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

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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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19

20

21 Running title: Black truffle genetic structure

## 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.

38

## 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 idiomorphs (*MATI-1*  
53 and *MATI-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 *MATI-1* or *MATI-2* (Rubini et al., 2011b; Murat et al., 2013; Taschen

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

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

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).

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 ( $A_c$ ) 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<sup>2</sup> 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 *MATI-2* with the exception of one ectomycorrhiza sampled in square F5  
156 formed by *MATI-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



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  $P_{sex}$  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  $P_{sex}$ ) 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),

189 also called unbiased gene diversity ( $H_e$ ), 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 *MATI-1* was found, and in one soil sample only,  
201 *MATI-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 *MATI-1* and *MATI-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  $P_{sex}$   
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

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

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

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*

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 ectomycorrhizas 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, saprotrophically, 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*

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<sup>2</sup> 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 Kulda, 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 Kulda, 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

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).



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

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  $\mu$ L 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  $\mu$ L of ethanol (70%), the pellet (DNA) was  
402 recovered in 50  $\mu$ L 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,

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 platform 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*

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 (P<sub>Sex</sub>) that copies of multilocus genotypes result from sexual reproduction or  
441 clonal spread. The threshold value (<0.05) for testing the significance of the P<sub>Sex</sub> for each  
442 genotype was estimated using 1000 simulations. When the P<sub>Sex</sub> 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  $(G-1)/(N-1)$  where G is the number of genotypes and N the number of samples (Dorken and  
451 Eckert, 2001) The clonal subrange representing the spatial scale over which genetic structure  
452 is affected by clonal processes (Alberto et al., 2005) was also calculated. Using Geneclone  
453 software (Arnaud-Haond and Belkhir 2007), the aggregation index (Ac) was calculated to test  
454 for the existence of spatial aggregation for mating types Arnaud-Haond et al., 2007). Ac index  
455 ranges from 0 (when the probability of identity between nearest neighbors does not differ  
456 from the average one) to 1 (when all nearest neighbors preferentially share the same  
457 genotype). The statistical significance of the Ac index was tested against the null hypothesis  
458 of spatially random distribution using a re-sampling approach based on 1000 permutations.  
459 The Ac was calculated using culled data for female genotypes and after consideration of the  
460 five seasons. In order to study a possible heterozygote deficit, the inbreeding coefficient (*F<sub>is</sub>*)  
461 was calculated as a difference of observed and expected heterozygosity, using the RClone  
462

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 ( $H_e=1-Q_{inter}$ ) 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](https://git.igzor.net/inra/iam_mapping.git)

477

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487

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- 615

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. Theoretical 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

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
<b>Female genotypes</b>						
Number of samples genotyped	17	42	30	98	51	238
Number of genotypes (number of genotypes with $P_{sex} < 0.05$ )	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 = $H_e$ )*	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)
<b>Male genotypes</b>						
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 $P_{sex} < 0.05$ )	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 = $H_e$ ) *	0.28/0.29	0.45/0.46	0.46/0.50	0.36/0.40	0.30/0.33	0.37/0.43

<b>Ectomycorrhiza (ECM) samples</b>						
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
<b>Hermaphrodite genotypes</b>						
Total number (number of genotypes with Psex <0.05)	1 (1)	2 (2)	2 (1)	6 (2)	4 (2)	8 (3)

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662 \* *Unbiased gene diversity was calculated with all samples and eliminating repeated multilocus genotypes*

663 \*\**Hermaphrodites are genotypes found as female and male genotypes*

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	<i>MAT 1-1</i>	<i>MAT 1-2</i>
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



Fig. 1

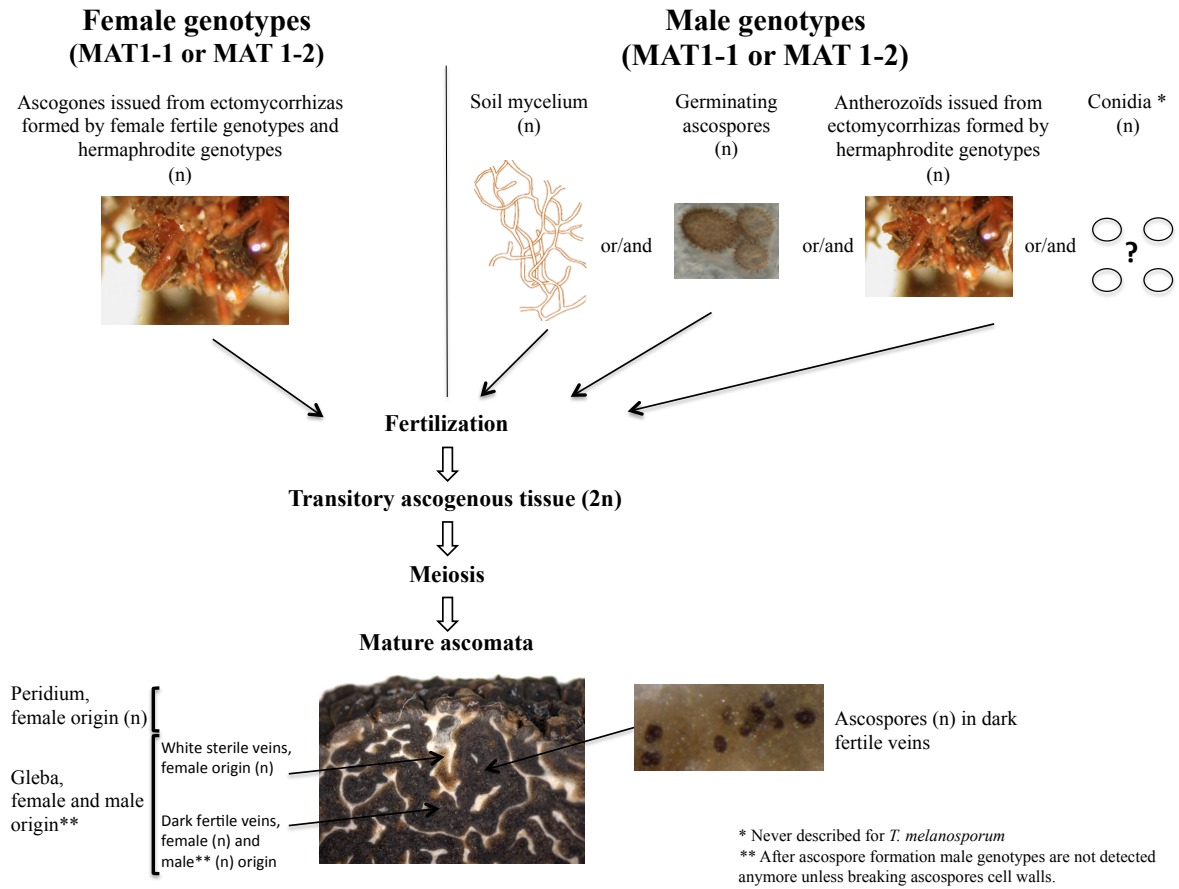
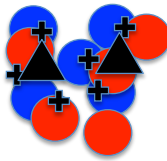


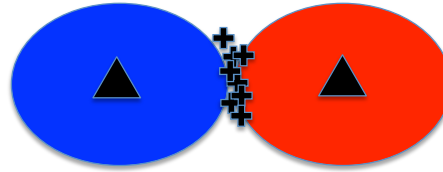
Fig. 2

Scenario 1



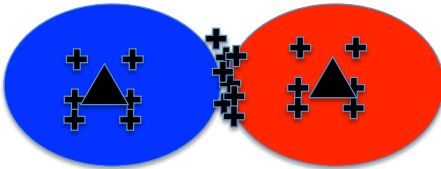
Mix of maternal genotypes of both mating type and hermaphroditism

Scenario 2a



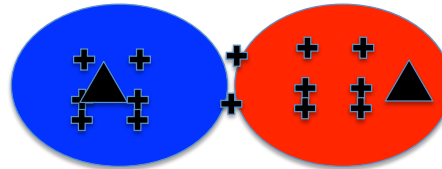
Large patches of maternal genotypes of the same mating type, hermaphroditism, and no dispersal of male genotypes

Scenario 2b



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

Scenario 2c



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

Fig. 3

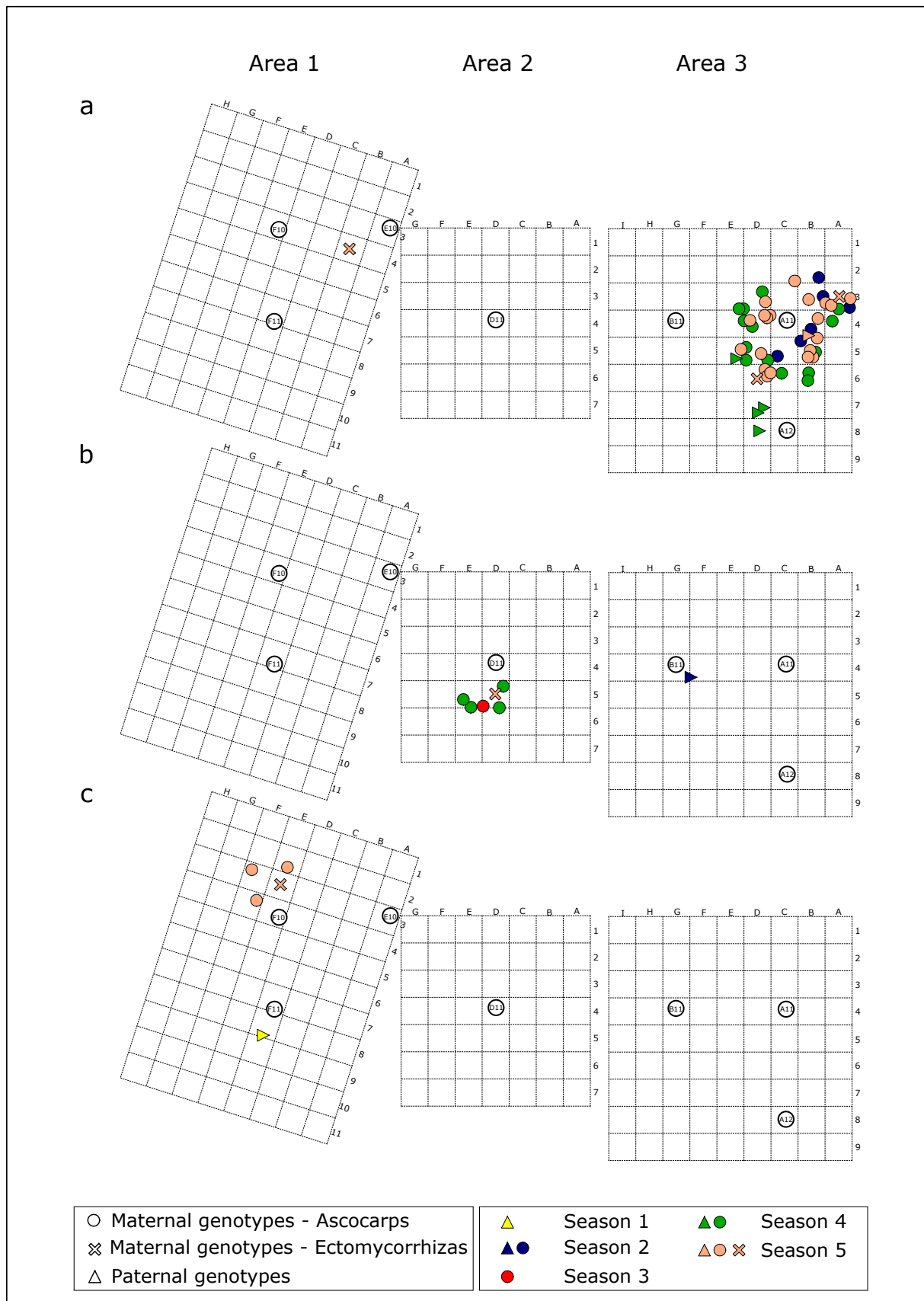


Fig. 4

