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► **To cite this version:**

Laure Mignerot, Chikako Nagasato, Akira F Peters, Marie-Mathilde Perrineau, Delphine Scornet, et al.. Unusual Patterns of Mitochondrial Inheritance in the Brown Alga *Ectocarpus*. *Molecular Biology and Evolution*, 2019, 10.1093/molbev/msz186 . hal-02269449

HAL Id: hal-02269449

<https://hal.science/hal-02269449>

Submitted on 22 Aug 2019

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

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12 **Abstract**

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

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

27 **Introduction**

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

41 In oogamous species, where the large female gamete (the egg cell) contributes more mitochondria to the
42 zygote than the small male gamete (sperm cell), a bottleneck phenomenon (Breton and Stewart 2015) could
43 explain the disappearance of the paternal mitochondria. However, uniparental mitochondrial inheritance is
44 also observed in isogamous species where the two gametes carry similar numbers of mitochondria, implying
45 the existence of specific mechanisms that eliminate the mitochondria of one parent. For example in the
46 unicellular green alga *Chlamydomonas reinhardtii* the mitochondrial genome contributed by the plus mating
47 type parent is specifically eliminated during zygote maturation (Nakamura 2010) and this appears to be
48 under genetic control (Nishimura et al. 2012). Specific mechanisms also exist to promote uniparental
49 mitochondrial inheritance in oogamous species (Mishra and Chan 2014; Greiner et al. 2015). These
50 mechanisms are highly diverse and can act either before or after zygote formation. Pre-zygotic mechanisms
51 include the elimination of mitochondria from male gametes, degradation of male gamete mitochondria
52 before fertilisation and prevention of male mitochondria from entering the egg cell during fertilisation.
53 Alternatively, selective degradation of the mitochondria or mitochondrial DNA of one parent can occur after
54 formation of the zygote and again this can occur via several different mechanisms involving, for example,
55 the ubiquitin-proteasome system or autophagy (Sato and Sato 2013).

56 There is accumulating evidence that many mitochondrial inheritance systems that have been classed as
57 uniparental actually exhibit some level of heteroplasmy (i.e. transmission of both parental mitochondrial
58 genomes to the offspring) or paternal leakage (Breton and Stewart 2015; Greiner et al. 2015). Strict
59 uniparental inheritance of mitochondria is expected to lead to the accumulation of deleterious mutations in

60 the mitochondrial genome due to the action of Muller's ratchet. It has been proposed that the mechanisms
61 that promote uniparental inheritance are periodically relaxed over evolutionary time to allow mitochondrial
62 genomes to recombine and thereby eliminate deleterious mutations (Takano et al. 2010; Greiner et al. 2015).
63 "Leakage" of paternal mitochondria through to the progeny is also expected to limit the effects of Muller's
64 ratchet but it is not clear whether leakage alone is sufficient. The broad diversity of the mechanisms by
65 which paternal mitochondria are eliminated (see above) is consistent with periodical relaxation of
66 uniparental inheritance in the sense that these mechanisms would need to re-evolve after each period of
67 relaxed inheritance.

68 A number of organisms exhibit patterns of mitochondrial inheritance that deviate from the usual situation
69 of uniparental maternal inheritance. These variations are of considerable interest because they can provide
70 insights into the evolutionary and molecular mechanisms underlying mitochondrial inheritance. Examples
71 include strict paternal inheritance in some plants including the sequoia tree (Neale et al. 1989), banana
72 (Fauré et al. 1994) and cucumber (Havey et al. 2004). In some organisms more than one mitochondrial
73 genome may be transmitted to the offspring. For example, stable inheritance of maternal heteroplasmy has
74 been described for terrestrial isopod crustaceans (Doublet et al. 2012). Biparental mitochondrial
75 transmission has been reported for several species but there do not appear to be any cases where both
76 maternal and paternal mitochondria are systematically transmitted to the zygote and then stably inherited
77 throughout development (Breton and Stewart 2015). Therefore, even when inheritance is biparental, there
78 are usually mechanisms that limit heteroplasmy, usually by ensuring that individual offspring carry either
79 the maternal or the paternal mitochondria, but not both. For example, in the fungus *Coprinopsis cinerea*
80 progeny can inherit mitochondria from either one or the other parent (Wilson and Xu 2012). This pattern of
81 inheritance occurs because heterokaryon formation involves an exchange of parental nuclei, but not
82 mitochondria, between mating partners. In this case, therefore, there is no stage where mitochondria from
83 both parents are mixed in a cell fusion product and therefore no need for selective elimination of
84 mitochondria derived from one of the parents. In the yeasts *Saccharomyces cerevisiae* and
85 *Schizosaccharomyces pombe* fusion of isogametes results in heteroplasmy but partitioning of mitochondria
86 during budding actively promotes the formation of homoplasmic daughter cells (Birky 2001). Several
87 bivalve species exhibit doubly uniparental inheritance, with mitochondrial being transmitted in a sex-
88 specific manner (Zouros 2013). The female (F) mitochondrial genome is transmitted to females and males
89 but the males do not transmit the F genome to their progeny, rather they transmit the male (M) genome,
90 which is transmitted uniquely through the male line. Finally, novel patterns of mitochondrial inheritance
91 have often been reported following inter-specific crosses in a broad range of eukaryotic taxa (Hoarau et al.
92 2009; Breton and Stewart 2015; Montecinos et al. 2017). Under these conditions, mitochondrial inheritance

93 systems may exhibit breakdown due to genome incompatibilities and therefore it is possible that the patterns
94 observed are dysfunctional.

95 Studies of mitochondrial inheritance in the brown algae have reported maternal inheritance (Motomura et
96 al. 2010). In oogamous species, male mitochondria are digested by lysosomes in the zygote (Motomura
97 1990), whereas in the anisogamous species *Scytosiphon lomentaria* male mitochondria persist until the four-
98 cell stage of sporophyte development (Motomura et al. 2010).

99 *Ectocarpus* is an emerging model species for the brown algae (Peters, Marie, et al. 2004; Cock et al. 2011).
100 An earlier study indicated that mitochondrial inheritance is strictly maternal in *Ectocarpus* (Peters, Scornet,
101 et al. 2004). However, a recent analysis of the species structure of the genus *Ectocarpus* (Montecinos et al.
102 2017) has indicated that the strains used in the 2004 study belonged to distinct cryptic species and therefore
103 that the crosses were inter-specific. Here we analysed mitochondrial inheritance in intra-specific crosses
104 using pairs of strains from two of the recently defined *Ectocarpus* species (Montecinos et al. 2017). For one
105 species (*Ectocarpus siliculosus*) we observed strict maternal inheritance, as reported previously, but,
106 surprisingly, crosses between different strains of a second species (*Ectocarpus* species 7) produced progeny
107 that exhibited either paternal uniparental inheritance or an unusual pattern of transmission where progeny
108 inherited either maternal or paternal mitochondria, but not both.

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

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

124 Results

125 Development of markers to follow mitochondrial inheritance in intraspecific crosses

126 A recent analysis by Montecinos et al. (2017) identified the presence of at least 15 cryptic species within
127 the genus *Ectocarpus* and indicated that an earlier study of mitochondrial inheritance in *Ectocarpus* (Peters,
128 Scornet, et al. 2004) was based on interspecific crosses. To determine whether the conclusions of the earlier
129 study held for intraspecific crosses, we developed molecular markers to distinguish between polymorphic
130 forms of the mitochondrial genome in two of the *Ectocarpus* species defined by Montecinos et al. (2017):
131 *E. siliculosus sensu stricto* (hereafter *E. siliculosus*) and *Ectocarpus* species 7. Note that *Ectocarpus* species
132 7, which corresponds to the reference genome species (Cock et al. 2010; Cormier et al. 2017), was earlier
133 referred to as *Ectocarpus siliculosus* under the older classification system but this nomenclature needs to be
134 revised.

135 The mitochondrial genome sequence of the male *Ectocarpus* species 7 strain Ec32, which had been initially
136 assembled using Sanger sequence data (deposited as *Ectocarpus siliculosus* with Genbank accession number
137 FP885846.1), was re-evaluated using high-coverage Illumina shotgun sequence data and two sequencing
138 errors were corrected. The corrected *Ectocarpus* species 7 strain Ec32 mitochondrial genome is available
139 through the accession number FP885846.2 (fig. 1A). The mitochondrial genome of the female *E. siliculosus*
140 strain EA1 was assembled using whole genome shotgun sequence data (supplementary table S1) and the
141 Ec32 genome as a guide (fig. 1A). The EA1 mitochondrial genome is available through the accession
142 number MK045263. Whole genome sequence data was then generated for independently isolated strains of
143 both species (RB1 and EcNAP-12-24 for *E. siliculosus* and Ec568 for *Ectocarpus* species 7) and each dataset
144 was mapped onto the corresponding, conspecific mitochondrial genome to identify intraspecific
145 polymorphisms. This analysis identified 28 and six intra-specific SNPs for the *E. siliculosus* and *Ectocarpus*
146 species 7 mitochondrial genomes, respectively (fig. 1A, supplementary table S2). Two additional
147 mitochondrial SNPs, between *Ectocarpus* species 7 male strain Ec246 and female strain Ec856 (a sister of
148 Ec568) were detected by Sanger sequencing of a region of the mitochondrial genome amplified by PCR
149 from strains Ec246 and Ec856. These latter SNPs correspond to A to G transitions at positions 31684 and
150 31744 (fig. 1A, supplementary table S2).

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

156 from the two parents (equivalent to biparental inheritance) provided they were in approximately equal
157 proportions (fig. 1B).

158 Mitochondria DNA inheritance in *E. siliculosus* and *Ectocarpus* species 7

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

165 dCAPS analysis of 20 sporophytes derived from a cross between the *E. siliculosus* strains EcNAP12-24 and
166 Ec236-191 (supplementary fig. S1) indicated that they had all inherited their mitochondrial genomes from
167 the mother (fig. 2 upper panel, supplementary table S1). This result was consistent with the maternal
168 uniparental inheritance pattern observed by Peters, Scornet, et al. (2004) following interspecific crosses.
169 Analysis of 13 sporophytes derived from crosses between the *Ectocarpus* species 7 female strains Ec721-
170 18-9 and Ec721-18-10 (both derived from the sporophyte Ec721, supplementary fig. S1, supplementary
171 table S1) and the male strain Ec246 also indicated uniparental inheritance but, surprisingly, all of the
172 progeny had inherited their mitochondrial DNA from the father (fig. 2 upper panel, supplementary table
173 S1). Uniparental inheritance was also observed in a second series of crosses between female strains derived
174 from the sporophyte Ec721 (sisters Ec568, Ec855 and Ec343) and an independently isolated male strain,
175 Ec32, but in this case a very unusual pattern of inheritance was observed, with about half the progeny
176 inheriting their mitochondria from the mother (8/15) but the others from the father (fig. 2 upper panel,
177 supplementary table S1). In *Ectocarpus* species 7, therefore, inheritance appears to be uniparental but two
178 unusual patterns of inheritance were observed, depending on the male parent used: either paternal
179 uniparental inheritance or a situation in which either the maternal or paternal mitochondrial genome was
180 retained, apparently at random.

181 Droplet digital PCR analysis indicates uniparental inheritance of mitochondria in *Ectocarpus*

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

196 Does male parthenogenetic capacity affect mitochondrial inheritance?

197 One difference between the gametes of male *Ectocarpus* species 7 strains Ec246 and Ec32 and those of the
198 male *E. siliculosus* strains RB1, Ec236-154 and Ec236-191 is that the former possess parthenogenetic
199 capacity (*i.e.* they can develop parthenogenetically if they fail to fuse with a female gamete) whereas the
200 latter do not (note that the gametes of all the female strains used, EA1, EcNAP12-24 and all female strains
201 derived from Ec721, are parthenogenetic; supplementary table S1). It is possible that the requirement for
202 functional mitochondria during parthenogenesis leads to the attenuation of mechanisms that would normally
203 prepare male mitochondria for destruction following zygote formation, resulting in a higher probability of
204 the male mitochondria being transmitted to heterozygous sporophyte offspring (Han et al. 2014). This is
205 because both zygote development and gamete parthenogenesis involve deployment of the sporophyte
206 developmental program. Consequently, if male mitochondria are marked in some way for destruction during
207 the development of the sporophyte, this process would have to be modified during parthenogenesis because
208 male-gamete-derived partheno-sporophytes possess only male mitochondrial. We hypothesised that the
209 emergence of mechanisms that promote the maintenance of mitochondria in male-gamete-derived partheno-
210 sporophytes might also have led to increased transmission of male mitochondria following sexual crosses.
211 In other words, paternal transmission of mitochondria during sexual crosses may occur as a result of
212 selection to maintain male mitochondria in male-gamete-derived partheno-sporophytes. To investigate this

213 hypothesis, we compared the number of mitochondria in female and male gametes for both *Ectocarpus*
214 species 7, using the parthenogenetic male strain Ec32, and for *E. siliculosus*, using the non-parthenogenetic
215 male strains EcNAP12-83, Ec236-191, EcNAP12-80 and Ec236-87 (supplementary table S1, fig. 4). This
216 analysis showed that, although female gametes (which are slightly larger than male gametes; Lipinska et al.
217 2015) from both species contained more mitochondria than male gametes on average (fig. 4), this difference
218 was significant for *E. siliculosus* (Kruskal Wallis test, $p\text{-value} < 2.2 \times 10^{-16}$; then Dun's post hoc-test, $p\text{-value} = 0$) but not for *Ectocarpus* species 7 (Dun's post hoc-test, $p\text{-value} = 0.052$). This observation suggested
219 a possible link between the number of mitochondria carried by the male gamete and transmission of the
220 male mitochondrial genome.
221

222 Parthenogenetic male *E. siliculosus* strains exhibit maternal uniparental inheritance

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

235 Life cycle mutants do not exhibit altered patterns of mitochondrial inheritance

236 In the unicellular green alga *Chlamydomonas reinhardtii*, the deployment of the diploid program following
237 gamete fusion is under the control of two genes called *GSP1* and *GSM1*, which encode three amino acid
238 loop extension homeodomain transcription factors (TALE HD TFs) (Lee et al. 2008). A *C. reinhardtii*
239 mutant in which a region of the genome including the gene *GSP1* was deleted exhibited aberrant biparental
240 inheritance of mitochondrial DNA rather than the usual uniparental inheritance (Nishimura et al. 2012).
241 There is accumulating evidence that *GSP1* and *GSM1* are derived from an ancient regulatory module and
242 that related regulatory systems play key roles following zygote formation in diverse eukaryotic lineages.
243 For example, genes related to *GSP1* and *GSM1* have been shown to be necessary for the deployment of the
244 diploid sporophyte generation following gamete fusion in the moss *Physcomitrella patens* (Sakakibara et

245 al. 2013; Horst et al. 2016) and a recent study has uncovered a similar role for two TALE HD TFs named
246 OUROBOROS (ORO) and SAMSARA (SAM) in *Ectocarpus* (Arun et al. 2019). Based on these
247 observations, we were interested to determine whether strains carrying mutations in the *ORO* or *SAM* genes
248 exhibited modified patterns of mitochondrial inheritance, in addition to their life cycle phenotypes. For this,
249 male strains carrying either *oro* or *sam* mutations were crossed with wild type *Ectocarpus* species 7 female
250 strains and the inheritance of mitochondrial DNA followed in the derived sporophyte generation. These
251 experiments indicated that the presence of the *oro* or *sam* mutations did not significantly modify the pattern
252 of mitochondrial inheritance. In both cases mitochondrial inheritance was uniparental with approximately
253 half of the sporophyte progeny inheriting the maternal mitochondrial genome and half inheriting the paternal
254 mitochondrial genome (fig. 2 lower panel).

255 Recombination between mitochondrial genomes

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

276 **Discussion**

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

291 The random inheritance of either maternal or paternal mitochondria observed following crosses with strain
292 Ec32 is particularly interesting. It seems unlikely that a mechanism that differentially marks mitochondria
293 from the two parents to allow selective destruction could act randomly to signal destruction of either the
294 maternal or the paternal mitochondria, depending on the diploid individual (and note that Kimura et al.
295 (2010) did not detect any evidence for differential methylation of mitochondrial DNA in female and male
296 gametes of the related brown alga *S. lomentaria*). A more likely explanation for the observed pattern of
297 mitochondrial inheritance in *Ectocarpus* would be the existence of a mechanism that tends to promote
298 homoplasmy (i.e. retention of only one mitochondrial genotype to avoid inter-genomic conflict) but which
299 does not distinguish between maternal and paternal mitochondria. The origin of the mitochondria that are
300 eliminated (i.e. whether they are maternal or paternal) might then simply be determined stochastically or
301 might be influenced by factors such as the relative numbers of mitochondria delivered by each of the two
302 fusing gametes into the zygote or the manner in which mitochondria segregate to daughter cells at each cell
303 division during the growth of the thallus. Note that the analyses carried out here did not provide information
304 about mitochondrial content during the early stages of development of the sporophytes because DNA
305 extractions were carried out after two to three months in culture.

306 One marked difference between the *E. siliculosus* and *Ectocarpus* species 7 strains that were used for these
307 crosses was that male gametes of the latter had been shown to be capable of parthenogenesis. We
308 hypothesised that the mechanisms that allow male gametes to retain functional mitochondria for

309 parthenogenesis may result in an increased likelihood of the male mitochondrial being transmitted through
310 sexual crosses. However, the identification of male *E. siliculosus* strains whose gametes were capable of
311 parthenogenesis and yet which exhibited a strict maternal pattern of mitochondrial inheritance argued
312 against this hypothesis. Nonetheless, we believe that this hypothesis would merit further investigation as it
313 is possible that the situation regarding male gamete parthenogenesis is not the same in the two species, in
314 the sense that parthenogenetic males appear to be rare in *E. siliculosus* but preliminary analyses indicates
315 that they may be a more common, and perhaps universal, phenomenon in *Ectocarpus* species 7. In other
316 words, an effect on mitochondrial inheritance may only be seen when male parthenogenesis become a fixed
317 characteristic within a species.

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

323 Finally, the intra-specific mitochondrial markers generated during this study were used to search for
324 evidence of recombination between mitochondrial genomes using collections of field isolates. This analysis
325 detected recombinant mitochondrial genomes at high frequencies in natural populations suggesting that
326 recombination may not be a rare event under field conditions. Note that the ddPCR analyses indicated that
327 the unusual patterns of mitochondrial inheritance observed in this study (paternal or randomly paternal or
328 maternal) all involve uniparental inheritance and, therefore, these inheritance patterns would not necessarily
329 be expected to influence the frequency of mitochondrial genome recombination.

330 In conclusion, we show here that patterns of mitochondrial inheritance vary across different *Ectocarpus*
331 isolates, with the commonly observed strict maternal inheritance pattern being observed in *E. siliculosus*
332 strains but unusual pattern of inheritance being observed in *Ectocarpus* species 7 strains. These observations
333 indicate that mitochondrial inheritance patterns can vary across related species within the same genus and
334 argue for broader analyses of inheritance using multiple strains. We would also like to underline the
335 importance of using intra-specific mitochondrial DNA polymorphisms, which allows the analysis of intra-
336 specific crosses and reduces the risk of observing aberrant inheritance patterns due to genome
337 incompatibilities, as may often be the case with inter-specific crosses. The pattern of randomly maternal or
338 paternal uniparental inheritance observed in *Ectocarpus* species 7 indicates that it is not essential that
339 mitochondria always be inherited from the same parent (i.e. only maternally or only paternally in all crosses)
340 provided that only one parent provides the mitochondria in each particular cross. Consequently, it seems
341 likely that the prevalence of strictly maternal and, more rarely, strictly paternal mitochondrial inheritance

342 across the eukaryotic tree may be the result of it being easier to evolve systems that consistently target the
343 mitochondria from the same parent rather than any fundamental requirement that mitochondrial inheritance
344 be limited to one specific sex or mating type.

345 **Material and methods**

346 *Ectocarpus* Strains, Culture Conditions and Crosses

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

361 *Ectocarpus* strains were cultured in autoclaved natural sea water supplemented with half strength Provasoli
362 solution (Starr and Zeikus 1993) at 13°C, with a light:dark cycle of 12h:12h (20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) using
363 daylight-type fluorescent tubes (Coelho, Scornet, Rousvoal, N. Peters, et al. 2012). All manipulations were
364 carried out under sterile conditions in a laminar flow hood. Crosses (listed in supplementary table S1) were
365 carried out using the protocol described by Coelho, Scornet, Rousvoal, N. Peters, et al. (2012). Sporophytes
366 derived from crosses were cultivated for two to three months before excision of material for DNA extraction.
367 Genomic sequence data accession numbers for strains Ec32, Ec568, EA1, RB1 and EcNAP-12-24 are
368 provided in supplementary table S1.

369 *Extraction of Genomic DNA and Identification of Heterozygous, Diploid Sporophytes*

370 Sporophyte tissue (10 to 20 mg wet weight) was frozen in liquid nitrogen and DNA was extracted using the
371 NucleoSpin® Plant II kit (Macherey Nagel) according to the manufacturer's instructions. As some

372 *Ectocarpus* gametes are able to undergo parthenogenesis to form haploid partheno-sporophytes, the ploidy
373 of the sporophytes derived from each cross was assessed using sex markers (supplementary table S3).
374 Diploid sporophytes are expected to carry both the female (U) and the male (V) sex chromosome whereas
375 partheno-sporophytes carry only one sex chromosome (U or V; supplementary fig. S2). Touchdown PCR
376 reactions, which consisted of 2 ng of DNA, 80 nM of primer mix, 0.2 mM dNTP, 2µl of 5X Go Taq® green
377 buffer (Promega), 2 mM MgCl₂, 2 mg/ml of BSA and 0.05 µl (0.25 units) of Taq polymerase (Promega),
378 were carried out in an Applied Biosystems thermocycler under the following conditions: 3 min at 95°C,
379 then 10 touchdown cycles of 30s 95°C; 30s at 65°C (-1°C/cycle) and 30s at 72°C followed by 25 cycles of
380 30s at 95°C, 30s at 55°C and 30s at 72°C and a final incubation at 72°C for 5 min before storage at 4°C.
381 After amplification, PCR products were separated and visualized on 2% agarose gels.

382 Mitochondrial Genome Assemblies and Detection of Intra-specific Mitochondrial DNA 383 Polymorphisms

384 An earlier assembly of the *Ectocarpus* species 7 strain Ec32 mitochondrial genome was re-evaluated using
385 high-coverage Illumina shotgun sequence data. A draft assembly of the *Ectocarpus siliculosus* strain EA1
386 genome, including organellar sequences, was generated using the CLC assembler (Qiagen Bioinformatics)
387 and Illumina shotgun DNA-seq data (supplementary table S1). Mitochondrial DNA scaffolds were
388 identified using the *Ectocarpus* species 7 mitochondrial genome as the query in a Blastn (Altschul et al.
389 1997) search. These scaffolds were then assembled manually to obtain the complete circular *Ectocarpus*
390 *siliculosus* strain EA1 mitochondrial genome. The genome was annotated by transferring annotation
391 information from the *Ectocarpus* species 7 strain Ec32 mitochondrial genome using Geneious R11.1.2
392 (<https://www.geneious.com>). Circular maps of the Ec32 and EA1 mitochondrial genomes were generated
393 using OGDRAW (Lohse et al. 2013).

394 Intra-specific mitochondrial DNA polymorphisms were identified either by mapping Illumina shotgun
395 DNA-seq data (supplementary table S1) against reference mitochondrial genome assemblies using Bowtie2
396 (Langmead et al. 2009) followed by manual SNP detection using GenomeView (Abeel et al. 2012) or by
397 PCR amplification and sequencing of targeted regions of the mitochondrial genome. For *Ectocarpus*
398 *siliculosus*, DNA-seq data for strains RB1 and EcNAP12-24 was mapped, individually, against the EA1
399 reference. For *Ectocarpus* species 7 strains Ec568 and Ec32, DNA-seq data for strain Ec568 was mapped
400 against the Ec32 reference. For both species, variants were detected with bcftools (Li et al. 2009) and
401 verified manually by visualisation of mapping data in GenomeView (Abeel et al. 2012). Mitochondrial
402 SNPs were detected for *Ectocarpus* species 7 strain Ec246 by Sanger sequencing of a region of the

403 mitochondrial DNA amplified using the oligonucleotide primers Trn (5'-
404 ATTGATTTAGCAAACCAAGGC-3') and Nad (5'-GGTAGYYTAGAATTGGGAATG-3').

405 Development of dCAPS Markers to Study Mitochondrial DNA Inheritance

406 Derived cleaved amplified polymorphic sequence-specific (dCAPS) markers (Neff et al. 1998) were
407 designed using dCAPS Finder2.0 (<http://helix.wustl.edu/dcaps/>) for the dCAPS primer and Primer 3
408 (<http://primer3.ut.ee/>) for the second primer of the primer pair. dCAPS primers allow the creation of a
409 diagnostic restriction enzyme recognition site specifically in the PCR product corresponding to one allelic
410 form of an SNP. Before use, dCAPS markers were tested on samples in which genomic DNA from the two
411 parental strains had been mixed in different proportions (1:2, 1:5, 1:10, 1:20). Touchdown PCR reactions
412 were carried out with dCAPS primer pairs in an Applied Biosystems thermocycler using the following
413 conditions for *Ectocarpus* species 7: 3 min at 95°C, then 10 touchdown cycles of 30s 95°C; 30s at 65°C (-
414 1°C/cycle) and 30s at 72°C followed by 25 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C and a final
415 incubation at 72°C for 5 min before storage at 4°C and the following conditions for *E. siliculosus*: 3 min at
416 95°C, then 10 touchdown cycles of 30s 95°C; 30s at 68°C (-0.1°C/cycle) and 30s at 72°C followed by 25
417 cycles of 30s at 95°C, 30s at 58°C and 30s at 72°C and a final incubation at 72°C for 5 min before storage
418 at 4°C. After amplification, 10 µl of PCR product was digested using five units of the relevant restriction
419 enzyme (supplementary table S3). Digestion products were separated and analysed on 2% or 2.5% agarose
420 gels. All marker genotyping tests were carried out twice to ensure that the result was reproducible.
421 Supplementary fig. S3 shows an example of typical dCAPS genotyping experiment.

422 Droplet Digital Polymerase Chain Reaction Assays to Detect Mitochondrial DNA

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

434 **Counts of Mitochondria using Confocal Microscopy**

435 A MitoTracker dye (MitoTracker® Orange CMTMRos ref MT7510, Invitrogen) was used to stain
436 mitochondria in freshly released gametes. Working solutions of MitoTracker dye were obtained by diluting
437 1 mM stock solution in DMSO to 0.166 μ M in freshly prepared Provasoli-enriched seawater. Gametophyte
438 filaments carrying plurilocular gametangia were allowed to release in 20 μ l of this solution on a clean
439 coverslip and the gametes were then fixed after 20 min at room temperature under low light by addition of
440 glutaraldehyde to a final concentration of 1%. Confocal microscopy was carried out with a Leica SP5
441 microscope (TCS SP5 AOBS, Merimage platform, Roscoff) and z-series of images were analysed with
442 ImageJ/Fidji to count the number of mitochondria in each gamete. The strains used for the mitochondrial
443 counts are indicated in supplementary table S1.

444 **Evaluation of Parthenogenetic Capacity**

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

448 **Data Availability**

449 All the sequence data used in this study has been submitted to public databases and can be recovered using
450 the accession numbers provided in supplementary table S1.

451 **Acknowledgements**

452 We thank Lucie Caradec for technical assistance. This work was supported by the Centre National de la
453 Recherche Scientifique, the Japan Society for Promotion of Science (JSPS), the Agence Nationale de la
454 Recherche (project Bi-cycle ANR-10-BLAN-1727), the European Research Council (SexSea grant
455 agreement 638240) and Sorbonne Université.

456 **Author contributions**

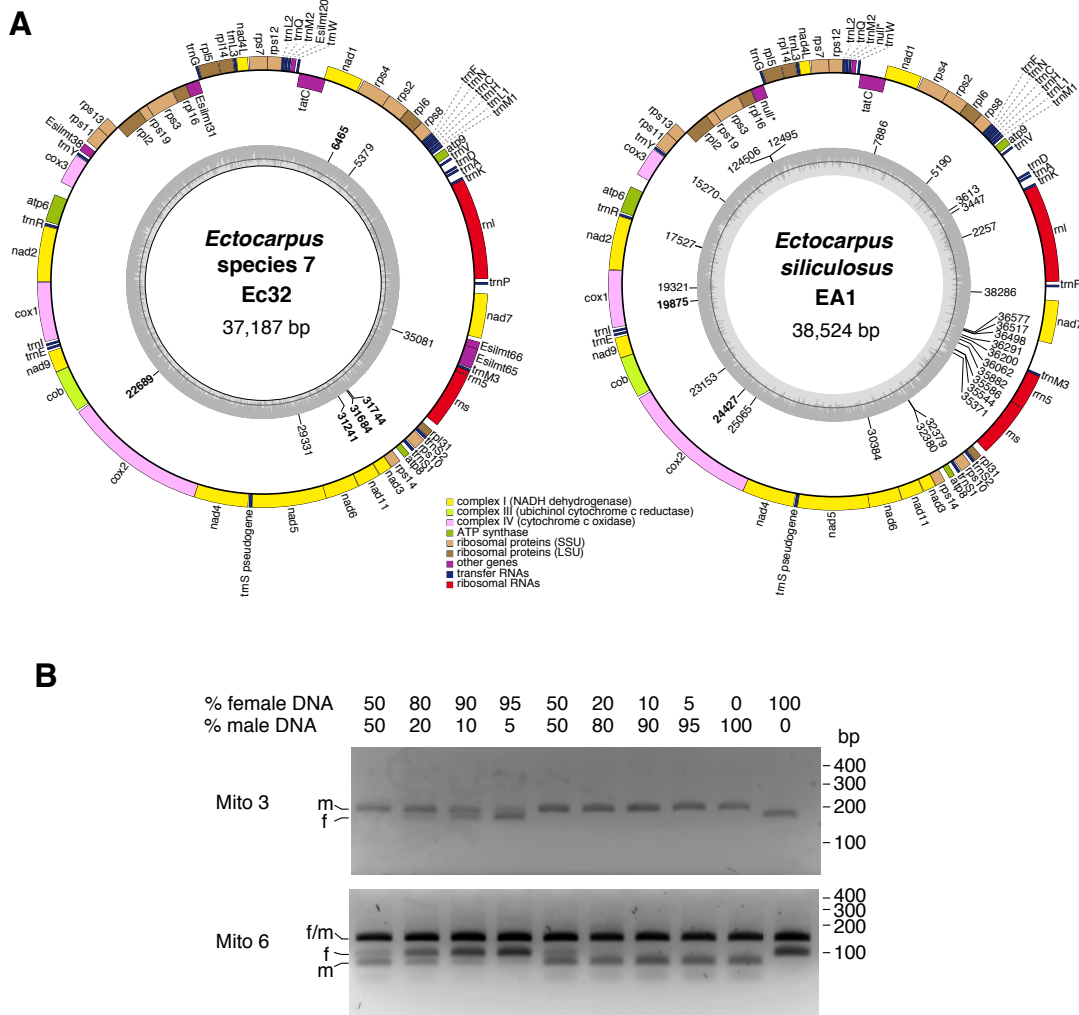
457 LM, CN, AFP, DS, FP, YB and TM prepared the biological material and performed experiments. LM,
458 MMP, DW and JMC performed the computational analysis. LM, CN, DS, FP, YB, DW, SMC and JMC
459 analysed data. JMC designed and coordinated the study. JMC wrote the manuscript with valuable input from
460 LM and SMC. All authors read and approved the final manuscript.

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570

571 **Fig. 1.** Mitochondrial genome analysis and development of dCAPS markers. (A) Mitochondrial genomes of

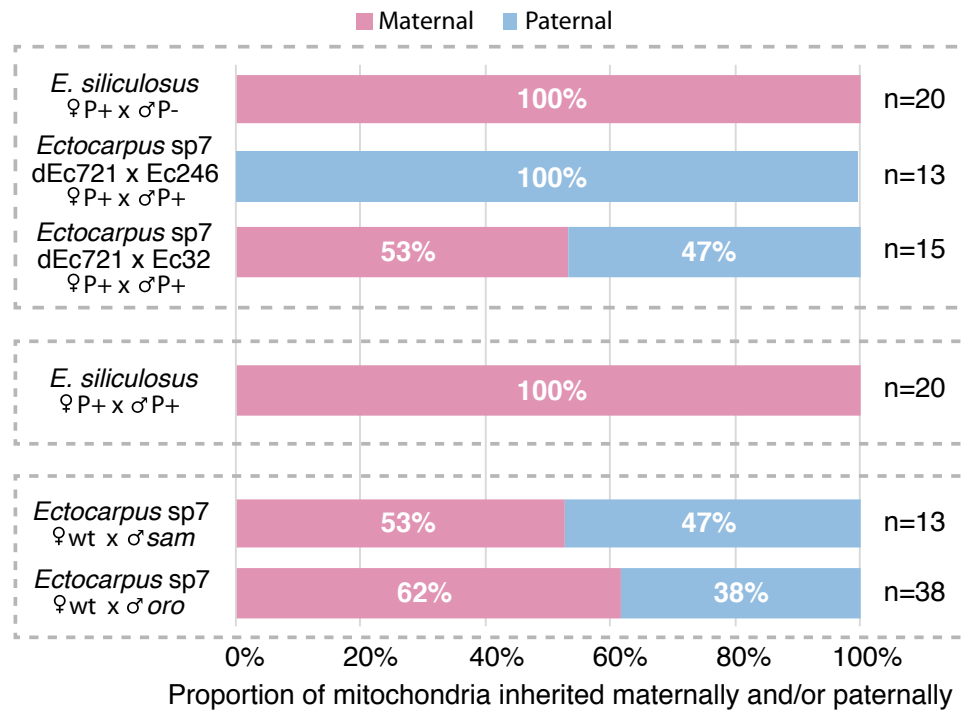
572 *Ectocarpus* species 7 strain Ec32 and *Ectocarpus siliculosus* strain EA1. The inner circles show GC content

573 and the positions of the intra-specific polymorphisms detected by this study. Polymorphisms indicated in

574 bold were used to develop dCAPS markers. (B) *In vitro* tests of dCAPS markers. PCR amplifications were

575 carried out using genomic DNA from the two parental strains mixed in different proportions. f, female; m,

576 male; bp, base pairs.



578

579 **Fig. 2.** Inheritance of mitochondrial genomes following different intra-specific crosses. Percentage of
 580 sporophyte progeny carrying maternal (pink) or paternal (blue) mitochondrial genomes. *Ectocarpus* sp7,
 581 *Ectocarpus* species 7; dEc721, daughters of the sporophyte Ec721; Ec32 and Ec246, male gametophytes
 582 corresponding to independently isolated strains; P+, parthenogenetic; P-, non-parthenogenetic; wt, wild
 583 type; *sam*, *samsara* mutants; *oro*, *ouroboros* mutant. See supplementary table S1 for further details about
 584 the genetic crosses.

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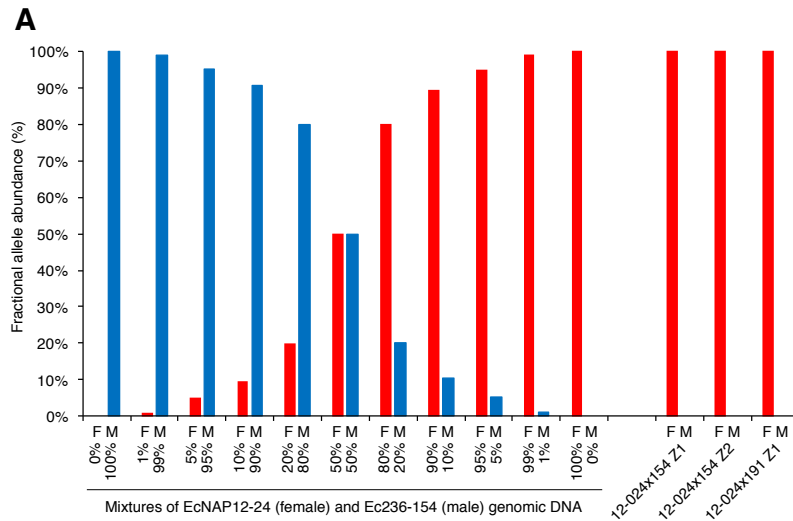
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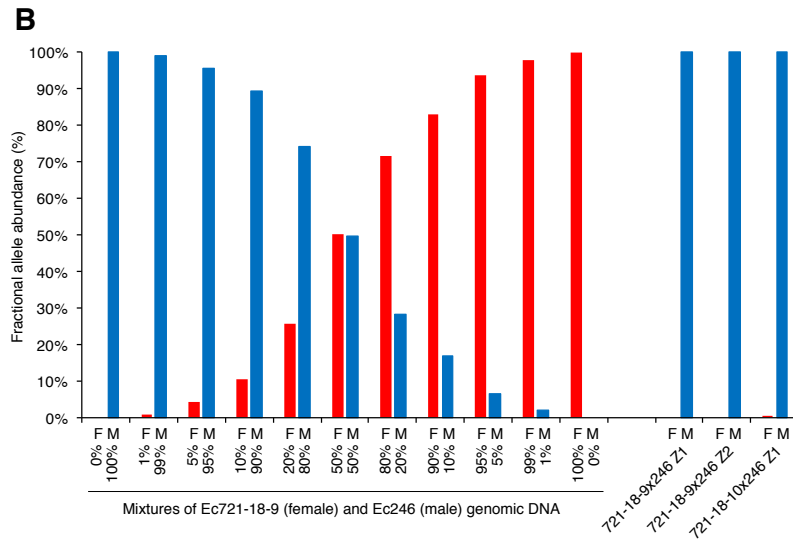
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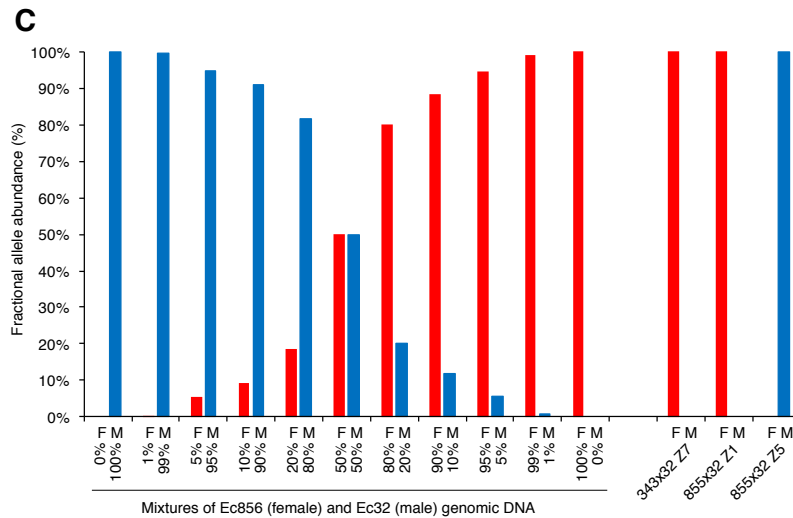
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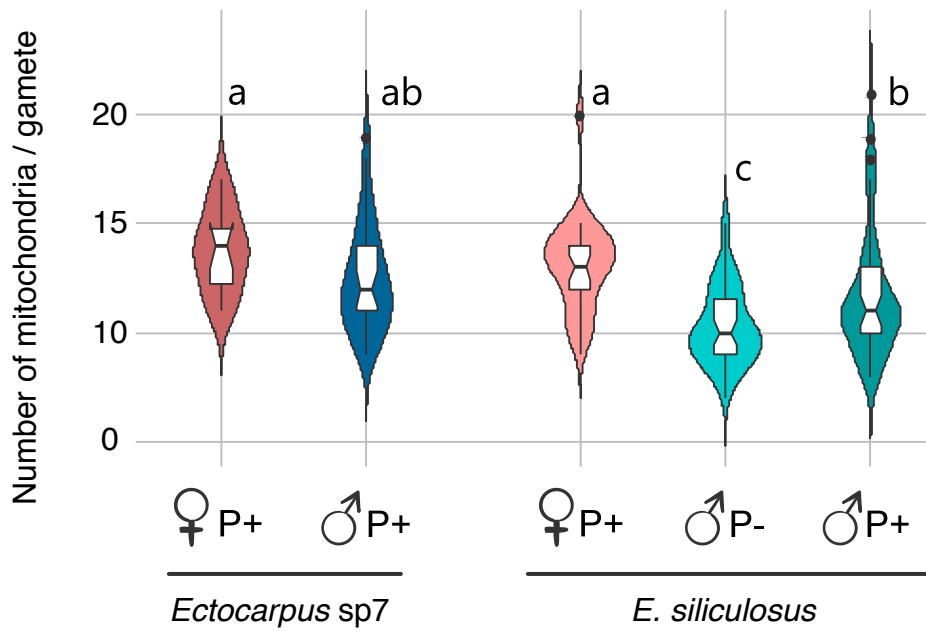
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611 **Fig. 3.** Droplet digital PCR assays indicate uniparental inheritance of mitochondrial DNA. Each panel
612 includes an *in vitro* test using genomic DNA from two parental strains mixed in different proportions on the
613 left and analyses of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

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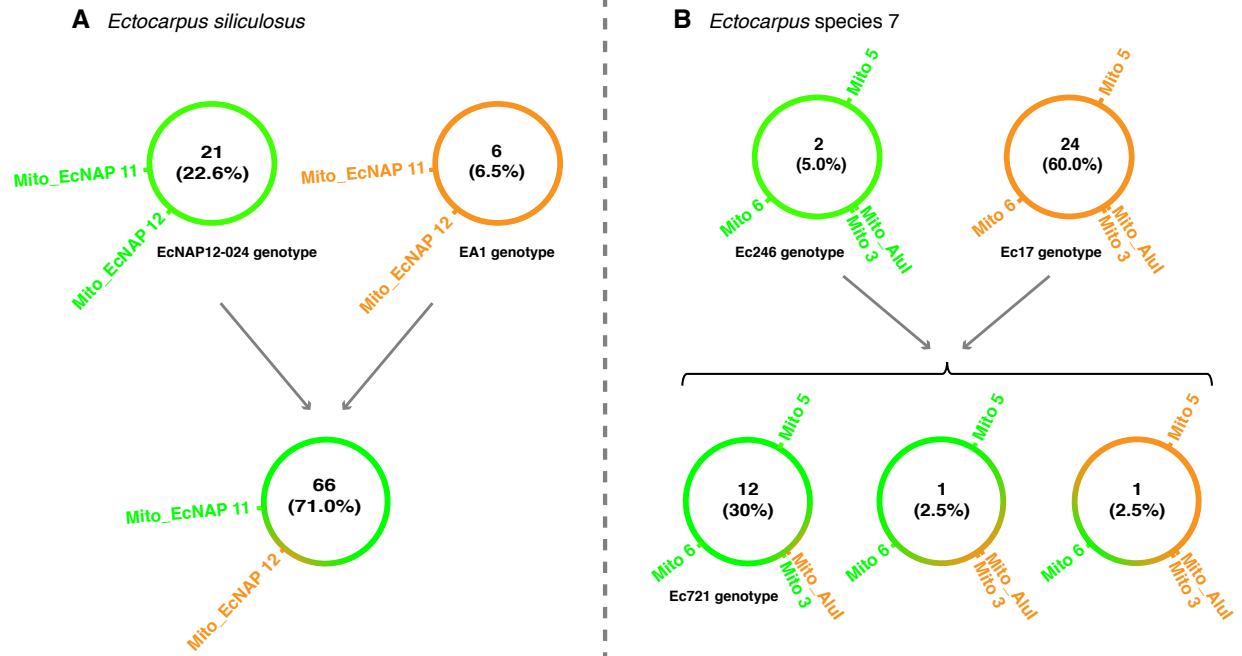


620

621 **Fig. 4.** Number of mitochondria in gametes of different *Ectocarpus* strains. Letters indicate significant
 622 differences (Kruskal Wallis test followed by Dun's post hoc-test). *Ectocarpus* sp7, *Ectocarpus* species 7;
 623 P+, parthenogenetic; P-, non-parthenogenetic. The strains analysed for each class of gamete are indicated
 624 in supplementary table S1.

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628 **Fig. 5.** Recombinant mitochondrial genomes detected in field isolates of (A) *Ectocarpus siliculosus* and (B)

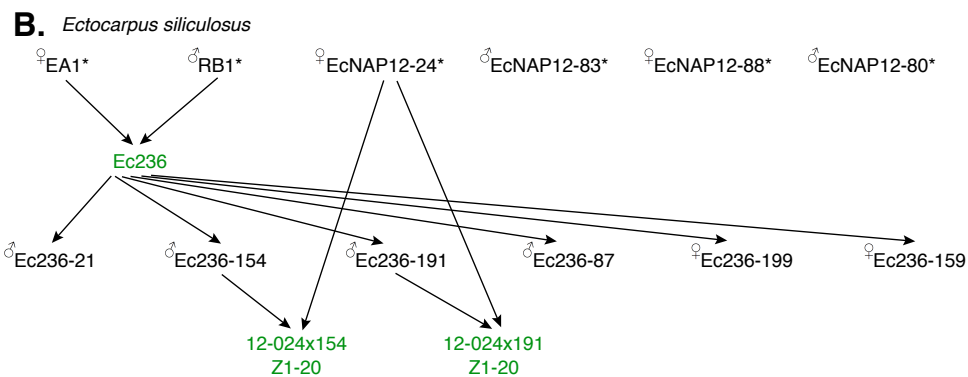
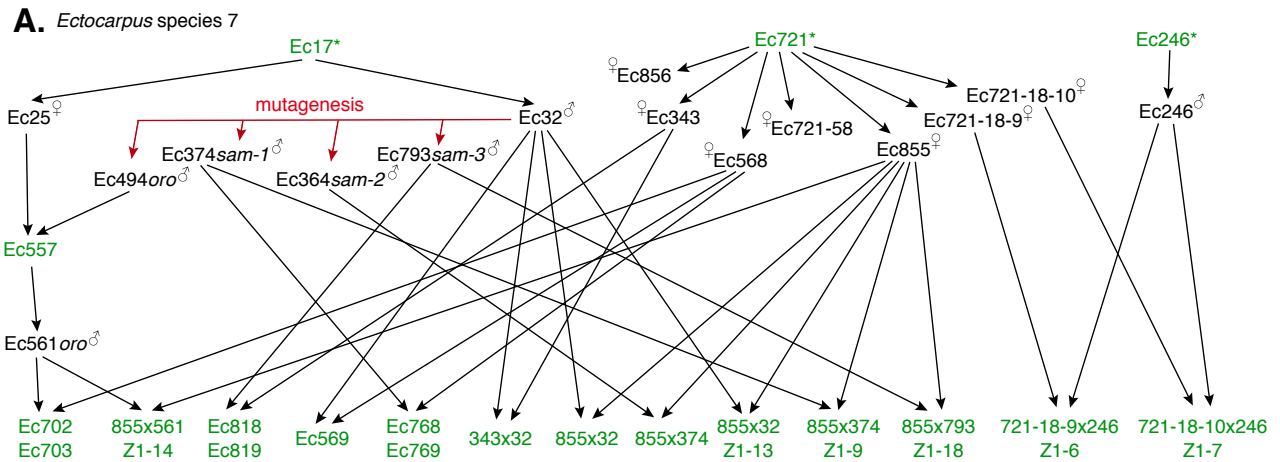
629 *Ectocarpus* species 7. Colours are used to indicate allelic variants corresponding to one or the other parental

630 genome. Numbers and percentages of each mitochondrial genotype are indicated, as are the genotypes

631 corresponding to strains used for the crossing experiments. See supplementary table S4 for information

632 about the field isolates.

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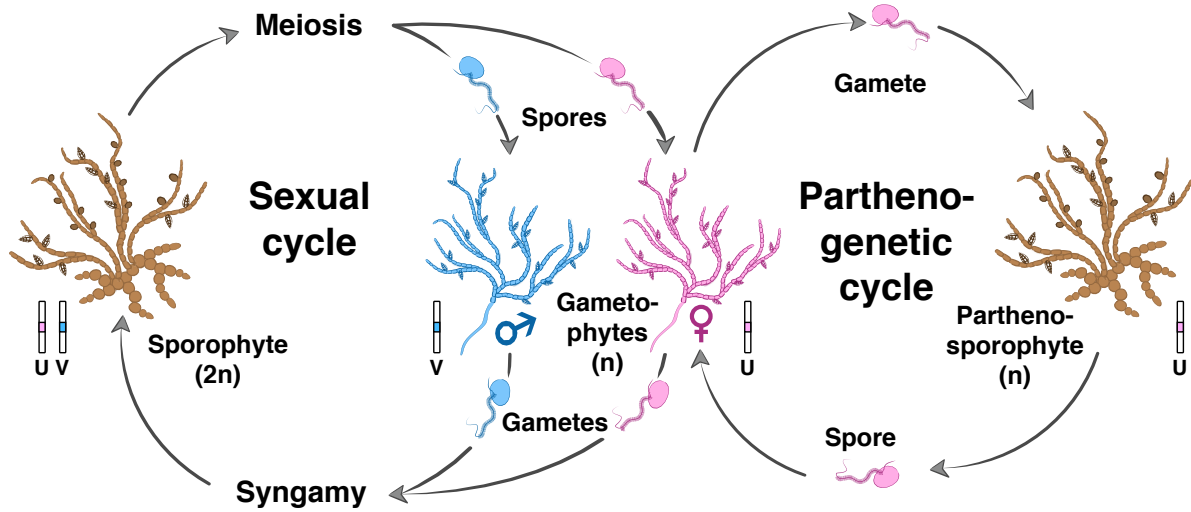


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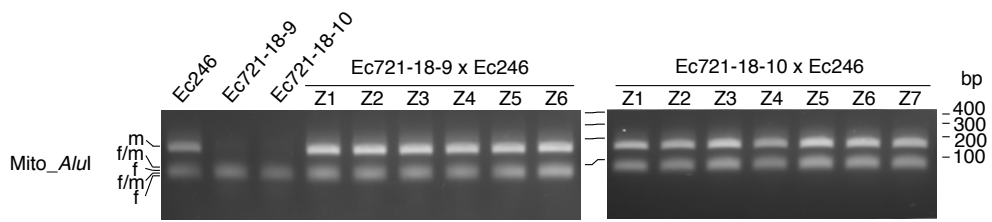
637 **Supplementary fig. S1.** Pedigrees of the *Ectocarpus* strains used in this study. (A) *Ectocarpus* species 7
 638 strains. (B) *E. siliculosus* strains. See supplementary table S1 for further information about the strains
 639 indicated. Asterisks indicate field-isolated strains, green indicates diploid sporophytes (apart from Ec246,
 640 which was probably isolated as a haploid partheno-sporophyte), black indicates gametophytes. *oro*,
 641 *ouroboboros* mutant; *sam*, *samsara* mutants; z, zygote.

642



643
 644 **Supplementary fig. S2.** *Ectocarpus* life cycle. The sexual cycle involves alternation between a diploid
 645 sporophyte and haploid, male and female gametophytes. Gamete parthenogenesis leads to the production of
 646 haploid partheno-sporophytes. White bars represent sex chromosomes with coloured boxes for the U or V
 647 sex-determining regions. Note that diploid sporophytes derived from zygotes carry both a U and a V
 648 chromosome whereas haploid partheno-sporophytes carry only one sex chromosome.

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650

651 **Supplementary fig. S3.** Example of dCAPS genotyping of mitochondrial genomes. Ec246, male parent
 652 gametophyte; Ec721-18-9 and Ec721-18-10, female parent gametophytes; Z1 to Z7, diploid sporophytes
 653 grown from zygotes generated by crossing either Ec721-18-9 or Ec721-18-10 with Ec246; f, m and f/m,
 654 DNA fragments corresponding to female, male or both mitochondrial genomes; bp, base pairs.

655

656 **Supplementary tables**

657

658 **Supplementary Table S1.** List of *Ectocarpus* strains used in this study.

659

660 **Supplementary Table S2.** Intra-specific mitochondrial DNA variants detected in this study.

661

662 **Supplementary Table S3.** PCR sex markers used to detect diploid individuals and dCAPS and ddPCR
663 markers used to genotype mitochondrial DNA in zygotes.

664

665 **Supplementary Table S4.** Mitochondrial genotypes of field-isolated *Ectocarpus* strains.