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

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Running title: Black truffle genetic structure

Summary

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

Introduction

The Périgord black truffle*, Tuber melanosporum,* is an ascomycete species that forms ectomycorrhizal symbiosis with trees and shrubs. For forty years, large-scale inoculation of tree seedlings with *T. melanosporum* ascospores has been used in nurseries to produced >300,000 symbiotic plantlets yearly (Chevalier and Grente, 1978; Murat, 2015). As a consequence, truffle orchards are now found worldwide (Europe, Australia, USA, South Africa, and New Zealand). In these truffle orchards, the production of ascocarps (fruiting bodies issued from sexual reproduction) faces several problems, one of them being the initiation of sexual reproduction (Chen et al., 2016). The impossibility of manipulating *in vitro* truffle sexual reproduction makes it difficult to unravel the factors involved in its induction (Le Tacon et al., 2015). Because this information is still missing, clear guidelines for truffle orchards' management are still missing.

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

et al., 2016). They give birth to the main structure of the ascocarp (the peridium and the unfertile tissues of the gleba) (Fig. 1). The origin of male gametes is still unknown, but it has been suggested to be germinating sexual ascospores, conidia, or persisting soil mycelium (Fig. 1) (Le Tacon et al., 2015; Taschen et al., 2016).

One approach to help understand the truffle's sexual reproduction is to characterize small-scale genetic structures for both female and male genotypes. Indeed, small-scale genetic structure depends on the distribution of genotypes in population and results from a combination of their propagation modes and demographic processes. This can be analysed by characterizing the persistence and size of the genotypes (Douhan et al., 2011). The isolation by distance (IBD) theory predicts that a small-scale genetic structure accounts for gene dispersion capacity (Vekemans and Hardy, 2004). The small-scale genetic structure of female *T. melanosporum* genotypes was studied by genotyping with microsatellites and mating type genes of ectomycorrhizas and ascocarps (Rubini et al., 2011a; Murat et al., 2013; Taschen et al., 2016). In natural stands and truffle orchards, a pronounced genetic structure was observed. Moreover, ectomycorrhizas and female genotypes formed patches containing only one of the two mating types (spatial segregation). This particular spatial distribution raises the questions about the origins and dynamics of the male gametes since a high density of ascocarps was not observed at the boundary of patches with opposite mating types. This observation also questioned the existence of hermaphroditism (capacity of one genotype to form either male or female gamete) for *T. melanosporum* (Fig. 2). Inside these mono mating type patches, up to 10 female genotypes were detected; some were perennial (found during several seasons and forming genotypes with size up to 4.7 m), while others were detected only in one season and formed genotypes less than one meter (Murat et al., 2013; Taschen et al., 2016). This pattern suggests a rapid turnover of the non-perennial female genotypes. Recently, Taschen and

colleagues (2016) also surveyed male genotypes in five brulés (areas without vegetation surrounding trees that are substantially mycorrhized by *T. melanosporum*) located on three plantations and two natural sites. The genetic diversity was higher for male than female genotypes, and most of the male genotypes were detected in only one ascocarp. Only a single male genotype occurring more than one year was identified. However, the male genotype small-scale genetic structure could not be assessed in that study.

The objective of the present study was to characterize the small-scale genetic structure of both male and female genotypes in order to clarify the *T. melanosporum* sexual reproduction strategy and, more particularly, the origin and the behavior of the male genotypes. This work addresses several main points: 1) the small-scale genetic structure of the male genotypes and their evolution according to the time (perennial versus transitory male genotypes); 2) the localisation in the field of male genotypes in ascocarps, ectomycorrhiza, and soil; 3) the possible origin of the male genotypes; and 4) the sexual reproduction strategy (hermaphroditism versus dioecy). To achieve these goals, we used a 5-year sampling of ascocarps, ectomycorrhizas, and soils under seven productive and contiguous trees in a 25-year-old established truffle orchard in northeastern France. We determined the genotypes of both female and male ascocarp partners in addition to that of mycorrhizas and the mating type present in the soil samples.

Results

Ascocarps and ectomycorrhiza samplings

A total of 241 ascocarps were harvested during five consecutive truffle production seasons (from November to March of the following year) from 2010/2011 to 2014/2015 (Table 1; Fig. 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* ectomycorrhiza per
122 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).

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

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

Characterization of male genotypes

We successfully genotyped 206 zygotes (a mix of female and male genotypes in ascocarps 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 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),

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

214 males and once as an ectomycorrhiza (Fig. 3A). The R060 genotype was found five times as
215 the female genotype, once as a male and once as an ectomycorrhiza (Fig. 3B). Finally, the
216 R068 was detected three times as the female genotype, once as a male, and once as an
217 ectomycorrhiza (Fig. 3C). These three hermaphrodite genotypes were detected as male and/or
218 female in 55 ascocarps (27%). Conversely, 135 genotypes were detected only as male (98%)
219 and 70 genotypes only as female (92%).
220

221 **Discussion**

222 In this study, ascocarps, ectomycorrhizas, and soil cores were harvested over a 5-year period
223 under *T. melanosporum* productive trees in a truffle orchard. The female and male genotypes
224 of 238 and 206 ascocarps, respectively, were successfully obtained. The genotypic diversity
225 was higher for male genotypes than female genotypes with numerous small size genotypes
226 suggesting an important annual turnover. However, a few larger and perennial female and
227 male genotypes were detected. A pronounced small-scale genetic structure was identified for
228 both female and male genotypes although IBD analysis indicated that the dispersal capacity
229 was similar for both. Most of the genotypes were detected only as female or male, and only
230 three genotypes (1.5%) were found as being both female and male (hermaphrodite). These
231 data allowed an *in situ* comparison of the temporal dynamic, genetic structure, and diversity
232 of both female and male genotypes providing new information on *T. melanosporum* sexual
233 reproduction.
234

235 *Strong small-scale genetic structure for both female and male genotypes*

236 According to the genotypic richness (R) and the Simpson's diversity index, the genotypic
237 diversity was higher for male than for female genotypes although the gene diversity (1-Qinter
238 index) was similar for both. Large and perennial genotypes were found for both male and

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

264 16.7 to 40 % of homozygote zygotes (Taschen et al., 2016). The small-scale genetic structure
265 due to limited dispersal capacities may explain the observed heterozygote deficit. An existing
266 genetic barrier impeding mating of unrelated female and male genotypes is unlikely since
267 26% of the zygotes have a negative kinship coefficient.
268
269 *Germinating ascospores could act as male genotypes*
270 For both female and male genotypes, we observed similar features with both co-occurrence of
271 small size genotypes, often detected transiently as a single ascocarp, and larger perennial
272 genotypes. These results suggest that for both female and male genotypes, there is a mix of
273 new genotypes recruited from ascospores and perennial genotypes that have been
274 disseminated by vegetative propagation. However, male genotypes presented a higher
275 genotypic diversity and were less perennial than female ones, suggesting that ascospore
276 recruitment is more important for male than for female genotypes. As already proposed by
277 Taschen and colleagues (2016), it is therefore tempting to hypothesize that most of the male
278 genotypes originate from germinating ascospores whose mycelium does not survive after
279 sexual reproduction. Indeed, sexual spores have been proposed as male gametes for
280 ascomycetes and basidiomycetes (Nieuwenhuis et al., 2011).
281 The inoculation of ascospores in truffle orchards in order to improve the production of
282 ascocarps has become a common practice (Olivier et al., 2012), but is totally empirical due to
283 lack of scientific background. This practice is not recent since Ciccarello (1564), Bradley
284 (1726) and Buffon (1749) proposed improving truffle production by inoculating pieces of
285 ascocarps under mature trees. The role of ascospores was not directly demonstrated in our
286 study but, in collaboration with truffle growers' dedicated experiments, have been initiated to
287 confirm their role.
288

Soil mycelium could also be a reservoir of male genotypes

In our study, 16 male perennial genotypes were found. One of them, genotype R102, was found in 18 ascocarps (8.7%) over four years (Fig. S5). Among these, only three corresponding to true hermaphrodite genotypes were present on ectomycorrhizas. We have made the assumption that these male genotypes have a poor ability to form associations with the host and that they likely survive as free-living mycelium in the soil or are associated with the roots of non-ectomycorrhizal plants. It has been demonstrated that roots of herbaceous plants can host truffle mycelium, but the nature of this interaction (such as colonization of the rhizosphere, endophytism,and endomycorrhiza as with orchids) is unknown (Gryndler et al., 2014). In our study, we detected the presence of both mating types in 16 out of 20 soil cores. This result demonstrates that close to the ectomycorhizas formed by the female genotype, mycelium of opposite mating type are present as mycelium and consequently the male genotype could survive in the soil through vegetative propagation, saprotrophycally, or in association with the roots of non-hosts.

In many ascomycetes, conidia (asexual spores) serve for vegetative propagation or as a male gamete (Nelson, 1996; Maheshwari, 1999). Urban et al. (2004) described the existence of anamorphous structures producing mitotic conidia in soils where *T. borchii* and *T. oligospermum* ascocarps were present. Healy et al. (2013) suggested that Pezizales mitospores, including *Tuber* mitospores, which failed to form ectomycorrhizas, could act as male gametes. Nevertheless, the question of whether conidia act as male gametes in the *Tuber* species in which they have been found remains unanswered and for the moment, conidia were never yet observed in *T. melanosporum.*

Sexual reproduction strategy in Tuber melanosporum

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

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

364

365 As described by Murat *et al.* (2013), the sampling started under trees F10, F11 and E10 (area
366 1) in the 2010-2011 season and under the trees A11, A12, B11 (area 3) and D11 (area 2) in
367 the 2011–2012 season (Fig. S1). The mature truffles were systematically harvested during the
368 production season with help from a well-trained dog, and at the time of harvest, they were
369 precisely mapped on the grid with 5 cm precision. The ascocarps were then washed to remove
370 soil particles and stored at -20°C for molecular analysis.
371 During this five-year interval, two ectomycorrhizal samplings were done. The first one was
372 done under F10/F11/E10 trees (area 1 in Fig. S1) in spring 2011 (season 1), and results on
373 that sampling were published by Murat et al. (2013). The second one was done in November
374 2014 (season 5), and 45 root samples were sampled all over the truffle orchard (Fig. S1). Root
375 samples were harvested from the first 10 cm of soil and mapped on the same grid used for the
376 positioning of the ascocarps. The ectomycorrhizas were carefully retrieved from the soil and
377 washed in water under a dissecting microscope. From each root sample, the *T. melanosporum*
378 ectomycorrhizas were identified as described by Zambonelli et al. (1993) and Rauscher et al.
379 (1995), and stored individually in microcentrifuge tubes at -20°C for molecular analyses.
380 In order to investigate the distribution and abundance of both mating types in the soil, 20 soil
381 cores were harvested in May 2015 (Fig. S1) at a 10–15 cm depth. All plant debris, stones, and
382 roots were discarded from the soil samples, and samples were kept at -20°C for DNA
383 extractions.
384

385 *DNA extractions*

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

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

414 Tm1_ATTG18, Tm75_GAAA14, Tm22_CCTCAT17 and Tm269_TGTTGC15) as described
415 by Murat et al. (2011; 2013). The genotyping was done by the INRA plateform Gentyane
416 (Clermont-Ferrand). The *T. melanosporum* mating types of all the samples were analysed
417 using specific primers for either the *MAT1-1-1* or the *MAT1-2-1* genes using the PCR
418 conditions described by Rubini et al. (2011b). Hereafter, according to Rubini et al. (2011b),
419 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^{TM} 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 (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 index 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 form 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

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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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. 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

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*

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

Fig. 1

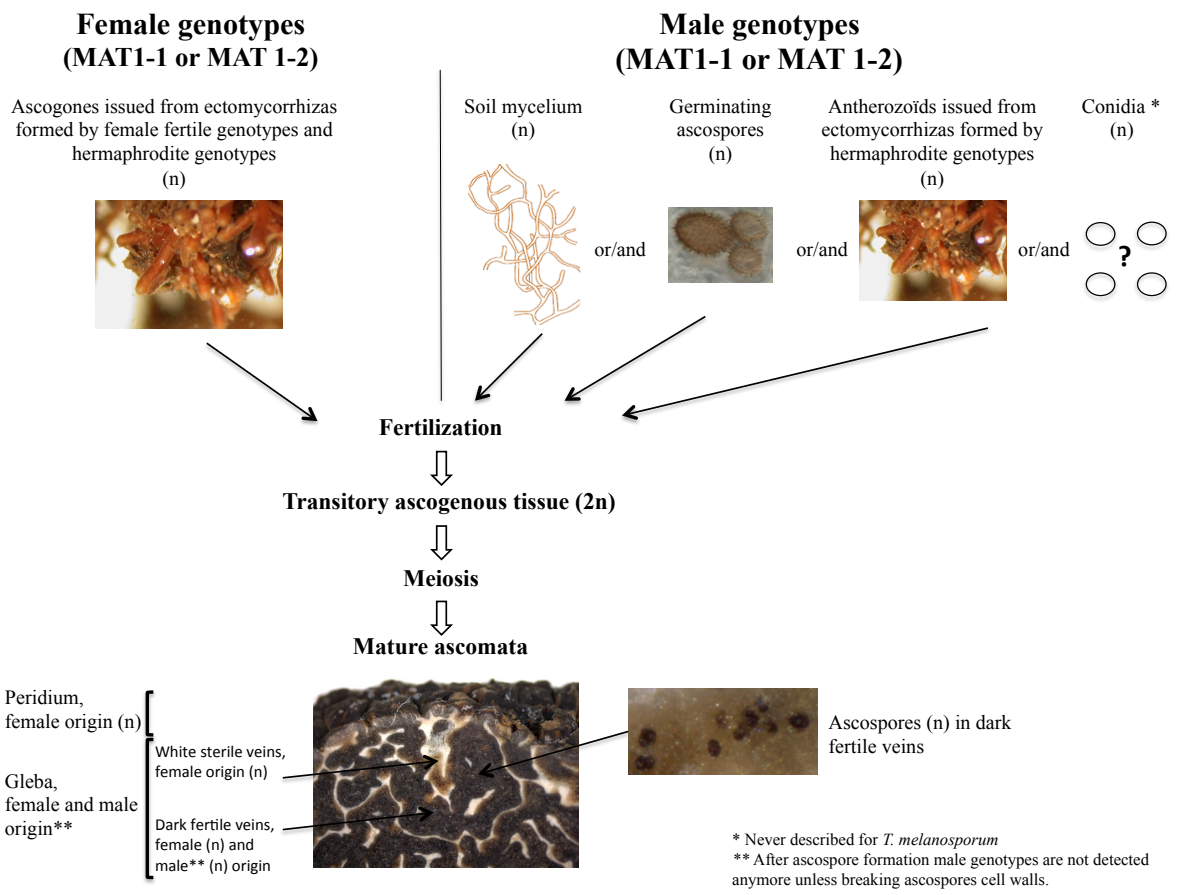
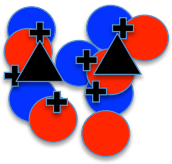

Female genotypes
(MAT1-1 or MAT 1-2)
Ascogones issued from ectomycorrhizas formed by female fertile genotypes and hermaphrodite genotypes
(n)
Male genotypes
(MAT1-1 or MAT 1-2)
Soil mycelium
(n)
or/and
Germinating ascospores
(n)
or/and
Antherozoïds issued from ectomycorrhizas formed by hermaphrodite genotypes
(n)
or/and
Conidia *
(n)
?
Fertilization
Transitory ascogenous tissue (2n)
Meiosis
Mature ascomata
Peridium, female origin (n)
Gleba, female and male origin**
White sterile veins, female origin (n)
Dark fertile veins, female (n) and male** (n) origin
Ascospores (n) in dark fertile veins
* Never described for T. melanosporum
** After ascospore formation male genotypes are not detected anymore unless breaking ascospores cell walls.

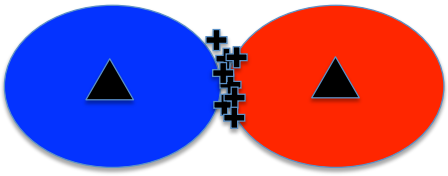
Fig. 2

Scenario 1



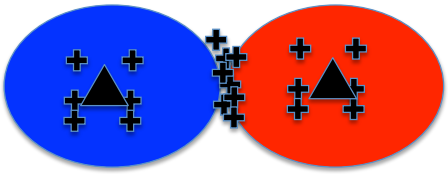
Mix of maternal genotypes of both mating type and hermaphrodism

Scenario 2a



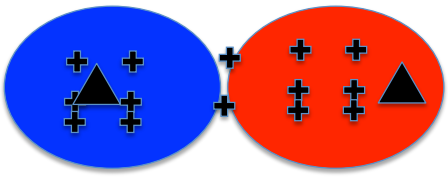
Large patches of maternal genotypes of the same mating type, hermaphrodism, and no dispersal of male genotypes

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

Fig. 3

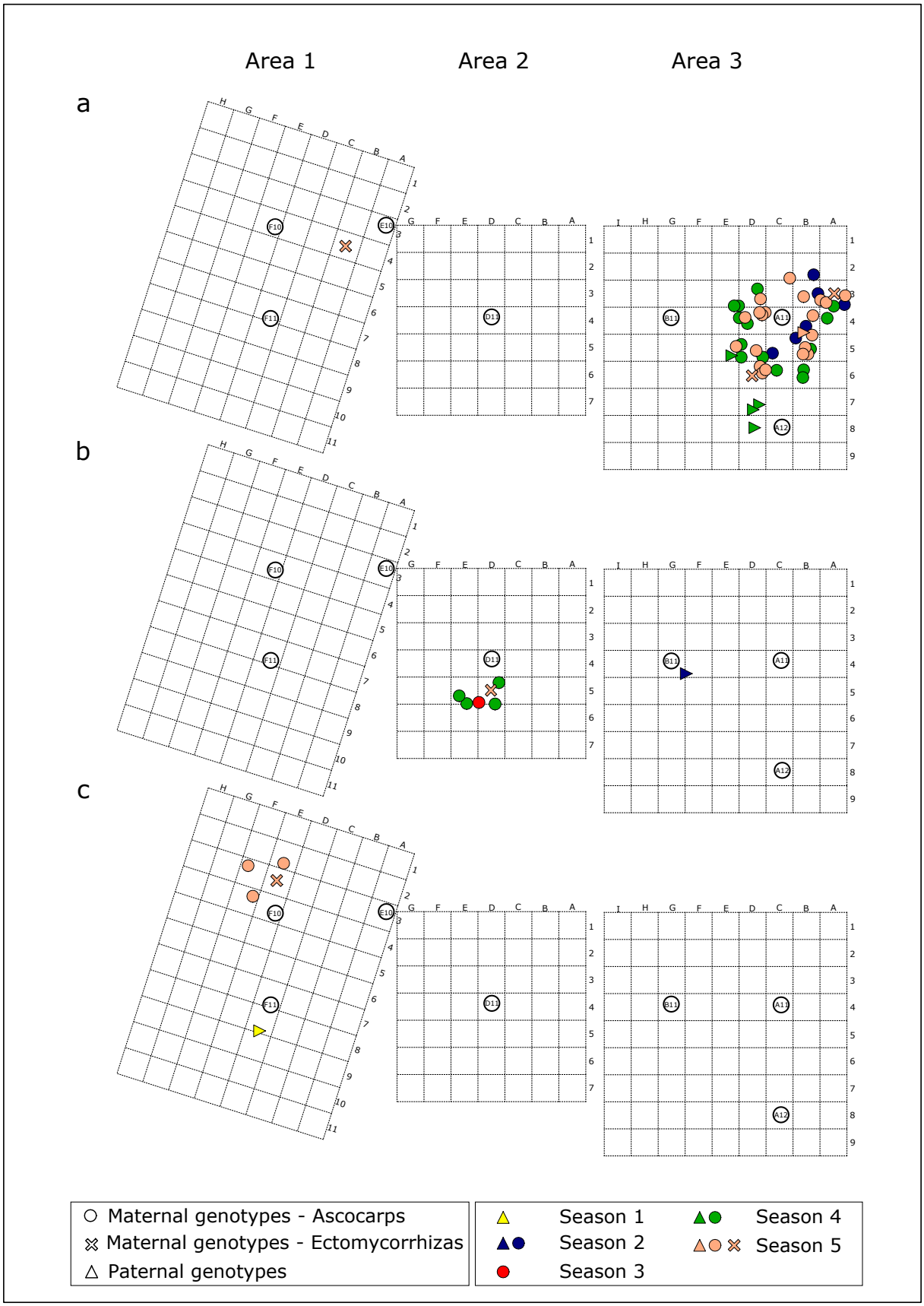
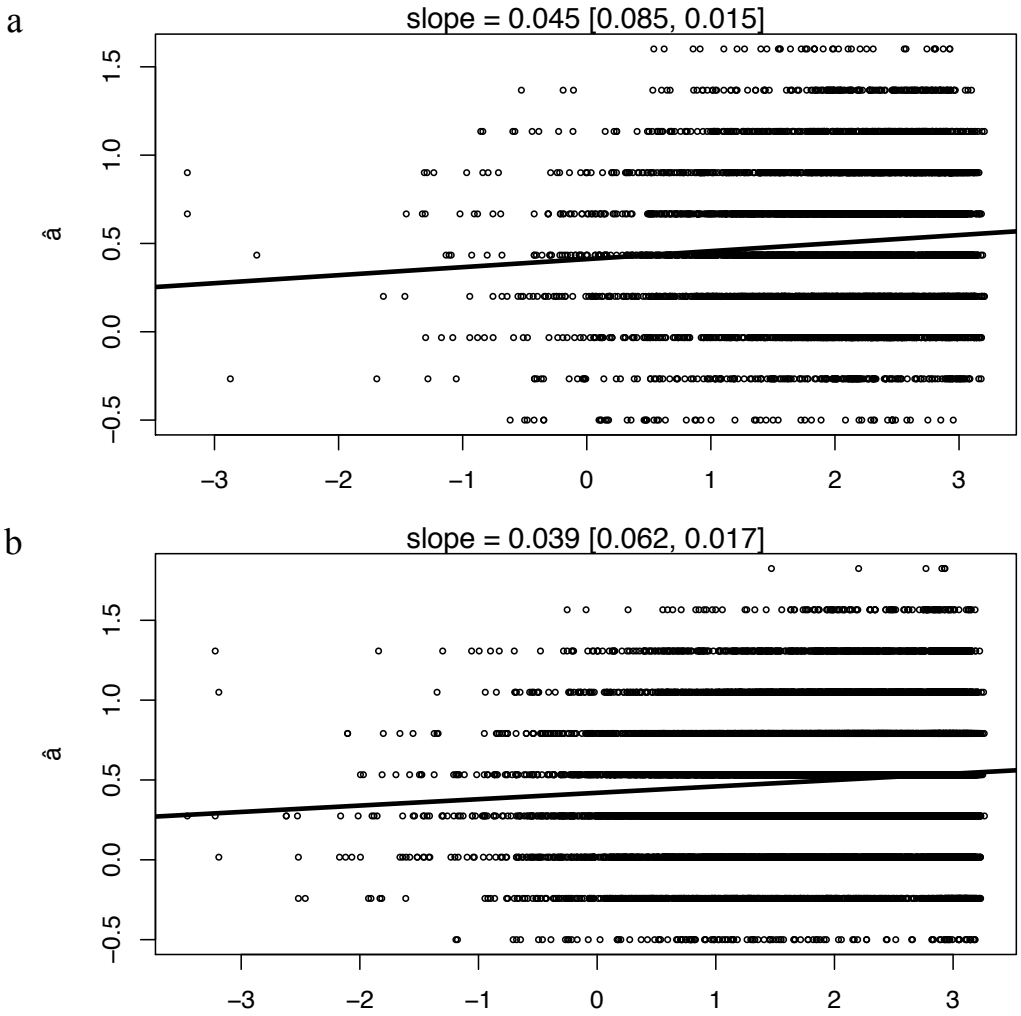

Area 1
Area 2
Area 3
a
b
c
Maternal genotypes - Ascocarps
Maternal genotypes - Ectomycorrhizas
Paternal genotypes
Season 1
Season 2
Season 3
Season 4
Season 5

Fig. 4


a
slope = 0.045 [0.085, 0.015]
â
1.5
1.0
0.5
0.0
−0.5
−3
−2
−1
0
1
2
3
b
slope = 0.039 [0.062, 0.017]
â
1.5
1.0
0.5
0.0
−0.5
−3
−2
−1
0
1
2
3