PCR products were separated and visualized on 2% agarose gels.

Mitochondrial Genome Assemblies and Detection of Intraspecific Mitochondrial DNA Polymorphisms

An earlier assembly of the *Ectocarpus* species 7 strain Ec32 mitochondrial genome was re-evaluated using high-coverage Illumina shotgun sequence data. A draft assembly of the *E. siliculosus* strain EA1 genome, including organellar sequences, was generated using the CLC assembler (Qiagen Bioinformatics) and Illumina shotgun DNA-seq data (supplementary table S1, Supplementary Material online). Mitochondrial DNA scaffolds were identified using the *Ectocarpus* species 7 mitochondrial genome as the query in a Blastn (Altschul et al. 1997) search. These scaffolds were then assembled manually to obtain the complete circular *E. siliculosus* strain EA1 mitochondrial genome. The genome was annotated by transferring annotation information from the *Ectocarpus* species 7 strain Ec32 mitochondrial genome

using Geneious R11.1.2 (https://www.geneious.com, last accessed August 22, 2019). Circular maps of the Ec32 and EA1 mitochondrial genomes were generated using OGDraw (Lohse et al. 2013).

Intraspecific mitochondrial DNA polymorphisms were identified either by mapping Illumina shotgun DNA-seq data (supplementary table S1, Supplementary Material online) against reference mitochondrial genome assemblies using Bowtie2 (Langmead et al. 2009) followed by manual SNP detection using GenomeView (Abeel et al. 2012) or by PCR amplification and sequencing of targeted regions of the mitochondrial genome. For *E. siliculosus*, DNA-seq data for strains RB1 and EcNAP12-24 was mapped, individually, against the EA1 reference. For *Ectocarpus* species 7 strains Ec568 and Ec32, DNA-seq data for strain Ec568 was mapped against the Ec32 reference. For both species, variants were detected with bcftools (Li et al. 2009) and verified manually by visualization of mapping data in GenomeView (Abeel et al. 2012). Mitochondrial SNPs were detected for *Ectocarpus* species 7 strain Ec246 by Sanger sequencing of a region of the mitochondrial DNA amplified using the oligonucleotide primers Trn (5′-ATTGATTTAGCAAACCAAGGC-3′) and Nad (5′-GGTAGYYTAGAATTGGGAATG-3′).

Development of dCAPS Markers to Study Mitochondrial DNA Inheritance

Derived cleaved amplified polymorphic sequence-specific (dCAPS) markers (Neff et al. 1998) were designed using dCAPS Finder2.0 (http://helix.wustl.edu/dcaps/, last accessed August 22, 2019) for the dCAPS primer and Primer 3 (http://primer3.ut.ee/; last accessed August 22, 2019) for the second primer of the primer pair. dCAPS primers allow the creation of a diagnostic restriction enzyme recognition site specifically in the PCR product corresponding to one allelic form of an SNP. Before use, dCAPS markers were tested on samples in which genomic DNA from the two parental strains had been mixed in different proportions (1:2, 1:5, 1:10, 1:20). Touchdown PCR reactions were carried out with dCAPS primer pairs in an Applied Biosystems thermocycler using the following conditions for *Ectocarpus* species 7: 3 min at 95 °C, then 10 touchdown cycles of 30 s 95 °C; 30 s at 65 °C (−1 °C/cycle) and 30 s at 72 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C and a final incubation at 72 °C for 5 min before storage at 4 °C and the following conditions for *E. siliculosus*: 3 min at 95 °C, then 10 touchdown cycles of 30 s 95 °C; 30 s at 68 °C (−0.1 °C/cycle); and 30 s at 72 °C followed by 25 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C and a final incubation at 72 °C for 5 min before storage at 4 °C. After amplification, 10 µl of PCR product was digested using five units of the relevant restriction enzyme (supplementary table S3, Supplementary Material online). Digestion products were separated and analyzed on 2% or 2.5% agarose gels. All marker genotyping tests were carried out twice to ensure that the result was reproducible. Supplementary figure S3, Supplementary Material online, shows an example of typical dCAPS genotyping experiment.

Droplet Digital Polymerase Chain Reaction Assays to Detect Mitochondrial DNA

Mitochondrial DNA genotyping was carried out using 10 ng of total DNA on a QX200 Droplet Digital PCR System with 5(6)-carboxyfluorescein (FAM) and hexachloro-fluorescein (HEX) labeled oligonucleotide probes (Bio-Rad, Hercules, CA). Oligonucleotide primers and probes (supplementary table S3, Supplementary Material online) were obtained from Bio-Rad. ddPCR reactions were carried out by Ingénierie et Analyse en Genome Editing (IAGE, Montferriez sur lez, France). A QX200 Droplet Generator (Bio-Rad) was used to distribute PCR components to individual reaction vessels. Droplets were generated by combining 70 µl of droplet generation oil with 22 µl of the PCR mix and 40 µl of resulting droplet reaction was subjected to amplification. The cycling conditions for the PCR reaction included an initial incubation for 10 min at 95 °C followed by 40 cycles of 94 °C for 30 s and 55 °C for 60 s. Amplified plates were transferred to a Droplet Reader (Bio-Rad) and the digital PCR data were analyzed with the Quanta Soft analytical software package (Bio-Rad).

Counts of Mitochondria Using Confocal Microscopy

A MitoTracker dye (MitoTracker Orange CMTMRos ref MT7510, Invitrogen) was used to stain mitochondria in freshly released gametes. Working solutions of MitoTracker dye were obtained by diluting 1 mM stock solution in DMSO to 0.166 µM in freshly prepared Provasoli-enriched seawater. Gametophyte filaments carrying plurilocular gametangia were allowed to release in 20 µl of this solution on a clean coverslip and the gametes were then fixed after 20 min at room temperature under low light by addition of glutaraldehyde to a final concentration of 1%. Confocal microscopy was carried out with a Leica SP5 microscope (TCS SP5 AOBS, Merimage platform, Roscoff) and z-series of images were analyzed with ImageJ/Fidji to count the number of mitochondria in each gamete. The strains used for the mitochondrial counts are indicated in supplementary table S1, Supplementary Material online.

Evaluation of Parthenogenetic Capacity

To evaluate parthenogenetic capacity, released gametes were allowed to settle in a Petri dish and parthenogenetic growth estimated after 15 days in culture. Strains were scored as parthenogenetic if >4% of parthenotes grew beyond the 10-cell stage (Mignerot et al. 2019).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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Author Contributions

L.M., C.N., A.F.P., D.S., F.P., Y.B., and T.M. prepared the biological material and performed experiments. L.M., M.M.P., W.D., and J.M.C. performed the computational analysis. L.M., C.N., D.S., F.P., Y.B., W.D., S.M.C., and J.M.C. analyzed data. J.M.C. designed and coordinated the study. J.M.C. wrote the article with valuable input from L.M. and S.M.C. All authors read and approved the final article.

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