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1 **Five years investigation of female and male genotypes in Périgord black truffle (*Tuber*
2 ***melanosporum*** **Vittad.) revealed contrasted reproduction strategies**

3

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21 Running title: Black truffle genetic structure

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22 **Summary**

23 The Périgord black truffle (*Tuber melanosporum* Vittad.) is a heterothallic ascomycete that
24 establishes ectomycorrhizal symbiosis with trees and shrubs. Small-scale genetic structures of
25 female genotypes in truffle orchards are known, but it has not yet been studied in male
26 genotypes. In this study, our aim was to characterize the small-scale genetic structure of both
27 male and female genotypes over five years in an orchard to better understand the *T.*
28 *melanosporum* sexual reproduction strategy, male genotype dynamics, and origins. Two-
29 hundred forty-one ascocarps, 475 ectomycorrhizas, and 20 soil cores were harvested and
30 genotyped using microsatellites and mating type genes. Isolation by distance analysis revealed
31 pronounced small-scale genetic structures for both female and male genotypes. The genotypic
32 diversity was higher for male than female genotypes with numerous small size genotypes
33 suggesting an important turnover due to ascospore recruitment. Larger and perennial female
34 and male genotypes were also detected. Only three genotypes (1.5 %) were found as both
35 female and male genotypes (hermaphrodites) while most were detected only as female or
36 male genotype (dioecy). Our results suggest that germinating ascospores act as male
37 genotypes, but we also proposed that soil mycelium could be a reservoir of male genotypes.

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39 **Introduction**

40 The Périgord black truffle*, Tuber melanosporum,* is an ascomycete species that forms
41 ectomycorrhizal symbiosis with trees and shrubs. For forty years, large-scale inoculation of
42 tree seedlings with *T. melanosporum* ascospores has been used in nurseries to produce in
43 France >300,000 symbiotic plantlets yearly (Chevalier and Grente, 1978; Murat, 2015). As a
44 consequence, truffle orchards are now found worldwide (Europe, Australia, USA, South
45 America, South Africa, and New Zealand). In these truffle orchards, the production of
46 ascocarps (fruiting bodies issued from sexual reproduction) faces several problems, one of
47 them being the initiation of sexual reproduction (Chen et al., 2016). The factors involved in
48 truffle sexual reproduction are difficult to investigate due to the impossibility of manipulating
49 truffle *in vitro* (Le Tacon et al., 2015). Because this information is still missing, clear
50 guidelines for truffle orchard management are still missing.

51

52 *Tuber melanosporum* is a heterothallic species having two mating type idiomophs (*MAT1-1*
53 and *MAT1-2*) (Martin et al., 2010; Rubini et al., 2011b). Mating in heterothallic ascomycetes
54 results from the union of female gametes (ascogonia) and male gametes of opposite mating
55 types. Male gametes can be formed in antheridia (haploid structure containing male gametes).
56 Conidia (haploid asexual spores) or haploid hyphae can also act as male gametes (Glass and
57 Kuldau, 1992; Leslie and Klein, 1996). In heterothallic species, male and female gametes can
58 be formed by the same haploid mycelium (one genotype) defining the so-called
59 hermaphroditism or monoecy. In another situation, male and female gametes are formed by
60 distinct male and female haploid mycelium (two different genotypes) corresponding to
61 dioecy. In *T. melanosporum*, the female gametes are ascogonia produced by the haploid
62 mycelium forming the ectomycorrhizal root tips. The ascogonia produced from this haploid
63 mycelium can be either *MAT1-1* or *MAT1-2* (Rubini et al., 2011b; Murat et al., 2013; Taschen

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64 et al., 2016). They give birth to the main structure of the ascocarp (the peridium and the
65 unfertile tissues of the gleba) (Fig. 1). The origin of male gametes is still unknown, but it has
66 been suggested to be germinating sexual ascospores, conidia, or persisting soil mycelium
67 (Fig. 1) (Le Tacon et al., 2015; Taschen et al., 2016).
68
69 One approach to help understand the truffle's sexual reproduction is to characterize small-
70 scale genetic structures for both female and male genotypes. Indeed, small-scale genetic
71 structure depends on the distribution of genotypes in population and results from a
72 combination of their propagation modes and demographic processes. This can be analysed by
73 characterizing the persistence and size of the genotypes (Douhan et al., 2011). The isolation
74 by distance (IBD) theory predicts that a small-scale genetic structure accounts for gene
75 dispersion capacity (Vekemans and Hardy, 2004). The small-scale genetic structure of female
76 *T. melanosporum* genotypes was studied by genotyping with microsatellites and mating type
77 genes of ectomycorrhizas and ascocarps (Rubini et al., 2011a; Murat et al., 2013; Taschen et
78 al., 2016). In natural stands and truffle orchards, a pronounced genetic structure was observed.
79 Moreover, ectomycorrhizas and female genotypes formed patches containing only one of the
80 two mating types (spatial segregation). This particular spatial distribution raises the questions
81 about the origins and dynamics of the male gametes since a high density of ascocarps was not
82 observed at the boundary of patches with opposite mating types. This observation also
83 questioned the existence of hermaphroditism (capacity of one genotype to form either male or
84 female gamete) for *T. melanosporum* (Fig. 2). Inside these mono mating type patches, up to
85 10 female genotypes were detected; some were perennial (found during several seasons and
86 forming genotypes with size up to 4.7 m), while others were detected only in one season and
87 formed genotypes less than one meter (Murat et al., 2013; Taschen et al., 2016). This pattern
88 suggests a rapid turnover of the non-perennial female genotypes. Recently, Taschen and

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89 colleagues (2016) also surveyed male genotypes in five brulés (areas without vegetation
90 surrounding trees that are substantially mycorrhized by *T. melanosporum*) located on three
91 plantations and two natural sites. The genetic diversity was higher for male than female
92 genotypes, and most of the male genotypes were detected once only. Only a single male
93 genotype occurring more than one year was identified. However, the male genotype small-
94 scale genetic structure could not be assessed in that study.
95
96 The objective of the present study was to characterize the small-scale genetic structure of both
97 male and female genotypes in order to clarify the *T. melanosporum* sexual reproduction
98 strategy and, more particularly, the origin and the behavior of the male genotypes. This work
99 addresses several main points: 1) the small-scale genetic structure of the male genotypes and
100 their evolution according to the time (perennial versus transitory male genotypes); 2) the
101 localisation in the field of male genotypes in ascocarps, ectomycorrhiza, and soil; 3) the
102 possible origin of the male genotypes; and 4) the sexual reproduction strategy
103 (hermaphroditism versus dioecy). To achieve these goals, we used a 5-year sampling of
104 ascocarps, ectomycorrhizas, and soils under seven productive and contiguous trees in a 25-
105 year-old established truffle orchard in northeastern France. We determined the genotypes of
106 both female and male ascocarp partners in addition to that of mycorrhizas and the mating type
107 present in the soil samples.
108

109 **Results**

110 *Ascocarps and ectomycorrhiza samplings*

111 A total of 241 ascocarps were harvested during five consecutive truffle production seasons
112 (from November to March of the following year) from 2010/2011 to 2014/2015 (Table 1; Fig.
113 S1). The ascocarps were randomly distributed around the productive trees and could be also

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114 found at four to five meters from the trunk in and between the zone of root extension (Fig.
115 S1). The productive area under each tree extended slowly over five years.
116 Four-hundred seventy-five ectomycorrhizal root tips were collected (205 in 41 root samples
117 with five *T. melanosporum* morphotypes each in 2011 and 270 in 45 root samples with six *T.*
118 *melanosporum* morphotypes each in November 2014) (Fig. S1). Only 37 ectomycorrhiza
119 samples did not amplify with the *T. melanosporum* specific primers, and the presence of *T.*
120 *melanosporum* ectomycorrhizas was not confirmed on five root samples (two in 2011 and
121 three in 2014). For microsatellite genotyping, only one *T. melanosporum* ectomycorrhizal root
122 tip per root sample was selected.
123
124 *Characterization of female genotypes (gleba and ectomycorrhiza)*
125 The female ascocarp genotypes (gleba) were successfully obtained for 238 out of the 241
126 ascocarps (Table 1 and Table S1). Seventy-three female multilocus genotypes (called
127 hereafter genotypes) with significant Psex (probability that two identical genotypes originated
128 from the same genet) value were found during the five years (Table 1, Tables S1 and Table
129 S2). Twelve genotypes were present in more than one season (Table S1). These genotypes
130 accounted for 26% to 94% of the ascocarps for seasons 3 and 1, respectively, for a total of
131 154 truffles (64 % of the total harvested ascocarps over the five seasons). One persistent
132 genotype (R002) was found throughout all of the seasons under the same tree (F11, area 3),
133 representing 15% of the total ascocarps harvested (Fig. S2 and Table S1). Another genotype
134 (R021) fructified under the A11 tree (in area 1) for three seasons and represented 16 % of the
135 total harvested ascocarps (Fig. 3 and Fig. S1). The maximum size (clonal subrange) of the
136 female genotypes was 22.36 m, which corresponded to the R002 genotype found in areas 1
137 and 3 of the truffle orchard (Table 1 and Fig. S2). Most female genotypes (58%) were found
138 in only one ascocarp (Table S1).

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139

140 One-hundred seventeen ectomycorrhizas representing nine and 14 genotypes for 2010 and
141 2014 samplings, respectively, were genotyped (Table 1 and Table S1). Five genotypes
142 (R001•– R004 and R007) were found in both ectomycorrhizal samplings in 2010 and 2014
143 (Table S1). The maximum ectomycorrhiza genotype size was 19.02 m for R021 (Table 1 and
144 Fig. 3). Eighty-six percent of ectomycorrhiza genotypes were also detected as female
145 genotypes in ascocarps, and were represented in 16 genotypes with significant Psex values
146 (Table S1). Similarly, 86% and 70% of the female genotypes were also detected in ECM
147 genotypes for seasons 1 and 5, respectively.

148

149 When considering only the mating type locus, the aggregation index (Ac) was 0.49 (p value =
150 0) for female genotypes, indicating that genotypes of the same mating type tend to aggregate
151 (Table 1). In the orchard, large patches from 5 to 20 m^2 of different genotypes of the same
152 mating type for the root colonization were observed (Fig. S3). A single tree can be colonized
153 by one patch (such as E10 and B11) or by two contiguous patches of opposite mating types
154 (F10, F11, D11, A11, and A12). Tree D11 harboured maternal genotypes and
155 ectomycorrhizas of *MAT1-2* with the exception of one ectomycorrhiza sampled in square F5
156 formed by *MAT1-1* mycelium (Fig. S3 and Table S1). This genotype was not detected in male
157 genotypes (see below). At the contact zone between patches of opposite mating types, no
158 differences in the ascocarp density were observed when compared to density within the patch
159 (Fig. S1 and Fig. S3).

160

161 *Characterization of male genotypes*

162 We successfully genotyped 206 zygotes (a mix of female and male genotypes in ascocarps
163 coming from the mate of female and male gametes) out of the 241 ascocarps (86.5 %; Table

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164 S1). By subtracting the female genotype, we were able to reconstruct male genotypes. A total
165 of 138 male genotypes were found (Table 1 and Table S1). Nine zygotes (4%) were
166 homozygous for all microsatellite loci (only mating type locus is heterozygous). In zygotes, a
167 significant heterozygote deficit was observed with F_{IS} values of 0.02 and 0.28 for seasons 1
168 and 3, respectively (Table 1). The relationship between female and male genotypes in zygotes
169 was investigated using the kinship coefficient calculation. The kinship coefficient varied from
170 -0.5 to 1.5 with a mean value of 0.25, indicating most of the female and male genotypes in
171 zygotes were genetically close (Fig. S4). Indeed, a kinship coefficient of 0.25 might
172 correspond to full-sibs (Loiselle et al., 1995). However, the kinship coefficient was negative
173 for 26% of the zygotes, indicating that in those cases female and male genotypes were not
174 genetically related.
175 Most of the male genotypes (75%) were transitory (found only in one ascocarp) (Table S1).
176 Only eight persistent male genotypes with significant Psex values were found. These
177 persistent genotypes produced 21% to 37% of the ascocarps (Table 1). Sixteen male
178 genotypes were found during several seasons and five (only one genotype with significant
179 Psex) were found under different trees (Table S1). One genotype (R102) was detected for four
180 seasons under A11 where it fertilized 8.7% of the ascocarps (Fig. S5). The maximum male
181 genotype size was 22.64 m for genotype R102 with detection of most of them in one single
182 ascocarp (Table 1).
183

184 *Small-scale genetic structure for both female and male genotypes*

185 The genotypic richness was always higher for male genotypes (from 0.70 to 0.85) than for
186 female genotypes (from 0.24 to 0.76). Depending on the season, the Simpson's diversity
187 indices ranged from 0.81 to 0.98, 0.98 to 0.99, and 0.79 to 0.99 for female, male, and
188 ectomycorrhizal genotypes, respectively (Table 1). The inter-individual diversity (1-Qinter),

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189 also called unbiased gene diversity (He), ranged from 0.24 to 0.46 and 0.28 to 0.46 for female
190 and male genotypes, respectively (Table 1). The small-scale genetic structure of the *T.*
191 *melanosporum* population was assessed using an IBD analysis with GenePop software. In
192 IBD, the slope indicates dissemination capacities (the higher the slope, the more the
193 dissemination capacities are reduced). Using the culled dataset, a significant genetic structure
194 was detected for both female and male genotype; the slope was higher for female than male
195 genotypes (0.045 versus 0.039), but not significantly different (Fig. 4).
196
197 *Quantity of mating type myceliums in soil samples*
198 In the 20 soil cores harvested in May 2015 (Fig. S1), both mating types were detected as
199 mycelium in 16 soil samples (Table 2). In one soil sample, neither of the both mating types
200 was detected, while in two soil samples only *MAT1-1* was found, and in one soil sample only,
201 *MAT1-2* was detected. The quantity of mycelium ranged from 0 to 7.4 mg of mycelium per g
202 of soil and 0 to 40.49 mg of mycelium per g] of soil for *MAT1-1* and *MAT1-2*, respectively.
203 With two exceptions (squares E4 and E8 in area 1), the most frequent mycelium corresponded
204 to that of female and ectomycorrhiza genotypes.
205
206 *Identification of hermaphrodite genotypes*
207 The hermaphrodite genotypes are those detected as both female and male genotypes. In our
208 dataset, eight genotypes (3.9%) were detected as both female and male. Only three genotypes
209 (1.5 % of the total number of female and male genotypes) were supported by significant Psex
210 values and thus considered true hermaphrodites (Table 1 and Fig. 3). The R021 genotype was
211 the most frequent female genotype (see above). It has been also detected in five ascocarps as
212 males and once as an ectomycorrhiza (Fig. 3A). The R060 genotype was found five times as
213 the female genotype, once as a male and once as an ectomycorrhiza (Fig. 3B). Finally, the

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214 R068 was detected three times as the female genotype, once as a male, and once as an
215 ectomycorrhiza (Fig. 3C). These three hermaphrodite genotypes were detected as male and/or
216 female in 55 ascocarps (27%). Conversely, 135 genotypes were detected only as male (98%)
217 and 70 genotypes only as female (92%).
218
219 **Discussion**
220 In this study, ascocarps, ectomycorrhizas, and soil cores were harvested over a 5-year period
221 under *T. melanosporum* productive trees in a truffle orchard. The female and male genotypes
222 of 238 and 206 ascocarps, respectively, were successfully obtained. The genotypic diversity
223 was higher for male genotypes than female genotypes with numerous small size genotypes
224 suggesting an important annual turnover. However, a few larger and perennial female and
225 male genotypes were detected. A pronounced small-scale genetic structure was identified for
226 both female and male genotypes although IBD analysis indicated that the dispersal capacity
227 was similar for both. Most of the genotypes were detected only as female or male, and only
228 three genotypes (1.5%) were found as being both female and male (hermaphrodite). These
229 data allowed an *in situ* comparison of the temporal dynamic, genetic structure, and diversity
230 of both female and male genotypes providing new information on *T. melanosporum* sexual
231 reproduction.
232
233 *Strong small-scale genetic structure for both female and male genotypes*
234 According to the genotypic richness (R) and the Simpson's diversity index, the genotypic
235 diversity was higher for male than for female genotypes although the gene diversity (1-Qinter
236 index) was similar for both. Large and perennial genotypes were found for both male and
237 female genotypes, but a more rapid annual turnover of male genotypes is suggested by the
238 higher number of small size genotypes (Table 1). A strong IBD and consequently a strong

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239 small-scale genetic structure was found for both female and male genotypes with IBD slope
240 value that was not significantly different between sexes (Fig. 4). This result suggests similar
241 dispersal capacities for both female and male genotypes. In fungi, two main dispersal
242 strategies have been well documented: 1) spread of epigeous species' spores by wind and 2)
243 passive dissemination of hypogeous species by animals. The strong IBD observed in our data
244 for both female and male genotypes reflects their low dispersal capacity. This strong genetic
245 structure is characteristic of hypogeous fungi that are expected to have reduced spore
246 dispersal compared to that of epigeous ones (Kretzer et al., 2005). Previous studies already
247 detected a strong small-scale genetic structure for female genotypes (Murat et al., 2013;
248 Taschen et al., 2016), but to our knowledge the male genotype genetic structure has not been
249 investigated to date.
250
251 *The strong genetic structure leads to heterozygote deficit*
252 A heterozygote deficit was observed in zygotes (F_{IS} value ranging from 0.02 to 0.28),
253 suggesting that female and male genotypes tend to be genetically related in zygotes.
254 Moreover, in nearly 4% of the zygotes, we found female and male genotypes that were
255 homozygous for all microsatellites loci and nearly 50% of the zygotes displayed a kinship
256 coefficient above 0.25 (full-sib level according Loiselle et al. 1995). This strong genetic
257 relatedness within the zygotes is in accordance with the small-scale genetic structure
258 identified and discussed above, suggesting that disseminations over large distances are rare
259 for both female and male genotypes as demonstrated by similar IBD slope values. As already
260 discussed previously, hypogeous fungi rely on animals for spore dissemination, and in most
261 case spores stay in the vicinity of the ascocarps. A more important heterozygote deficit was
262 recently identified in five brulés for *T. melanosporum* with F_{IS} varying from 0.30 to 0.68 and
263 16.7 to 40 % of homozygote zygotes (Taschen et al., 2016). The small-scale genetic structure

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264 due to limited dispersal capacities may explain the observed heterozygote deficit. An existing
265 genetic barrier impeding mating of unrelated female and male genotypes is unlikely since
266 26% of the zygotes have a negative kinship coefficient.
267
268 *Germinating ascospores could act as male genotypes*
269 For both female and male genotypes, we observed similar features with both co-occurrence of
270 small size genotypes, often detected transiently as a single ascocarp, and larger perennial
271 genotypes. These results suggest that for both female and male genotypes, there is a mix of
272 new genotypes recruited from ascospores and perennial genotypes that have been
273 disseminated by vegetative propagation. However, male genotypes presented a higher
274 genotypic diversity and were less perennial than female ones, suggesting that ascospore
275 recruitment is more important for male than for female genotypes. As already proposed by
276 Taschen and colleagues (2016), it is therefore tempting to hypothesize that most of the male
277 genotypes originate from germinating ascospores whose mycelium does not survive after
278 sexual reproduction. Indeed, sexual spores have been proposed as male gametes for
279 ascomycetes and basidiomycetes (Nieuwenhuis et al., 2011).
280 The inoculation of ascospores in truffle orchards in order to improve the production of
281 ascocarps has become a common practice (Olivier et al., 2012), but is totally empirical due to
282 lack of scientific background. This practice is not recent since Ciccarello (1564), Bradley
283 (1726) and Buffon (1749) proposed improving truffle production by inoculating pieces of
284 ascocarps under mature trees. The role of ascospores was not directly demonstrated in our
285 study but, in collaboration with truffle growers' dedicated experiments, have been initiated to
286 confirm their role.
287
288 *Soil mycelium could also be a reservoir of male genotypes*

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289 In our study, 16 male perennial genotypes were found. One of them, genotype R102, was
290 found in 18 ascocarps (8.7%) over four years (Fig. S5). Among these, only three
291 corresponding to true hermaphrodite genotypes were present on ectomycorrhizas. We have
292 made the assumption that these male genotypes have a poor ability to form associations with
293 the host and that they likely survive as free-living mycelium in the soil or are associated with
294 the roots of non-ectomycorrhizal plants. It has been demonstrated that roots of herbaceous
295 plants can host truffle mycelium, but the nature of this interaction (such as colonization of the
296 rhizosphere, endophytism,and endomycorrhiza as with orchids) is unknown (Gryndler et al.,
297 2014). In our study, we detected the presence of both mating types in 16 out of 20 soil cores.
298 This result demonstrates that close to the ectomycorhizas formed by the female genotype,
299 mycelium of opposite mating type are present as mycelium and consequently the male
300 genotype could survive in the soil through vegetative propagation, saprotrophycally, or in
301 association with the roots of non-hosts.
302
303 In many ascomycetes, conidia (asexual spores) serve for vegetative propagation or as a male
304 gamete (Nelson, 1996; Maheshwari, 1999). Urban et al. (2004) described the existence of
305 anamorphous structures producing mitotic conidia in soils where *T. borchii* and *T.*
306 *oligospermum* ascocarps were present. Healy et al. (2013) suggested that Pezizales
307 mitospores, including *Tuber* mitospores, which failed to form ectomycorrhizas, could act as
308 male gametes. Nevertheless, the question of whether conidia act as male gametes in the *Tuber*
309 species in which they have been found remains unanswered and for the moment, conidia were
310 never yet observed in *T. melanosporum.*
311
312 *Sexual reproduction strategy in Tuber melanosporum*

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313 In our study, a non-random distribution of female genotypes according to their mating type
314 was observed to form large patches from 5 to 20 m^2 of different genotypes of the same mating
315 type. This result had already been found for *T. melanosporum* (Rubini et al., 2011a; Murat et
316 al., 2013; Taschen et al., 2016) and *T. aestivum* (Molinier et al., 2016). This aggregation was
317 stable over five years despite genotype turnover (Fig. S3). Interestingly, few ascocarps were
318 harvested in the contact zone of either mating type, suggesting that hermaphroditism, or
319 monoecy, is not widespread in *T. melanosporum* (Fig. 2). Indeed, we found only three
320 genotypes that were detected in 27% of the ascocarps as female or male genotypes, which can
321 be considered as true hermaphrodites (scenario 2c in Fig. 2). It is not surprising to identify
322 hermaphrodite genotypes since for heterothallic ascomycete hermaphroditism is the common
323 rule (Glass and Kuldau, 1992; Nieuwenhuis and Aanen, 2012). However, most genotypes
324 were identified as either female (92%) or male (98%) genotype, suggesting a specialisation in
325 one sex leading to subsequent dioecy. In the population of *T. melanosporum* surveyed in the
326 present study, a mix of a few hermaphrodites genotypes with a majority of female and male
327 genotypes (dioecy) co-occurred suggesting trioecy. Trioecy is known in plants (Joseph and
328 Murthy, 2014; Mirski and Brzosko, 2015) and animals (Weeks et al., 2006; Chaudhuri et al.,
329 2011). In fungi, trioecy was reported for the ascomycete *Triceromyces* (Benjamin, 1986), and
330 it can exist for *F. fujikuroi* (Leslie, 1995), but it does not seem to be a widespread occurrence.
331 In animals, trioecy can occur when environmental conditions change or when a species
332 colonizes a new habitat, leading to a transition from hermaphroditism to dioecy or *vice versa*
333 (Weeks et al., 2006). Trioecy is therefore a transitory status, and in *Caenorhabditis elegans* it
334 seems evolutionarily unstable (Chaudhuri et al., 2011). In heterothallic ascomycetes,
335 hermaphroditism could be the ancestral status since it is expected for most of the species
336 (Glass and Kuldau, 1992). It is therefore tempting to hypothesize that hermaphroditism has
337 been lost in *T. melanosporum* in order to favour female and male genotypes. But

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338 unfortunately, without *in vitro* tests, which are not yet available for *T. melanosporum*, the
339 likelihood of hermaphroditism *versus* dieocy cannot be formally demonstrated.
340
341 In conclusion, sexual dimorphism could be more frequent in fungi than expected, and
342 progress in genome sequencing could allow for its investigation. Indeed, in contrast to
343 animals and plants, dioecious fungi often are morphologically similar, and sexual dimorphism
344 can be detected only at genomic or agene regulation levels (Samils et al., 2013). The
345 understanding of economically interesting fungal species' sexual reproduction (such as those
346 producing edible mushrooms) is a major issue for a better control of their life cycles,
347 cultivation, and domestication. It appears that re-inoculation of planted *T. melanosporum*
348 mycorrhizal trees with ascospores leads to an increased number of male gametes (Taschen et
349 al., 2016), improving their dispersal, and favouring the turnover of ectomycorrhizas
350 producing female gametes. Consequently, the re-inoculation of spores is considered as one of
351 the management strategies aiming to decrease the time of appearance for the first
352 fructifications after plantation and increase the number of ascocarps after the initiation of the
353 sexual reproduction.
354
355 **Experimental procedures**
356 *Truffle orchard and sampling*
357 Ascocarps and ectomycorrhizas were sampled at a long-term experimental site located at
358 Rollainville in north-eastern France. The work site has been described in a previous work by
359 Murat *et al.* (2013). Samples and trees were identified by a letter and number and mapped on
360 a grid of 1 m x 1 m squares set up with camping pickets (Fig. S1). Three different grids were
361 made to identify three areas (areas 1–3) that cover all of the productive zones of the plantation
362 (Fig. S1).

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363

364 As described by Murat *et al.* (2013), the sampling started under trees F10, F11 and E10 (area
365 1) in the 2010-2011 season and under the trees A11, A12, B11 (area 3) and D11 (area 2) in
366 the 2011–2012 season (Fig. S1). The mature truffles were systematically harvested during the
367 production season with help from a well-trained dog, and at the time of harvest, they were
368 precisely mapped on the grid with 5 cm precision. The ascocarps were then washed to remove
369 soil particles and stored at -20°C for molecular analysis.
370 During this five-year interval, two ectomycorrhizal samplings were done. The first one was
371 done under F10/F11/E10 trees (area 1 in Fig. S1) in spring 2011 (season 1), and results on
372 that sampling were published by Murat et al. (2013). The second one was done in November
373 2014 (season 5), and 45 root samples were sampled all over the truffle orchard (Fig. S1). Root
374 samples were harvested from the first 10 cm of soil and mapped on the same grid used for the
375 positioning of the ascocarps. The ectomycorrhizas were carefully retrieved from the soil and
376 washed in water under a dissecting microscope. From each root sample, the *T. melanosporum*
377 ectomycorrhizas were identified as described by Zambonelli et al. (1993) and Rauscher et al.
378 (1995), and stored individually in microcentrifuge tubes at -20°C for molecular analyses.
379 In order to investigate the distribution and abundance of both mating types in the soil, 20 soil
380 cores were harvested in May 2015 (Fig. S1) at a 10–15 cm depth. All plant debris, stones, and
381 roots were discarded from the soil samples, and samples were kept at -20°C for DNA
382 extractions.

383

384 *DNA extractions*

385 Genomic DNA from the gleba (inner part of the ascocarps), and ectomycorrhizal tips were
386 extracted by using the DNeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France), following
387 the manufacturer's instructions. According to Paolocci and colleagues (2006), when using this

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388 protocol, only the haploid female genotype DNA is isolated since spores are not disrupted. To
389 have access to male genotype from each ascocarp, a mixture of spores was isolated, and their
390 DNA was extracted as described below. Thin slices of each ascocarp were put onto a layer of
391 water in a Petri dish to let spores be released into the water in order to isolate the pool of
392 spores. The liquid was collected in a 1.5 mL tube and centrifuged at 14,000 rpm for 5 min.
393 The supernatant was then discarded in order to obtain a mixture of asci and spores. DNA from
394 the isolated mixture of spores from each ascocarp was extracted as described in Rubini et al.
395 (2011b) with some modifications. First, to each pool of spores, we added 300 mL of NTE
396 buffer (Tris-HCl 200 mM, NaCl 250 mM EDTA 25 mM), and two tungsten beads after which
397 the spores were disrupted with a Tissue Lyser (Qiagen) for 10 minutes at 30 Hz. The tubes
398 were then centrifuged at 14,000 rpm for 10 min, and the recovered supernatant was added to a
399 new tube. Thirty microliters of NaAc (3M) and 330 µL isopropanol were added, and those
400 tubes were mixed and centrifuged again at 14,000 rpm for 10 min. After discarding the
401 supernatant and cleaning the pellet with 200 µL of ethanol (70%), the pellet (DNA) was
402 recovered in 50 µL TE. DNA extracts were stored at -20°C.
403 Total DNA from soil samples was extracted using the Power Soil® DNA extraction Kit
404 (MoBio, Laboratories, Carlsbad, CA) following manufacturer's protocol and stored at -20°C
405 for molecular analysis.
406
407 *Molecular genotyping*
408 All extracted DNA samples were amplified using species-specific *T. melanosporum* primers
409 (Paolocci *et al*. 1999) in order to check the species and DNA quality.
410 All of the samples in which *T. melanosporum* identity was confirmed were genotyped by
411 using primer pairs corresponding to ten microsatellite markers (Tm16_ATA12,
412 Tm241_TAA17, Tm2_TAT15, Tm98_TAT15, Tm112_TAT19, Tm9_ATCA12,

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413 Tm1_ATTG18, Tm75_GAAA14, Tm22_CCTCAT17 and Tm269_TGTTGC15) as described
414 by Murat et al. (2011; 2013). According to Murat et al. (2011), the use of ten microsatellites
415 allows to reach a genotypic diversity of 0.99. The genotyping was done by the INRA
416 plateform Gentyane (Clermont-Ferrand). The *T. melanosporum* mating types of all the
417 samples were analysed using specific primers for either the *MAT1-1-1* or the *MAT1-2-1* genes
418 using the PCR conditions described by Rubini et al. (2011b). Hereafter, according to Rubini
419 et al. (2011b), the mating types are termed *MAT1-1* and *MAT1-2*.
420 From the mix of DNA spores, we obtained genotypes of zygote (the mating of female and
421 male gametes for each ascocarp) from which the male genotype was deduced by subtraction
422 of the corresponding female genotype.
423 The mycelium from soil of both mating types was quantified by quantitative real time PCR
424 (qPCR) using a protocol developed from the international patent (EP2426215 A1) in a
425 confidential research program with ALCINA sarl (Montpellier, France). qPCR reactions were
426 carried out with a StepOne Plus™ Real-Time PCR System machine provided with the
427 StepOne software v. 2.3 (Life Technologies, Carlsbad, CA). Two standard curves (R^2=0.99;
428 Eff = 96.99% and R^2=0.99; Eff = 99.67% for *MAT 1-1* and *MAT 1-2,* respectively) were
429 obtained, as described in Parladé et al. (2013), by mixing a known amount of soil harvested in
430 a cereal field close to the truffle orchard (in which the absence of *T. melanosporum* was
431 confirmed) with a known amount of fresh immature ascocarp of *T. melanosporum* belonging
432 to one or the other mating type. DNA from the mixture was extracted as all the other soil
433 samples and serial tenfold dilutions were done to obtain a standard curve for each mating
434 type. Absolute quantification of mating types in soil samples was obtained by interpolating
435 their threshold cycle (Ct) values on the corresponding standard curve.
436
437 *Data analyses*

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438 All the samples with null alleles were discarded from our analyses. MLGsim 2.0 (Stenberg et
439 al., 2003) was used for the multilocus genotype identification and the calculation of the
440 likelihood (PSex) that copies of multilocus genotypes result from sexual reproduction or
441 clonal spread. The threshold value (<0.05) for testing the significance of the PSex for each
442 genotype was estimated using 1000 simulations. When the PSex values fell below the
443 threshold value (<0.05), it was concluded that identical genotypes originated from the same
444 genet (clonal multiplication).
445
446 In order to analyse clonal and spatial genetic structure of this *T. melanosporum* population,
447 different clonal related indices were estimated. Using the RClone package (Bailleul et al.,
448 2016), the Simpson's diversity index modified for finite sample sizes (1-D) was used as an
449 estimation of genotypic diversity to calculate if two randomly selected samples from the
450 population have the same genotype. The genotypic richness R was also calculated as (G-
451 1)/(N-1) where G is the number of genotypes and N the number of samples (Dorken and
452 Eckert, 2001) The clonal subrange representing the spatial scale over which genetic structure
453 is affected by clonal processes (Alberto et al., 2005) was also calculated. Using Geneclone
454 software (Arnaud-Haond and Belkhir 2007), the aggregation index (Ac) was calculated to test
455 for the existence of spatial aggregation for mating types Arnaud-Haond et al., 2007). Ac index
456 ranges from 0 (when the probability of identity between nearest neighbors does not differ
457 from the average one) to 1 (when all nearest neighbors preferentially share the same
458 genotype). The statistical significance of the Ac index was tested against the null hypothesis
459 of spatially random distribution using a re-sampling approach based on 1000 permutations.
460 The Ac was calculated using culled data for female genotypes and after consideration of the
461 five seasons. In order to study a possible heterozygote deficit, the inbreeding coefficient (*Fis*)
462 was calculated as a difference of observed and expected heterozygosity, using the RClone

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463 package. SPAGeDi 1.3 software (Hardy and Vekemans, 2002) was used to estimate the
464 kinship coefficient of Loiselle et al. (1995), as estimator of the genetic relatedness between
465 female and male genotypes in zygotes. Unbiased gene diversity (He=1-Qinter) and isolation
466 by distance (IBD) analysis were obtained with GenePop software v4.2 online (Rousset, 2008).
467 The significance of IBD was tested using Mantel test with 10,000 permutations. All values
468 were calculated for all the data and also separately for female, male, and ECM data. A culled
469 dataset was constructed to reduce the bias due to sampling the same genotype several times.
470 For each season, the isobarycentre was considered for samples sharing the same genotype and
471 having significant Psex. If two samples with the same genotypes were found in different areas
472 of the truffle orchard (areas 1–3), they were separated. Similarly, we did not calculate the
473 isobarycentre with samples sharing the same genotype from different years.
474 All the maps were obtained with a dedicated python program developed for this study. The
475 mapping program can be downloaded using a Unix terminal and the following command: git
476 clone https://git.igzor.net/inra/iam_mapping.git
477

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486 in the truffle orchard. We have no conflict of interest to declare.
487

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616 **Tables and Figure legends**

617 Table 1. Sampling details, genotypic, and genetic diversity for female and male genotypes and
618 ectomycorrhiza over five years from the Rollainville orchard.

619

620 Table 2. Quantity of soil mycelium of both mating types in mg of mycelium/g of soil. The
621 position in the truffle orchard is indicated in addition to the maternal mating type in the
622 sampling square. For squares without ascocarps, the most frequent mating type of maternal
623 tissue in the surrounding square is indicated (identified by an asterisk).

624

625 Fig. 1. Schematic representation of sexual reproduction for the heterothallic ascomycete *T.*
626 *melanosporum*. In order that sexual reproduction occurs female and male genotypes needs to
627 mate. In nature, the female genotype is found in the host tree root system as ectomycorrhiza,
628 although the male genotype could have different origins: germinating ascospores, soil
629 mycelium (free-living or associated to ectomycorrhiza for hermaphrodite genotype), or
630 conidia. Conidia have never been observed for *T. melanosporum,* but they have been
631 described in other *Tuber* species. After the fertilization, a diploid transitory phase occurs (that
632 cannot be detected in mature ascocarps), followed by a meiosis phase that will end in the
633 formation of a mature ascocarp. The structures of ascocarps (peridium, gleba. and ascospores)
634 are represented in the photographs.

635

636 Fig. 2. Theorical scenarios for the localisation of ascocarps for an ectomycorrhizal
637 heterothallic ascomycete according to the sexual strategy. The trees are indicated as triangle
638 and ascocarps as crosses. Scenario 1 considered that on the root system female genotypes of
639 both mating type are intermixed and they are hermaphrodites. This scenario is not true for *T.*
640 *melanosporum* since genotypes of the same mating type tend to aggregate (Rubini et al.,
641 2011a; Murat et al., 2013; Taschen et al., 2016 and this study). Scenarios 2a–c considered that

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642 the aggregation of genotypes in the root system was observed in *T. melanosporum* and less or
643 more hermaphroditism and male dispersion.

644
645
646 Fig. 3. Map of distribution of the hermaphrodite genotypes R021, R060, and R068 from the
647 Rollainville orchard. Representation of the distribution of the hermaphrodite genotypes along
648 the different seasons from the 2010–2011 (season 1) to the 2014–2015 (season 5) and in the
649 different areas of the orchard. a. Distribution of genotype R021. b. Distribution of genotype
650 R060. c. Distribution of genotype R068. Dots represent female ascocarp genotypes, crosses
651 represent ectomycorrhiza genotypes and triangles represent paternal genotypes. Each season
652 is represented by a different colour.

653

654 Fig. 4. Isolation by distance analysis (IBD) representing genetic *versus* geographic distance
655 for female and male genotypes. IBD analyses obtained with female (a) and male (b)
656 genotypes using culled data to avoid oversampling of the same genotype. The slope with 95
657 % interval confidence is indicated for each graph.

658

659 Table 1. Sampling details, genotypic and genetic diversity for female, male genotypes and ectomycorrhiza for five years in the Rollainville orchard.

660

	2010-2011	**2011-2012**	**2012-2013**	**2013-2014**	**2014-2015**	**All seasons**
Number of sampled ascocarps	17	42	30	101	51	241
Female genotypes						
Number of samples genotyped	17	42	30	98	51	238
Number of genotypes (number of genotypes with Psex < 0.05)	7 (6)	21 (8)	23 (7)	31 (12)	13 (10)	73 (21)
Maximum number of ascocarps per genotype	4	6	3	18	20	40
Number of genotypes represented by a single ascocarp	2	11	15	12	3	42
Number of persistent genotypes (found in 2–5 seasons)						12 (6; 3; 2; 1)
Percent of ascocarps produced by persistent genotypes	94.12%	45.24%	26.67%	63.34%	88.24%	63.87%
Clonal Subrange (m)	3.74	3.23	4.06	20.46	22.36	22.71
Simpson's diversity index (D)	0.88	0.94	0.98	0.92	0.81	0.99
Genotypic richness (R)	0.38	0.49	0.76	0.31	0.24	0.30
Unbiased gene diversity (1-Qinter = He)*	0.32/0.38	0.38/0.41	0.46/0.51	0.37/0.43	0.24/0.37	0.37/0.46
Aggregation index (Ac) (p-value)						0.49 (0)
Male genotypes						
Number genotyped zygotes	14	35	28	78	51	206
Number of homozygous zygotes (% genotyped)	1 (7.14%)	1 (2.85%)	1 (3.57%)	2 (2.56%)	4 (7.84%)	9 (4.37%)
Zygotes F_{IS}	0.02	0.18	0.28	0.18	0.15	0.21
Number of genotypes (number of genotypes with Psex < 0.05)	12 (1)	33 (4)	24 (3)	56 (11)	36 (10)	138 (15)
Max number of ascocarps per genotype	2	3	3	10	4	18
Number of male genotypes represented by a single ascocarp	8	26	18	39	19	110
Number of persistent genotypes (found in 2; 3; 4 or 5 seasons)						16 (12; 1; 3; 0)
Percent of ascocarps produced by persistent genotypes	23.53%	21.43%	33.33%	28.72%	37.26%	29.83%
Clonal Subrange (m)	0.52	1.13	17.95	12.56	22.64	23.18
Simpson's Diversity index (D)	0.98	0.99	0.98	0.98	0.98	0.99
Genotypic richness (R)	0.85	0.94	0.85	0.71	0.70	0.67
Unbiased gene diversity (1-Qinter = He) *	0.28/0.29	0.45/0.46	0.46/0.50	0.36/0.40	0.30/0.33	0.37/0.43

Ectomycorrhiza (ECM) samples						
Number of genotyped ECM	81				36	117
Number of genotypes (number of genotypes with Psex < 0.05)	9(9)				23(14)	27 (18)
Clonal Subrange (m)	4.58				19.02	19.02
Simpson diversity index (D)	0.79				0.99	0.87
Aggregation index (Ac) - ECM (p-value)	1 (0)				0.22 (0)	0.85 (0)
Number of ECM genotype occurring as female/male/both						16/3/3
Hermaphrodite genotypes						
Total number (number of genotypes with Psex <0.05)	1 (1)	2 (2)	2 (1)	6 (2)	4 (2)	8 (3)

** Unbiased gene diversity was calculated with all samples and eliminating repeated multilocus genotypes*
***Hermaphrodites are genotypes found as female and male genotypes*

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664 Table 2. Quantity of soil mycelium of both mating types in mg of mycelium/g of soil. The position in the
665 truffle orchard is indicated as well as the female mating type in the sampling square. The female mating
666 type was inferred according to the ascocarps harvested in the corresponding square. For squares without
667 ascocarps the most frequent mating type of female tissue in the surrounding square is indicated (identified
668 by an asterisk).

Area	**Square**	**Female MAT**	***MAT 1-1***	***MAT 1-2***
1	A4	2	0.428	16.37
1	A8	2*	0.402	21.58
1	C3	2*	0.444	18.22
1	C6	2	0.408	23.03
1	E4	1	0.753	40.49
1	E8	1*	0	0.021
1	G3	1	6.14	0
1	G6	1*	7.4	0.04
2	B4	2	0.535	25.39
2	D2	2	0	0
2	D6	2*	0.11	5.6
2	F4	2*	0.022	1.13
3	A2	2	0.171	9.51
3	B7	2*	0.179	9.009
3	C4	2	0.567	31.56
3	D5/6	2	0.112	4.32
3	D9	1	3.55	0.04
3	E2	2	0.464	38.58
3	F7	1	1.87	0
3	G4	2*	0.224	10.79

669
670

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Fig. 1

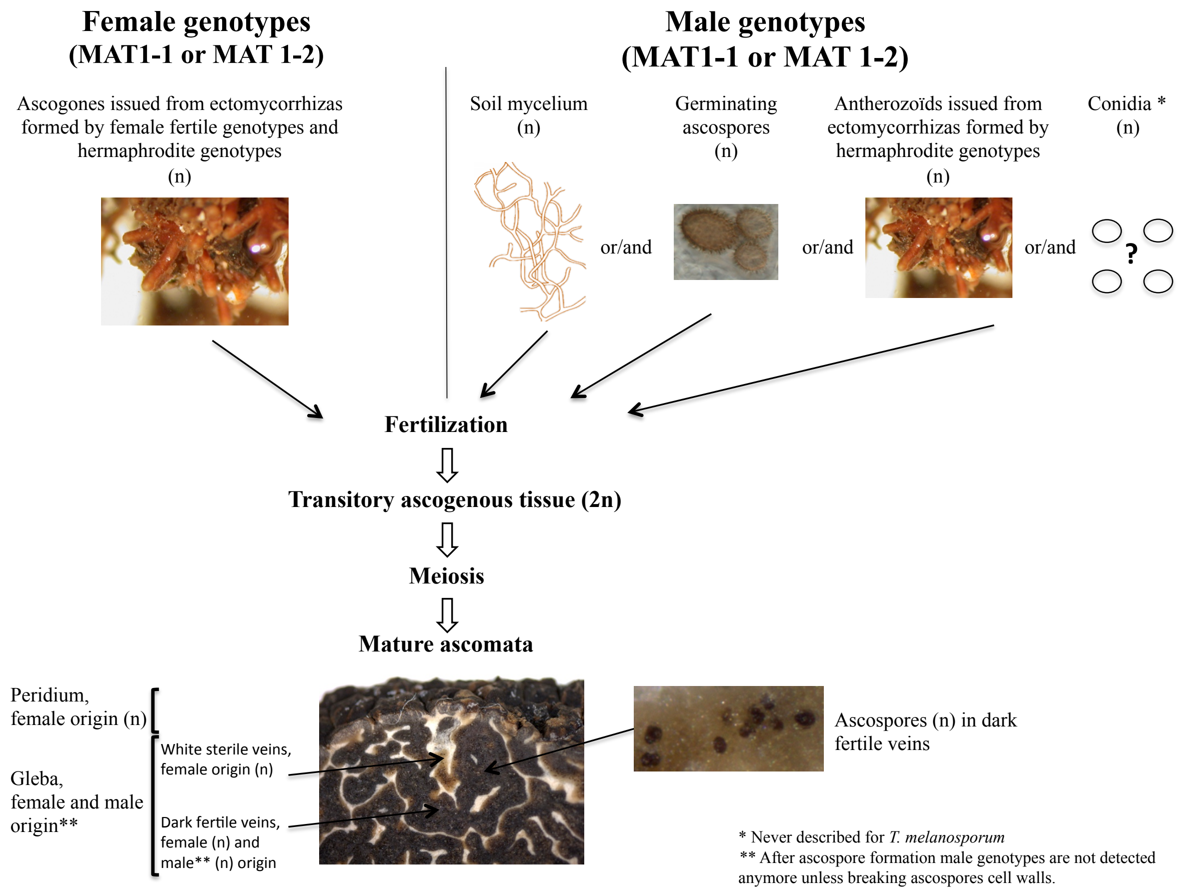

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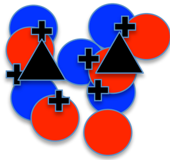
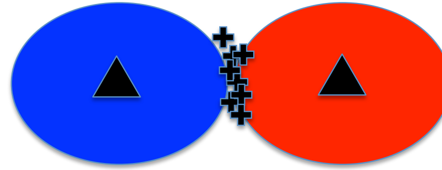
Fig. 2

Scenario 1



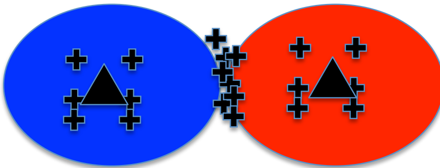
Mix of maternal genotypes of both mating type and hermaphrodism

Scenario 2a



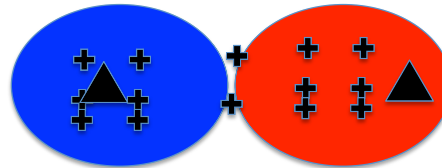
Large patches of maternal genotypes of the same mating type, hermaphrodism, and no dispersal of male genotypes

Scenario 2b



Large patches of maternal genotypes of the same mating type, hermaphrodism and dispersal of male genotypes

Scenario 2c



Large patches of maternal genotypes of the same mating type, no or few hermaphrodism and dispersal of male genotypes

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Fig. 3

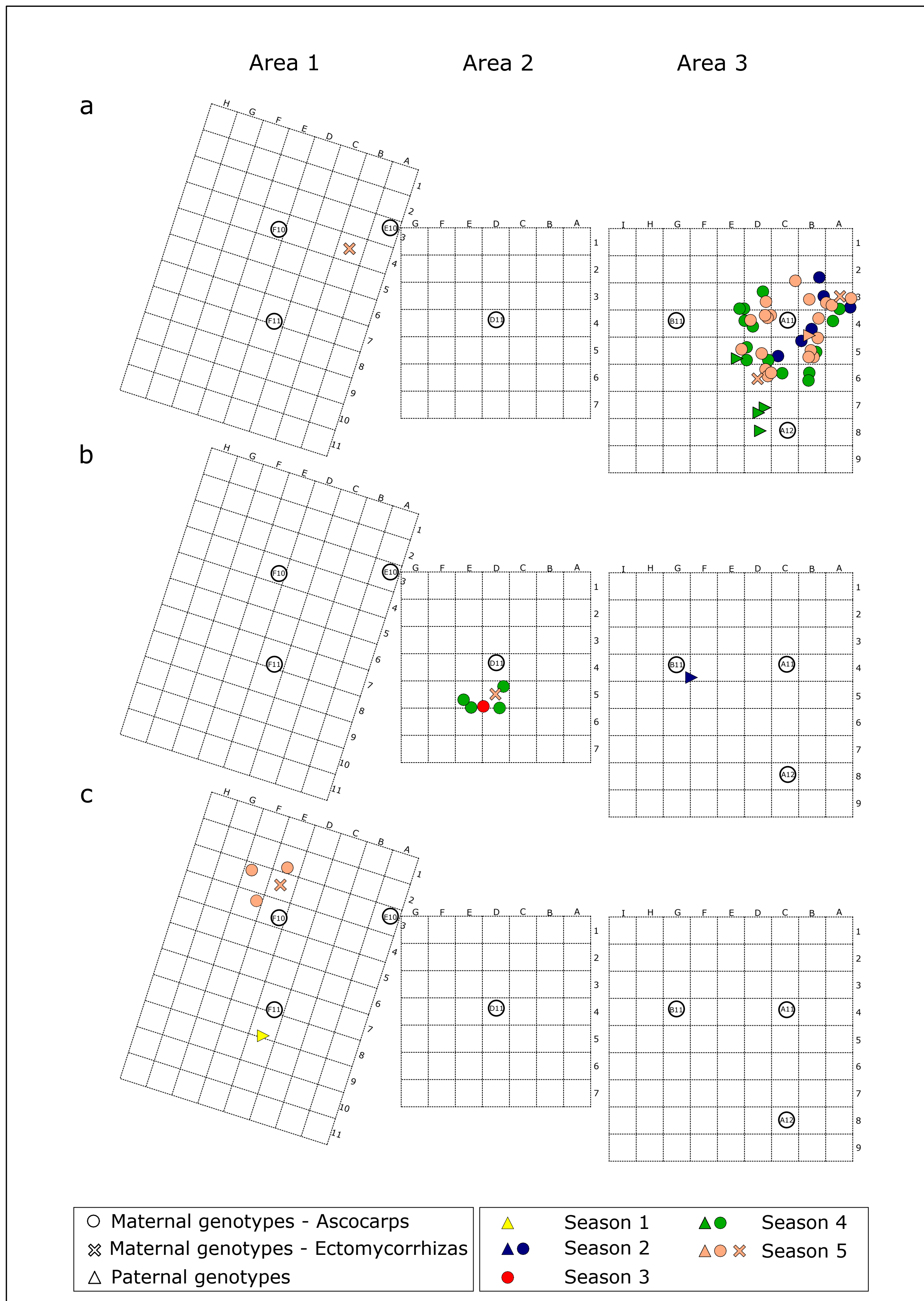

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Fig. 4