Complex genetic determination of male-fertility restoration in the gynodioecious snail *Physa acuta*

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Abstract

Male fertility in plants is often controlled by the interaction between mitochondrial and nuclear genes. Some mitotypes confer cytoplasmic male sterility (CMS), making the individual male-sterile, unless the nuclear background contains alleles called restorers, that suppress the effects of CMS and restore the hermaphroditic condition. Restorers in cultivated crops are often alleles with strong and dominant effect, but in wild plants, data often suggest more complex systems. Here, we characterized the inheritance and specificity of restoration in a new CMS model, the freshwater snail *Physa acuta*. We explored two different populations (i) a naive population i.e., without contact CMS in the past 80 generations, (ii) a non-naive population, where CMS is present and largely restored. We found that restoration potential was indeed heritable in both contexts and specific to a CMS type. However our results suggest that restoration of male fertility could be of a different nature depending on population history: in naïve populations not coevolved with CMS the background variation may include alleles that happen to act as weak quantitative modifiers of the penetrance of CMS, while in populations coevolved with CMS, selection may have favored, when such variants were available, the emergence of strong alleles with a dominant effect.

Key words: cytoplasmic male sterility, restoration, genetic determination, *Physa acuta*

Introduction

Gynodioecy is a sexual polymorphism, common in angiosperms (Dufay et al., 2014; Caruso et al., 2016), whereby hermaphrodites coexist with male-sterile individuals (i.e., functionally females, Saumitou-Laprade et al., 1994). Gynodioecy represents a well-known example of genetic conflict (Burt & Trivers, 2006) because sex determination in gynodioecious systems is often cyto-nuclear. In that case, cytoplasmic male sterility (CMS) genes, usually associated with the mitochondrial genome, suppress pollen production and render an individual functionally female unless CMS is counteracted by one or more nuclear genes that restore the male function (Cosmides & Tooby, 1981; Saumitou-Laprade et al., 1994; Werren & Beukeboom, 1998). An individual is phenotypically female if it carries CMS without the proper nuclear gene known as a restorer. It is a hermaphrodite if it carries either (i) a male-fertile cytoplasm, or (ii) a CMS cytoplasm paired with the proper nuclear restorer. CMS has been documented in 140 species of angiosperms (Laser & Lersten, 1972), and twenty-eight CMS genes from 13 crop species have been identified (Chen & Liu, 2014).

It appears that cyto-nuclear gynodieocious systems often contain multiple forms of CMS (e.g. *Oryza sativa, Zea mays*, see Chen and Liu 2014 for a review), each requiring its own mode of restoration (A. de Haan et al., 1997; Charlesworth & Laporte, 1998; van Damme et al., 2004). Fourteen restorer genes (*Rf*) from nine crops have been isolated (see Table 1 Kim & Zhang, 2018), with the majority of them encoding PPR (Pentatricopeptide Proteins Repeat). However, *Rf* genes encoding for other proteins have been discovered, including peptidase in sugar beet (Yamamoto et al., 2008; Matsuhira et al., 2012) and aldehyde dehydrogenase in maize (Cui et al., 1996). In most cases, a *Rf* gene is specific to one CMS, whereas a CMS gene can be affected by multiple *Rf* genes (see Table 1 Chen and Liu 2014). For example, in *Z. mays* the CMS-T is restored by both *Rf1* and *Rf2* (Cui et al., 1996; Dewey et al., 1987; Dill et al., 1997) and in *Plantago coronopus* at least five restorer alleles seem to be involved in the restoration of CMS (Koelewijn & Van Damme, 1995). Cases in which a gene restores more than one CMS mitotype are rare. Such an example is found in the common rice, where *Rf5* and *Rf6* (initially identified in CMS-HL individuals) are also able to restore CMS-BT mitotype (Huang et al., 2012, 2015; Kim & Zhang, 2018).

The maintenance of cyto-nuclear polymorphism has been extensively modelled (e.g. (Charlesworth & Ganders, 1979; Couvet et al., 1998; Gouyon et al., 1991 ; Bailey et al., 2003 ; Dufaÿ et al., 2007) and theoretical studies have shown that the stability of this polymorphism depends on a variety of factors, including the positive pleiotropic effects

of CMS alleles (i.e., seed fitness advantage of females, or "female advantage", Lewis, 1941) and negative pleiotropic effects of restorer alleles ("cost of restoration", Charlesworth & Ganders, 1979; Delannay et al., 1981; Frank, 1989; Gouyon et al., 1991). Models often assume that restoration of male fertility is achieved through one allele (but see Frank 1989) and a variety of assumptions regarding its dominance of restoration have been modeled (Delph et al., 2007). The majority of those models considered the presence of dominant restorer alleles with only a couple a cases using a recessive inheritance (Ross & Gregorius, 1985). Regarding empirical studies, most of the crop species exhibited dominant restorer alleles (see Table 2 Delph 2007 for a review but see also *Plantago lanceolata*, van Damme, 1984 ; de Haan et al., 1997). However, in natural populations the restoration of male fertility suggests a more complex genetic determination (Charlesworth & Laporte, 1998; Koelewijn, 2003; Touzet et al., 2004). Indeed, several nuclear loci may act in an additive manner (Charlesworth & Laporte, 1998; Dufay et al., 2008) and/or through a threshold effect (Ehlers et al., 2005). For example, Koelewijn & van Damme (1995) suggested that at least five restorer loci were involved in restoring male sterile cytoplasms of *Plantago coronopus*, and Dudle et al., (2001)found that sex determination in *Lobelia siphilitica* seemed to vary among cytoplasms with one CMS gene being restored by a single dominant allele, while restoration of other CMS could only be explained by the action of several nuclear loci and/or epistatic effects. Inspired by those examples, Elhers et. al (2005) developed a threshold model for sex-determination in gynodioecious species and showed that treating restoration of CMS as a quantitative trait can be an alternative way of explaining complicated patterns of restoration in CMS species. Following on those steps, Bailey & Delph (2007) described a model of cyto-nuclear controlled gynodioecy, in which sex was determined by two CMS types each one interacting with multiple restorer alleles. The used assumptions reflected several well-studied cases of gynodioecy, such as, *P. coronopus, P. lanceolata, S. vulgaris*, and *T. vulgaris* and showed that females can be maintained in the population (Belhassen et al., 1991; de Haan et al., 1997; Koelewijn, 2003; Mccauley & Olson, 2003).

A mix of several determinisms is an interesting situation to consider. Dominant and specific restorers acting on one or a few CMS mitotypes, found in present-day plant populations, are strong-effect alleles that may result from a history of coevolutionary arms-race with their specific CMS gene (e.g. Sloan et al., 2012), or at least from the selective sweep of a favorable mutant (Bergero et al., 2019). When a new CMS type first appears, it is unlikely that such strong-effect specific restorer alleles are already ready and waiting.

However, the standing variation may contain genotypes that happen to reduce the penetrance of CMS, not necessarily in a way that fully restores male-fertility, and not necessarily in a specific way. Such a background variation may contribute to the cases of complex inheritance in plants, but is expected to be more visible when there are not yet specific large-effect restorers segregating in the population, a relatively rare condition (refs).

As illustrated above, CMS is well understood theoretically and prevails in plants but is exceedingly rare among animals (Vellnow et al., 2017). However, CMS has been recently reported in one animal: *Physa acuta*, a freshwater snail (David et al., 2022). In this species, two mitochondrial types conferring male sterility, D and K, have been discovered so far, both of them showing an extreme molecular divergence from one another, as well as from normal, fertile cytotypes (collectively called N), at all mitochondrial genes. The D mitochondrial type was discovered first, but evidence for corresponding nuclear restoration is still lacking (David et al. 2022). Recently however, the second CMS mitotype K revealed a different situation, with evidence that most K individuals captured in natural populations have a male-fertile phenotype, i.e., there exist genes that restore male fertility in K-mitotype snails, while male-sterility reappears when the K mitotype is introgressed into a "naïve" laboratory genetic background maintained without contact with CMS (Laugier et al., 2024).

In this study, we explored the inheritance and specificity of restoration in *Physa acuta*. We aimed to characterize the variation in restoration for the K mitotype, specifically to test if the data are consistent with the existence of dominant-restorer alleles in natural populations where K is present. We also tested whether these restorers are specific to K or also have an effect on male-fertility of the D mitotype. Finally, we were interested in the determination of male-fertility in our laboratory population, where the K-mitotype, although mostly male-sterile, still maintains a fraction of male-fertile individuals. This population may represent a truly "naïve" state, i.e. the state of a population in which CMS would be newly introduced and confronted to standing variation; or it may have inherited, from many generations ago, the same restorer alleles as in natural populations where K is present, but these alleles would now be at a low frequency.

To achieve these objectives, we phenotyped CMS K- and D-mitotype individuals within two different nuclear backgrounds (Lyon and Montpellier). The Lyon population has been in contact with both K and D, while the Montpellier has not. We used two different approaches to reveal genetic variation in restoration potential within Lyon and Montpellier population: we produced populations selected against and for restoration, and we analysed inbred lines. This approach enables us to explore restoration in various contexts and

131 characterize it accordingly. Answers to these questions will provide insight into the CMS
132 evolutionary dynamics and, for the first time, explore genetic variation for CMS restoration
133 in animals.
134
135 **Material and methods**

136 ***Study system***
137 *Physa acuta* is a cosmopolitan, hermaphroditic, predominantly outcrossing, freshwater snail,
138 very common in natural freshwaters worldwide, and easy to raise in the laboratory. The
139 generation time is about 6-8 weeks in the laboratory at 25°C ; we maintain this snail in
140 standard conditions (25°C, 12/12 photoperiod) using ground water changed at least once a
141 week, and grinded boiled lettuce ad libitum as food. Cytoplasmic male sterility has been first
142 described by David et al. (2022) in this species, later completed by Laugier et al. (2024).
143 Briefly two very divergent mitogenomes, named D and K, have been found to coexist with
144 the normal (N) type in wild populations near Lyon (France). While N individuals are regular
145 hermaphrodites, D and K are associated with male-sterile phenotypes, unable to play the
146 male role in sexual interactions and to sire offspring. Nearly all the D (92%, David et al.
147 2022) individuals are male-sterile. In contrast, nuclear backgrounds restoring male-fertility
148 of K snails are found in natural populations where K is present in Lyon (France). However
149 the same K mitotype, when introgressed into the nuclear background of a population from
150 Montpellier (hereafter « naive », maintained for >80 generations in the laboratory without
151 contact with CMS), produces around 70% male-sterile phenotypes (Laugier et al., 2024).
152 The nature of the ~30% residual fertility is still hypothetical, it could be either that restorer
153 genes are segregating at low frequency in the naive population (despite the absence of K
154 mitotypes) ; or that the penetrance of CMS is incomplete, always leaving a fraction of
155 individuals with nonzero male fitness.
156
157 ***General structure of the experiments***
158 Our objective was to characterize, within both the naive (Montpellier) and the non-naive
159 (Lyon) backgrounds, genetic variation for the potential to restore male fertility in CMS
160 mitotypes. We did this in two ways (i) first, we tested whether we could select for high versus
161 low restoration potential within the naive population (ii) second, we produced inbred lines
162 from both populations and characterized genetic variance in restoration potential among
163 lines (trying, in particular, to find lines fixed for either restorer or maintainer alleles). In both

164 cases, lines were of the N mitotype, and their restoration potential was evaluated by crossing
165 them with either a K or a D snail, and evaluating the male fertility of the offspring. In these
166 crosses, the paternal strains were N while the maternal ones were K or D. As mentioned
167 above, all (or nearly all) our D individuals are un-restored, mostly male-sterile individuals.
168 However, two K strains are available, one with the original background from Lyon (with
169 high restoration potential and mostly male-fertile, noted KF) and one with the naive
170 background from Montpellier (low restoration potential, mostly male-sterile, noted KS). We
171 used both types to be in position to observe the effects of potential restorer alleles provided
172 by the paternal strain either in a predominantly homozygous (cross with KF) or heterozygous
173 (cross with KS) form.
174
175 *Source populations and their history in the laboratory*
176 The Montpellier population has been founded in 2007 by a mix of ten natural populations
177 sampled around Montpellier, and maintained in the laboratory, in high numbers, since then
178 (Figure 1). The albino stock was separated from the main population in the first few
179 laboratory generations (see Noël et al., 2016). It has been maintained as an autonomous
180 large outbred population in aquaria to this day (estimated age in 2023: 96 generations) and
181 is entirely of the N mitotype and male-fertile. Albinism is coded by a recessive allele, and
182 albino mothers produce pigmented babies when inseminated by a wild type snail. More
183 recently, a new type of albinotic mutants appeared within the Montpellier stock, with a
184 slightly different phenotype (see Figure S1 and S1). After some crosses (see S1) we were
185 able to establish it as an independent outbred stock (called alb-2) that complements with the
186 first albino stock (now called alb-1): alb-1 and alb-2 are mutated at different loci, and crosses
187 between them produce 100% pigmented individuals, as well as crosses between any of the
188 two and pigmented homozygous snails.
189 The Lyon populations have been founded from two populations sampled in 2019 near
190 Lyon (Irigny and Erevan, see Laugier et al. 2024) mixed together. They initially contained a
191 mix of K and N mitotypes, the former with mostly male-fertile phenotypes (see above) and
192 all pigmented (wild-type). After mass-mating, we collected progenies from individual
193 mothers that were typed by PCR as either K or N; and the K and N progenies were pooled
194 into two separate tanks, to initiate pure-K and pure-N stocks (Figure 1). Each stock was kept
195 as a separate population (hereafter KF and NF populations) and maintained as a large outbred
196 population to this day (in 2023, KF and NF were at generation 20 after foundation). Each of
197 the outbred populations (alb-1, alb-2, NF, KF) was maintained throughout as a

metapopulation of 5 aquaria, each with 100-150 snails at reproductive stage, freely mating (« mass-mating »), with regular exchange of ~10% individuals among aquaria; the eggs are separated from the parents to hatch as soon as there are enough on the aquarium walls, and a new cohort is started. Aquaria are fed once to twice a week depending on life stage.

A last source of snails for our experiments is a set of lines in which the D or the K mitotypes have been introgressed into the naive Montpellier alb-1 background (David et al 2022, Laugier et al 2024). Briefly this introgression takes place in sets of « matrilines » each founded by a D or K ancestor from Lyon (wild population) and propagated by maternal descent. At each generation each snail from a matriline is inseminated by an individual taken at random from the alb-1 stock so that over generations the genome of the founder is progressively replaced by the alb-1 background. At the time of the experiment the D matrilines were at the 41th generation and the K matrilines at the 20th generation, their genome can be considered as totally replaced. Matrilines are maintained pigmented and heterozygous to distinguish them from their albino mates: at each generation we obtain a mix of albino and pigmented progeny, of which we keep only the latter to propagate the matriline. For the experiment however it was practical to use the albino progeny. Both K and D matrilines are mostly male-sterile (as mentioned above); for this reason we will refer to them as KS and DS respectively (by contrast, KF snails are restored and mostly male-fertile).

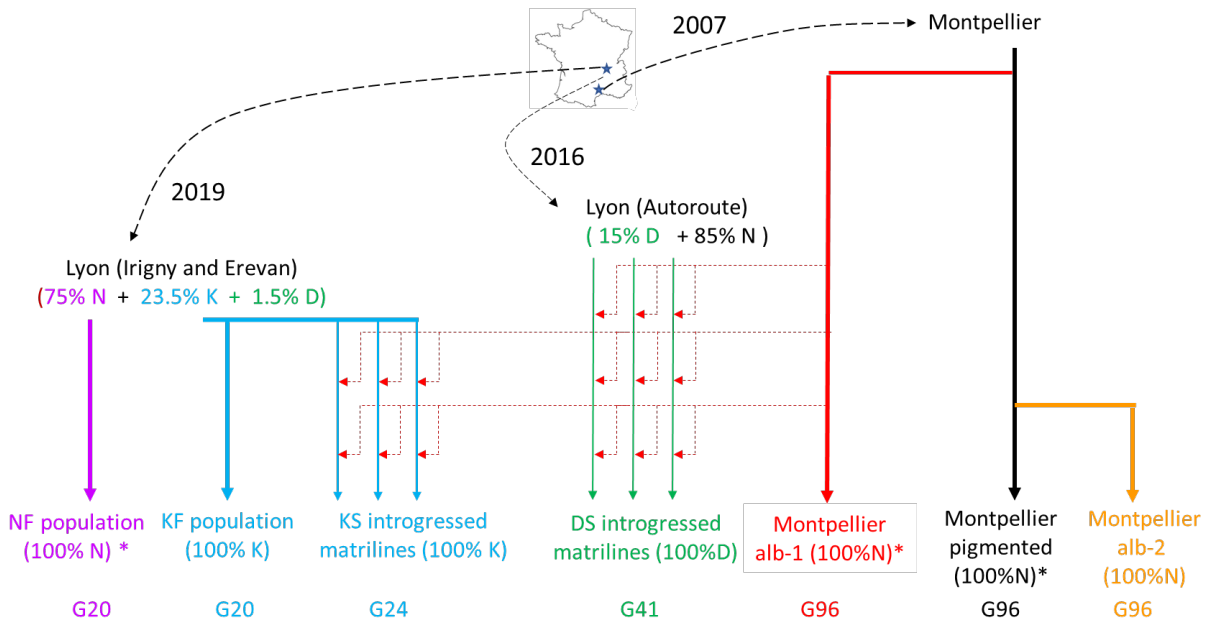

Figure 1: **Source populations and their history in the laboratory since extraction from natural populations (indicated by dashed arrows, with year of sampling).** Thick lines

221 indicate propagation in large outbred populations in aquaria. Fine lines indicate propagation
222 in matrilines, with insemination at each generation by sperm donors from another population
223 (dotted arrows). The percentages of each mitotype (K, D or N) are indicated for the natural
224 populations at sampling (from David et al.2022, Laugier et al. 2024) and for populations
225 kept in the laboratory. The number of generations elapsed since extraction from natural
226 populations in late 2022 – early 2023 (when most of the experiment took place) is indicated
227 as G_{XX} below each stock. The numbers of independent matrilines are 12 for KS and 11 for
228 DS. Populations from which inbred lines were extracted are indicated by a star (*). The
229 coordinates of the populations of origin can be found in Noël et al. 2016 (Montpellier), David
230 et al. 2022 (Lyon, Autoroute), and Laugier et al. 2024 (Lyon, Irigny and Erevan)
231
232 ***Selection for high and low frequency of restoration of the K mitotype***
233 The aim of this selection was to mimick the arrival of a CMS mutant in a naive population
234 and test whether there was variation from which restoration potential could be rapidly
235 selected in such a situation. This was envisaged for the K mitotype because the penetrance
236 of the male-sterile phenotype in KS individuals (mitotype K introgressed into the naive alb-
237 1 background) is around 60-70% ; if the residual fertility is genetic it can increase or decrease
238 by selection. On the other hand, the residual fertility of DS snails is too low to start a similar
239 selection.
240 The selection protocols are detailed in supplementary S2. In short, to select against
241 restoration, we kept the (N-mitotype) alb-1 sperm donors that, when crossed with a KS snail,
242 produced only male-sterile K daughters. The selected sperm donors where then grouped and
243 constituted the first generation of selection for low-frequency-of-restoration (LFR). We re-
244 used offspring of these individuals as sperm donors for a second round of selection. The LFR
245 individuals used here are therefore considered to be at their second generation of selection
246 against restoration. The selection for high frequency of restoration (HFR) is simpler because
247 selecting for fertility is easier than for sterility: we simply kept the offspring sired by male-
248 fertile KS individuals at each generation. The HFR population was selected for two
249 successive generations, as the LFR. Expected results of such a selection in the simplest
250 scenario (fully penetrant, monodominant restorers) are given in S2.
251
252 ***Constitution of inbred lines***
253 Inbred lines allow in principle to fix restoring and non-restoring genotypes in the
254 homozygous form. We created inbred lines from N-mitotype ancestors originating from

either the Lyon or the Montpellier laboratory populations (more lines were made from Montpellier where restoration is supposed to be rare). Each ancestor produced a line by at least two generations of selfing (enforced by letting a virgin snail reproduce in isolation) and single-individual descent. Lines are not fully homozygous at the second generation (there remains ¼ of the initial heterozygosity) but we had to find a compromise with the time required and with the potential loss of lines by inbreeding depression over generations. In this experiment we used 9 lines from Lyon and 40 from Montpellier.

Crosses

In total we tested the restoration potential of focal individuals from two selection lines (HFR and LFR) and from 49 inbred lines of two origins: Montpellier (naive population= and Lyon (experienced population). To that end these focal individuals were used to inseminate three types of individuals: DS, KS and KF. The resulting progeny has the focal individual as father and DS, KS or KF mother. In each progeny we grew one to three G1 offspring until maturity and tested them for male fertility. If the father transmits restorer alleles, these alleles are present in the tested offspring either in a mostly heterozygous form (mothers DS or KS, with absent or rare restoration), or in a mostly homozygous form (mothers KF, with a high rate of restoration). The total number of progenies tested was 1508, with the repartition indicated in Table 1.

Table 1: Sample sizes by category. For each combination of paternal and maternal types, we provide N_1/N_2, where N_1 is the number of G_1 individuals successfully assessed for male fertility, and N_2 is the number of G_0 mothers (each mother produced 1 or 2 G_1 individual).

		Maternal			
		KS	KF	DS	Total
	Lyon inbred lines	164/82	36/21	65/47	265/150
	Montpellier inbred lines	350/231	220/130	211/168	781/529
	Total inbred lines	524/313	256/140	276/215	1056/668
Paternal	HFR selected population	52/31	49/27	32/22	133/80
	LFR selected population	248/105	39/20	32/25	319/150
	Total selected populations	300/136	88/47	64/47	452/230

281 ***Assessment of male sterility***

282 The assessment of male sterility of an individual is made by pairing it to a virgin partner for
283 three days, letting the latter lay for three days in isolation, and observing the progeny. The
284 studied individual is said to be male-sterile if either the partner does not lay any egg (failure
285 to stimulate egg-laying, which is a normal outcome of insemination) or it lays eggs but the
286 juveniles are 100% self-fertilized. Partners of male-fertile individuals in this situation
287 normally produce several tens of eggs and juveniles are nearly all outcrossed. A small
288 number of individuals had an intermediate status: outcrossed individuals were obtained but
289 in abnormally low numbers (<10) ; in this case we recorded them as semi-sterile. In the
290 analyses we grouped these together with fully male-sterile individuals (without appreciable
291 consequence on the results). It is, in any case, important to realize that the male-fertility test
292 is not perfect: even male-fertile individuals (with fertile mitotype) occasionally fail to
293 inseminate their partner during pairing, with a probability <10% in our lab conditions.

294 We used body pigmentation to recognise outcrossed offspring from self-fertilized
295 ones. Depending on the paternal pigmentation genotype, we used either albino alb-1 or alb-
296 2 partners in such a way that any pigmented offspring they produced must have been sired
297 by the tested individuals. We therefore simply counted the number of pigmented offspring
298 in the progeny after incubating clutches for 13 days (we stopped counting at 20 and noted
299 20+ if all offspring were pigmented, and counted exhaustively all pigmented and albino
300 offspring otherwise, including those that were well-developed though not yet hatched, if
301 any). In some cases (heterozygous individuals tested with an alb-1 partner) outcrossed
302 offspring were expected to be 50% pigmented, 50% albino instead of 100% pigmented; in
303 this case we set the threshold for semi-sterility to 5 pigmented (i.e. the expectation for 10
304 outcrossed offspring) instead of 10 (see above).

305 We tested male fertility in G1 individuals and in their DS, KS, KF mothers too. Many
306 of the latter were pigmented and mated with albino N-mitotype fathers; thus the father could
307 act as a partner for testing the male-sterility of the mother, and we kept the father's progeny
308 to that end. In case the father was a pigmented individual and/or the mother an albino, this
309 was not possible so we provided a second partner to the mother, after she had laid eggs, and
310 this second partner was alb-2 so that outcrossed offspring would be recognised (alb-1 and
311 alb-2 are complementary lines and produce pigmented babies upon cross-fertilization).
312 Because of the size of the experiment, it was spread over nearly two years from 2022 to early
313 2024, with four phases treated as temporal blocks.

314

315 ***Statistical analyses***

316 We analysed separately the differences between the selected populations (HFR and LFR) on
317 one hand, and the variation among inbred lines on the other hand. The male-fertility status
318 of the G_1 individuals from selected lines was treated by GLMMs as a binary 0/1 variable.
319 The main model included the maternal type (KF, KS or DS), the selected line (HFR or LFR)
320 and their interaction as fixed effects, and the identity of the mother and the block as random
321 effects. We also made more detailed models including the maternal status (fertile or sterile)
322 in the fixed effects. The male-fertility status of the G_1 individuals from inbred lines was
323 treated in a similar way, except that the fixed effects included: the geographic origin of the
324 inbred line (Montpellier versus Lyon), the maternal type (KF, KS or DS) and their
325 interaction; and the random effects included the identity of the inbred line, in interaction
326 with maternal type, in addition to block and mother identity. All tests were performed by
327 likelihood-ratio chi-squares, comparing models with and without the effect of interest with
328 all other effects kept. The models were run using the `lme4` package in R (Bates et al., 2015).
329

330 ***Predictions***

331 The selected lines inform on the nature of the variation in male-fertility observed when CMS
332 is introduced into a naive background (the Montpellier laboratory population), i.e. why is
333 there a residual male fertility in K individuals introgressed into this background ? If there
334 exist restorer alleles with large effects, we expect the selection protocol to be efficient, and
335 the frequency of male-fertility of K individuals sired by HFR fathers should exceed that of
336 their congeners with LFR fathers. Depending on dominance, this effect should be more
337 visible in different circumstances. A dominant allele transmitted by fathers makes a
338 difference when the mother does not herself bring the same allele (in our case, with male-
339 sterile mothers, and/or with mothers from the KS strain). A recessive allele, on the contrary,
340 would have more effects when the mother is male-fertile and/or from the KF strain. Finally,
341 the specificity of restoration with respect to the mitotype can be tested by checking whether
342 the LFR/HFR difference, if any, affects male-fertility when the mother is of the D mitotype.
343 The inbred lines allow to document the nature and specificity of restoration in both
344 the Montpellier and Lyon populations. Based on previous studies (Laugier et al. 2014) we
345 expect that K-mitotype individuals sired by Lyon lines (a largely restored population) will
346 have more male-fertile offspring than those sired by Montpellier. We expect, if restoration
347 has a simple genetic basis, that some lines will be homozygous for restorers and others for
348 maintainer alleles, generating high variation in male-fertility among lines, either when

crossed with KS / male-sterile mothers (if restorers are dominant) or when crossed with KF / male-fertile mothers (if recessive). Similar effects could be observed upon crossing with DS if there exist restorers efficient against CMS brought by the D mitotype; restoration may be specific (in that case, the same lines should restore fertility in both D and K) or not.

Results

Male-sterility in the maternal generation

The phenotypes of the mothers used to test the restoration potential in inbred lines and selected (HFR/LFR) lines were distributed in the same way and conformed to expectations based on previous studies (see Material and methods): mothers with CMS introgressed into the alb-1 Montpellier background were mostly male-sterile with (in total) 5.7% of male-fertile individuals among D-mitotype mothers (DS) and 27.2% in K-mitotype ones (KS). In contrast nearly all (92.3%) mothers of the KF type were male-fertile. In both datasets the proportion of male-fertile individuals differed very significantly among types (Binomial GLMMs, χ^2_{1df} =119.9, and 36.6 respectively for the inbred lines and selected lines datasets, both $p < 0.001$).

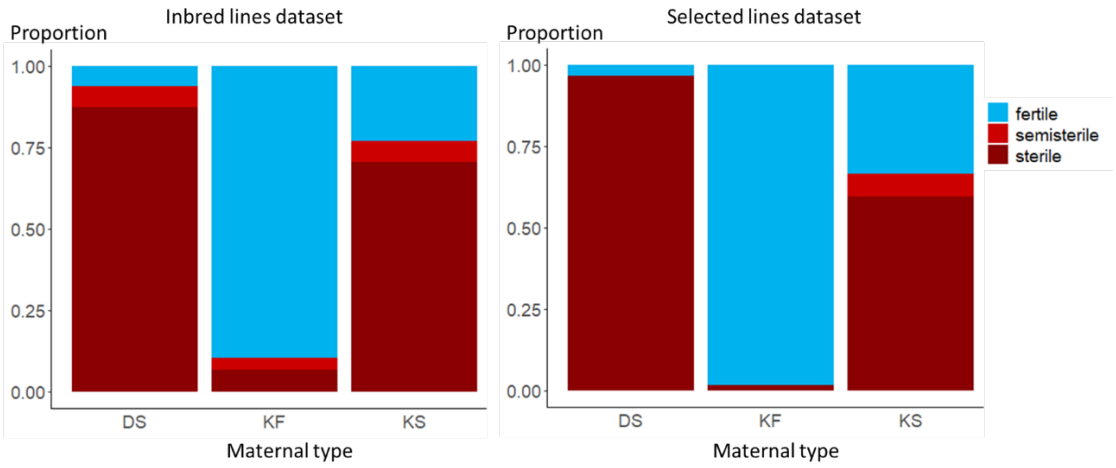

Figure 2: Proportions of individuals with male-sterile, male-fertile and male-semi-sterile phenotype in the maternal generation. Proportion are represented for both datasets as a function of maternal type DS: D-mitotype introgressed into the alb-1 background from Montpellier; KF : K-mitotype within its native background (Lyon) ; KS : K-mitotype introgressed into the alb-1 background from Montpellier. The two datasets are presented separately because they were made at different times.

375 ***Male sterility in the offspring generation: LFR versus HFR***

376 The proportions of male-fertile offspring closely mirror those observed in their mothers
377 (Figure 3, compare with Figure 2), and the maternal type (DS, KS, or KF) accordingly has a
378 very significant effect on the probability of being male-fertile (p <0.001, Table 2). The
379 paternal line (HFR or HFR) has a weakly significant effect (p = 0.039, Table 2), and this
380 effect is perceptible only when the mother has a K mitotype introgressed into the alb-1
381 Montpellier background (KS mothers, Table 2). In this category the proportion of male-
382 fertile offspring is slightly higher when the father comes from the LFR line than when the
383 father comes from the HFR line.

384 The effect of the maternal phenotype (i.e. whether the mother was a male-sterile or a
385 male-fertile snail) cannot be studied in the DS and KF categories because of insufficient
386 numbers (only one DS individual with a male-fertile mother; only two KF individuals with
387 a male-sterile mother). In the KS category however, numbers are more balanced (98
388 individuals with male-fertile mothers, versus 202 with male-sterile or -semisterile mothers),
389 and the maternal phenotype partially predicts the offspring's one. Indeed, male-fertile
390 mothers tend to have more male-fertile offspring, whatever the paternal line; the influence
391 of the paternal line remains significant, and in the same direction as previously, when the
392 maternal phenotype is taken into account (GLMM: effect of paternal line χ^2_{1df} = 4.65, p =
393 0.031 ; effect of maternal phenotype χ^2_{1df} = 4.48, P = 0.034 ; interaction χ^2_{1df} = 0.15, p =
394 0.70 ; Figure 3).

395 Note that full-sibs (offspring sharing the same mother and father) tend to have similar
396 phenotypes as attested by significant effects of mother identity (Table 2) on top of what is
397 already explained by the fixed effect of maternal type. This mother effect is not completely
398 predicted by maternal phenotype (among KS mothers) as it remains approximately the same
399 in the model with the maternal phenotype included as a fixed effect (χ^2_{1df} = 4.80, variance =
400 0.72, p = 0.014). Note that the mother effect is also, surprisingly, significant within the KF
401 category (Table 2) ; this is because among the few (four) individuals with KF mother and
402 HFR father observed to be male-sterile, two have the same mother which is unlikely by
403 chance.

404

405 **Table 2: Generalised linear mixed models on the binomial male-fertility variable**
406 **(fertile versus sterile or semi-sterile).** LRT tests are given for fixed and random factors
407 (χ^2_{df} and corresponding p-values). For random factors we also provide estimates of variance

(v, on logit scale) and *p*-values account for the one-sided alternative (variances cannot be negative). Note that the very high « maternal ID » variance in the KF category is an effect of the logit scale when proportions are close to 0 or 100% (here 92% individuals are fertile): in these conditions a very large variance in the logit scale makes a small variance in actual proportions. The block random factor was omitted in this table because in all models the estimated variance was 0.00. *p*-values are bolded when <0.05.

Data	Fixed effects			random effects
	maternal type (DS, KF, KS)	paternal type (HFR, LFR)	Interaction	maternal ID
All	$\chi^2_{df2} = 64.36$ $p = \mathbf{1.06\ 10^{-14}}$	$\chi^2_{df1} = 4.25$ $p = \mathbf{0.039}$	$\chi^2_{df2} = 0.91$ $p = 0.634$	$\chi^2_{df1} = 6.79$, v = 1.01 $p = \mathbf{0.0045}$
only DS		$\chi^2_{df1} = 0.356$ $p = 0.551$		$\chi^2_{df1} = 0.00$, v = 0.00 $p = 0.500$
only KF		$\chi^2_{df1} = 0.001$ $p = 0.970$		$\chi^2_{df1} = 10.07$, v =91.72 $p = \mathbf{0.008}$
only KS		$\chi^2_{df1} = 4.23$, $p = \mathbf{0.039}$		$\chi^2_{df1} = 6.79$, v = 0.76 $p = \mathbf{0.010}$

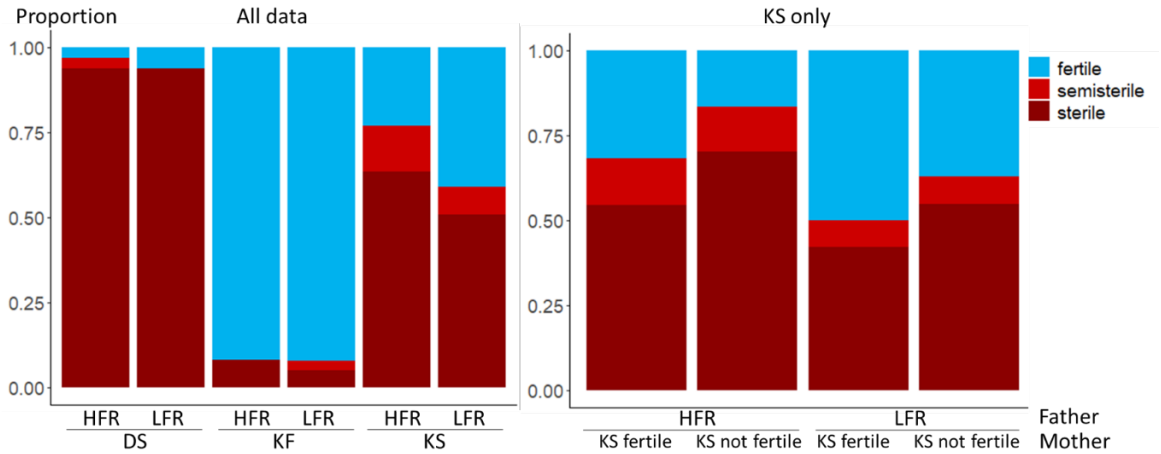

Figure 3: Proportions of individuals with male-sterile, male-fertile and male-semi-sterile phenotype. Left: proportions of male-fertile and male-sterile phenotypes as a function of paternal selection line (HFR versus LFR), maternal type (DS, KF, KS). Right: detailed view of proportions in offspring of KS mothers, as a function of paternal line and

maternal phenotype (male-fertile versus not male-fertile). Similar divisions were not considered in the DS and KF category because of the low numbers of male-fertile and non male-fertile mothers respectively.

Male-sterility in the offspring generation: inbred lines

As previously, maternal types (DS, KS or KF) induced large contrasts in the proportion of male-sterility, with DS being predominantly male-sterile, KF predominantly male-fertile, and KS in an intermediate situation (Table 3, Figure 4). Only in the KS category, the origin of the paternal inbred line made a difference: paternal lines from Montpellier (laboratory population entirely of the N mitotype) had largely male-sterile offspring, while paternal lines from Lyon (where K and D were initially found) had more than 50% male-fertile offspring. In these KS offspring, the variance explained by paternal lines was significant (Table 3), on top of what was already explained by the geographic origin, and the among-lines variance, when estimated separately, was higher in the Lyon population compared to Montpellier (Table 3). This can be visualised by representing BLUPs (Best Linear Unbiased Predictors) associated with paternal lines in the linear models: the Montpellier inbred lines span a range of male-fertility between 0 and 50% while the Lyon lines cover essentially the whole range (0-100%) (Figure 4). The apparent continuity of the distribution does not seem to be an artefact of the GLMM (which assumes an underlying normal distribution on the logit scale for random effects) as it is also observed when plotting the raw percentages of male-fertile individuals (supplementary Figure S4). In contrast with KS, the variance among paternal inbred lines is estimated to be zero in the KF category. However it is significant in the DS category, in which the distribution of BLUPs spans the 0-50% interval with an apparently random distribution of lines from Montpellier and Lyon. The predicted male-fertility of offspring of an inbred line in a DS context is not significantly correlated to the same line in a KS context (Spearman $\rho = 0.22$, NS, Figure 4).

Models including the maternal phenotype (male-fertile or not) in the predictors, in addition to paternal population of origin, showed no significant effect in KS ($\chi^2_{1df} = 0.018$, $p = 0.895$), and a weakly significant effect in the DS context ($\chi^2_{1df} = 4.37$, $p = 0.037$) where male-fertile mothers had more male-fertile daughters than male-sterile mothers (see plots in S3). The number of male-sterile mothers was insufficient to test a difference in the KF category.

455 **Table 3: Generalised linear mixed models on the binomial male-fertility variable**
456 **(fertile versus sterile or semi-sterile), inbred lines dataset. Legend as in Table 2.** The
457 block random factor was omitted because the estimated variance was 0.00. (1) the test gives
458 the significance of the variance explained by the paternal line, in interaction with maternal
459 type. Because of the significance of the interaction, the variances (ni logit scale) explained
460 by paternal line are examined within each maternal type separately. In each case we also
461 provide separate estimates of variance for the inbred lines derived from Montpellier and
462 Lyon populations.
463

Data	Fixed effects			Random effects	
	maternal type (DS, KF, KS)	paternal origin (Lyon, Montpellier)	interaction	maternal ID	paternal line
all	$\chi^2_{df2} = 80.61$ $p <$ **10^{-15}**	$\chi^2_{df1} = 9.73$ $p =$ **0.002**	$\chi^2_{df2} =$ 5.37 $p = 0.068$	$\chi^2_{df1} = 7.16$ v = 0.92 $p =$ **0.0037**	$\chi^2_{df5} = 16.75$ $p =$ **0.005** (1)
only DS		$\chi^2_{df1} = 1.439$ $p = 0.203$		$\chi^2_{df1} = 0.18$ v = 0.29 $p = 0.387$	$\chi^2_{df1} = 6.58$, v = 0.80 $p =$ **0.005** (in Montpellier: v= 0.77, in Lyon : v=0.79)
only KF		$\chi^2_{df1} = 0.28$, $p = 0.597$		$\chi^2_{df1} = 0.89$ v = 1.32 $p = 0.174$	$\chi^2_{df1} = 0.00$, v = 0.00, $p = 0.5$ (in Montpellier: v = 0.00, in Lyon : v = 0.00)
only KS		$\chi^2_{df1} = 14.84$, $p =$ **0.0001**		$\chi^2_{df1} = 6.54$, v = 1.00 , $p =$ **0.005**	$\chi^2_{df1} = 32.4$ v = 1.58 , $p =$ **$6.2\ 10^{-9}$** (in Montpellier: v = 1.45, in Lyon : v= 1.97)

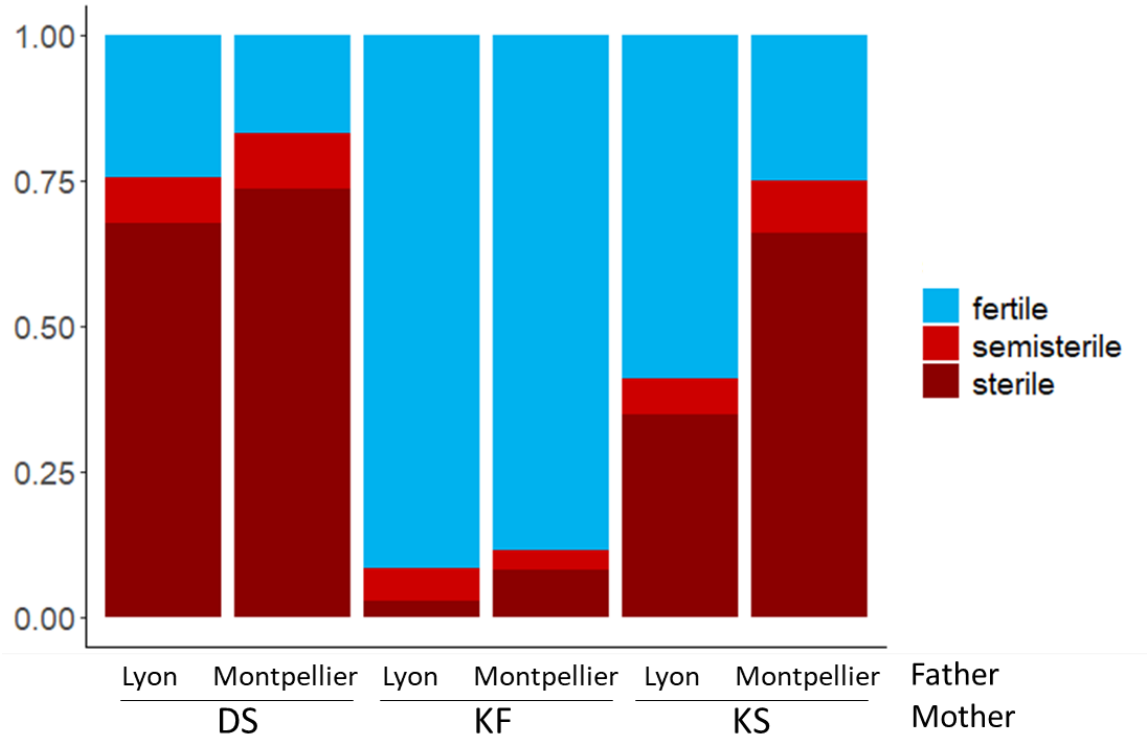

Figure 4: Proportions of male-fertile and male-sterile phenotypes as a function of the origin of the paternal inbred line (Lyon or Montpellier) and the maternal type (DS, KF, KS).

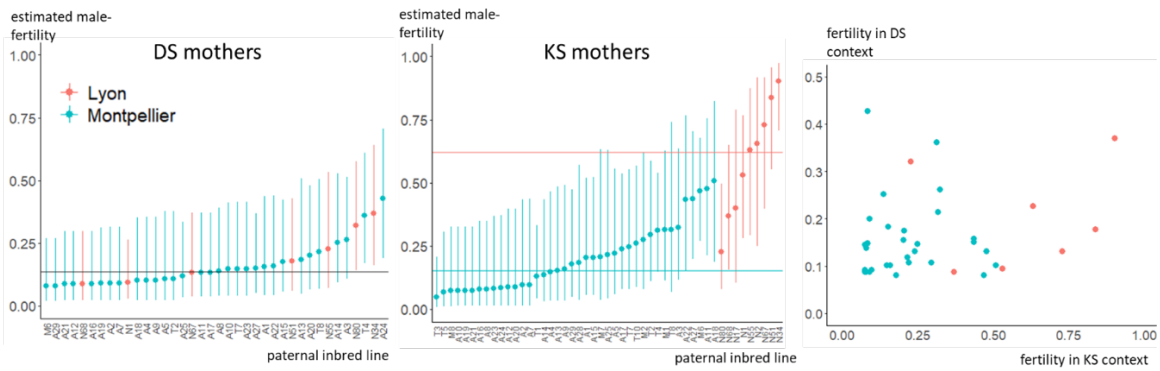

Figure 5: Proportions of male-fertile individuals in offspring sired by various inbred lines from Montpellier (red) and Lyon (blue), in a DS maternal context (left) or KS maternal context (middle). The estimates are extracted from binomial GLMMs (BLUPs), de-logited to put them back into natural scale, and presented with 95% confidence intervals. The lines are arranged by increasing male-fertility, and separated by origin in the KS context, as the population of origin has a significant effect in this context (in DS context, this is not the case). Horizontal lines provide mean proportions of male-fertile individuals estimated by

the model, over all paternal lines (DS context) or separately for each population of origin (KS context). Note that there are more paternal lines tested in KS than in DS context. The performance of lines tested in the two contexts are plotted together in the right panel (each dot is a paternal inbred line). The correlation is not significant (Spearman's $\rho = 0.22$, $p = 0.184$).

Discussion

This experiment was designed to characterize the restoration in *P. acuta* using two different nuclear backgrounds (i) a naive background, originated from Montpellier, which has never been in contact with N individuals at least during the previous 90 generations, (ii) a non-naive background, originated from Lyon, which has been in contact with the K and the D mitotypes in its recent history. Our results show that the two nuclear backgrounds had different genetic determination and that restoration, when present, was mitotype-specific, as in most plant examples.

Male fertility is restored by two different systems in Physa acuta

In the Montpellier background the K mitotype induced male sterility with an average penetrance close to 70%. Results from the selection experiment point to a significant but limited heritability of this penetrance, as (i) within the KS stock (Montpellier background), male-fertile mothers produced slightly more male-fertile daughters than male-sterile mothers, regardless of the father, and (i) fathers from the two selected populations, both derived from the same background after two generations, also differed in their restoration potential. However the latter difference was counter prediction as the lines selected to have a low restoration frequency (LFR) sired more male-fertile offspring than the lines assumed to have a high restoration frequency (HFR). This means that selection on the LFR and the HFR lines was ineffective, and has probably been overwhelmed by drift. This outcome is unexpected if restoration depends on fully-penetrant, dominant alleles, in which case large differences among LFR and HFR, in the direction of selection, would be expected (see supplement S2). However, if genetic variation is not large, and is expressed as a quantitative degree of penetrance (rather than binary 100% sterile or 100% fertile), the male-fertility phenotype of a few offspring becomes an imprecise indicator of the breeding value of each individual, lowering the efficiency of our selection process relative to drift, especially with the low numbers of individuals that passed the selection criteria. The distribution of male fertility in offspring of inbred lines derived from the Montpellier population strengthens this

512 view, as overall, we have no convincing indication that any of the lines produced more than
513 50% male-fertile daughters, and the distribution seemed continuous between 0 and 50%,
514 though mostly concentrated near 0.

515 From these results, we concluded that male-fertility of the K mitotype in the
516 Montpellier nuclear background was expressed as a quantitative trait with a small amount of
517 genetic variance that can be transmitted both by fathers and mothers, and some nongenetic
518 variance (i. e. the same genotype can be male-fertile or male-sterile, with a genetically
519 determined proportion, as in the threshold trait model Ehlers et al., 2005). In addition, we
520 did observe cases of partial male-sterility ("semi-steriles") although their low number did
521 not allow a separate analysis, suggesting that binary classification is a simplification of true
522 phenotypic distributions. Indeed, in case of polygenic determination of restoration,
523 individuals carrying CMS may rarely be fully restored and male fitness may vary
524 quantitatively among restored CMS hermaphrodites (e.g. Dufay et al., 2008). This result is
525 similar to previous studies on non-crop species that rejected Mendelian models of restoration
526 (e.g. *Thymus vulgaris,* Belhassen et al., 1991; Charlesworth & Laporte, 1998; *Lobelia*
527 *siphilitica,* Dudle et al., 2001). Ehlers et al. (2005) suggested that scenarios considering
528 restoration of male fertility as a threshold trait may fit these data better than multilocus
529 Mendelian models while producing overall similar expectations as single-allele evolutionary
530 models regarding sex-ratio dynamics.

531 Results obtained from the crosses with KF mothers (i.e. with a background from
532 Lyon) suggested that male-fertility of the K-mitotype has a very different genetic basis in
533 the original Lyon population (a population where K naturally occurs) compared to the
534 laboratory Montpellier population. The results are consistent with one or a few dominant
535 restorer alleles acting on the K mitotype fixed or segregating at very high frequency in the
536 Lyon nuclear background. Indeed, mothers from the KF population were nearly all (>90%)
537 male-fertile and their daughters too, even when their father came from the Montpellier
538 population (HFR, LFR or Montpellier inbred lines) where there is no or rare restoration (as
539 discussed above), as expected from transmission of dominant restorers by mothers.
540 Reciprocally, mothers from Montpellier, although predominantly male-sterile themselves,
541 produced high frequencies of male-fertile daughters when fertilized by fathers from inbred
542 lines derived from the Lyon population. It is interesting to note however, that if dominant
543 restorers were fixed in Lyon, we would expect all these inbred lines to restore fertility with
544 high efficiency ; this is the case of only three of the 9 lines (75 to 100% fertility) , the rest
545 being distributed in the 25%-75% range, with an overall estimated mean of 62%. The

546 contrast in male-fertility frequencies in Montpellier-Lyon crosses, depending on whether the
547 father or the mother comes from the Lyon population, is likely to reflect the recent history
548 of the Lyon stock in the laboratory. The KF mothers come from a 100% K population
549 maintained autonomously for 20 generations, during which all sperm donors must have been
550 of the K-mitotype. Therefore the frequency of any restorer of male-fertility was positively
551 selected, and maintained high, or perhaps increased, compared to the natural population. On
552 the other hand, fathers from inbred lines from Lyon were derived from a population that,
553 although founded from the same natural population, was maintained in parallel with 100%
554 N mitotype. In these conditions, restorers were silent (in the sense that all individuals were
555 male-fertile anyway) and if they were costly, may have decreased in frequency. This
556 hypothesis of a divergent selection on restorers depending on mitotype frequencies will
557 however have to be checked by looking at more replicates than just one population of each
558 type. Interestingly, the inbred lines from Lyon do not show a strikingly bimodal distribution,
559 suggesting that several loci with cumulative effects may be involved in restoration.
560 Our results highlighted two different restoration systems in *P. acuta*. In plants,
561 restorer alleles with different effects have been reported. For example, in *Plantago* genus,
562 multiple independent restoration systems were identified with both dominant and recessive
563 effects sometimes involving more than one locus (Koelewijn & Van Damme, 1995; E.
564 van Damme, 1984). In *Lobelia siphilitica* one CMS gene is restored by a single dominant
565 allele, while restoration of other CMS is controlled by several loci and/or epistatic effects
566 (Dudle et al. 2001). In these examples, one restoration system corresponds to one CMS type,
567 however in *P. acuta* we found that two restoration systems acting together on the same CMS
568 type K.
569
570 *The two restoration systems are specific of the K mitotype*
571 The D-mitotype individuals were on average 90 – 100% male-sterile irrespective of the
572 nuclear background. The low proportion of male-fertile individuals was consistent with the
573 previous study of David et al. (2022) which found 8% of male-fertile D individuals in the
574 original population. Here, we found no evidence for a dominant restorer with large
575 penetrance; however we observed the existence of genetic variation for penetrance among
576 inbred lines from both Montpellier and Lyon, without evidence for a difference between the
577 two origins (we would expect a higher rate of male-fertility in Lyon if this variation has been
578 specifically maintained by selection for restoring male fertility, as the Montpellier
579 population had no recent contact with the D mitotype). This lack of difference between

580 Montpellier and Lyon, and the fact that the lines with the highest male-fertility rate in the
581 context of the D mitotype were not necessarily the ones that efficiently restore fertility in the
582 K-mitotype, suggest that the two types of variation act in a mitotype-specific manner. The
583 specificity of the restorers has been also observed in plants where different CMS genes can
584 coexist within a population each with its own specific nuclear restorer alleles (e.g.
585 van Damme & van Delden, 1982; Manicacci et al., 1997).
586 In the wild the D mitotype seems rare as it was found only in one population and at
587 low frequency (14%, David et al. 2022). Thus, suppression of the male sterility may not be
588 frequent enough for a restorer to be selected. The high evolutionary rate detected in the D
589 mitotype (David et al. 2022) could result from an arms race with a restorer gene which is
590 now extinct or rare, or has lost its ability to restore fertility following a recent evolution of
591 the D mitochondrial genome itself. According to the frequency-dependent model, as soon as
592 a CMS gene is present in a population, there is a strong selection for any nuclear mutation
593 that counteracts the phenotypic effects of CMS. Restorers should spread rapidly and if they
594 have no or little fitness cost, the polymorphism is transient. However, nuclear restoration is
595 expected to be rare under the hypothesis of a high cost of restoration (Gouyon et al., 1991;
596 Dufay et al., 2007). Founder effects causing a reduction of the nuclear diversity could also
597 lead to the local absence of certain nuclear restorers in a recent founded population (e.g.
598 Manicacci et al. 1996; Laporte et al., 2001). Therefore, we can hypothesize that the evolution
599 of restorers specific to the D mitotype might be constrained by a high cost or founder effects.
600
601 *Two stages in the maintenance of cyto-nuclear polymorphism*
602 Most models have studied the maintenance of the cytoplasmic and nuclear genetic variation
603 in gynodioecious populations assuming strong and dominant restorer alleles at a few loci.
604 Such alleles are indeed the most likely to be under strong selection and undergo selective
605 sweeps in populations invaded by new CMS genes. Depending on their associated costs,
606 they may either reach fixation ("hard-sweep") or stabilize at intermediate or cyclically
607 variable frequencies ("soft-sweep") together with their corresponding CMS cytotypes
608 (Gouyon et al., 1991; Dufaÿ et al., 2007). Gynodioecious populations observed today are
609 mostly in the polymorphic state and the restorers found indeed include large-effect alleles
610 with dominant action (e.g. de Haan et al., 1997; Dufay et al., 2008, 2009). In addition this
611 action is usually specific to each cytotype, revealing either a arms-race coevolutionary
612 history specific to each cytotype-restorer pair, and/or the fact that each new CMS cytotype
613 has a mode of action that differs so strongly from previous ones that existing restorers are

unlikely to neutralize it. In *Physa acuta,* the K mitotype, and the restorers present in the natural population where it was found, are likely to be in this situation, as we found restoration to be strong (though possibly distributed into several loci), dominant (same effect in the heterozygous state in daughters, than in homozygous state in mothers) and specific (no cross-mitotype effect).

However, while such situations are expected to be frequent in stable gynodioecious populations, they are probably not representative of initial stages when a new CMS mutant appears in a "naïve" population or species, where no selection has ever taken place on nuclear genes to suppress its effect. According to Lewis (1941), the spread of CMS in the population will in this case become limited only by pollen limitation when females become so abundant that many of them lose their ovules by lack of pollen to fertilize them. Our results suggest however a way by which such populations may lower the impacts of pollen (or, in the case of *Physa*, sperm) limitation: CMS may not be fully penetrant, leaving a residual production of male gametes that is at least sufficient to fertilize the eggs of mates that have not had previous contacts with male-fertile congeners. It is likely that even in a naïve population, pre-existing quantitative genetic variation includes variants that act on this penetrance. This probably corresponds to the situation of the K-mitotype when introgressed into the "naïve" Montpellier population, and of the D mitotype in both the Montpellier and Lyon populations. Here, we mean by "naïve" that it has either never had any history of selection in presence of the K and D mitotypes, or that the contact is too recent for large-effects restorers to have emerged, or too ancient for them to have persisted if they ever existed. The background variation acting on incomplete penetrance of CMS could produce a reservoir of variation within the population, by which male fertility can start to increase, until the appearance of complete penetrant restorers.

The emergence of restoration could therefore often take place in two stages: a first stage with predominantly quantitative, weak modifiers of CMS penetrance, and a second stage when dominant, strong restorer alleles appear and are selected at one or several loci, ending up with either fixation or a mixed determinism of male-fertility. Although theoretical models generally consider a simple genetic determination for restoration (but see Frank, 1989; Bailey & Delph, 2007), empirical studies on wild plants have often rejected this assumption as mentioned above (e.g. Charlesworth & Laporte, 1998; Koelewijn, 2003). A complex determination of restoration implies that within a population, CMS individuals may be partially restored (i. e. male fertility may be more of a continuous than binary 0/1

distribution) and this could affect the degree of selection for restorer alleles. Then, the positive selection of restorers could slow down and ultimately modify the conditions of maintenance of cytonuclear polymorphism, thereby favouring the maintenance of females in gynodioecious populations (Bailey & Delph, 2007).

Conclusion

This study shows that as in plants, the genetic determination of male-sterility in *Physa acuta* is complex and suggests that it encompasses two different layers: one of weak, quantitative modifiers of the penetrance, not necessarily coevolved with CMS, the other of strong-effect restorer alleles with dominant action, that were selected in the presence of CMS. Moreover, the way the phenotype is measured could influence our perception of the restoration potential. Indeed, the male fertility status was evaluated in the absence of competition as individuals were always paired with virgin mates; the restoration of the male fertility might be considered less efficient when measured in a population with competition for the male function. Thus, it will be interesting to evaluate male fitness in competition, e.g. when partners are not virgin, and have been first paired with a male-fertile partner before being paired with focal individuals. The genetic determinism of the restoration may influence the maintenance of the cyto-nuclear polymorphism by acting on the strength of the restoration cost. This could be evaluated by looking at the correlations between the penetrance of the nuclear backgrounds and the cost of restoration on male and/or female fitness. However, studies performed in plants suggest that costs are difficult to detect (but see de Haan et al., 1997; Bailey, 2002; Dufay et al., 2008). Finally, because our results suggest that the restoration of the male function is a quantitative trait, identification of QTLs for male-fertility restoration would be necessary.

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