Investigation of the sexual reproduction strategy of the Périgord black truffle (*Tuber melanosporum* Vittad.) revealed trioecy

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Summary

Fungi display a rich variety of sexual reproduction systems and are therefore good models to investigate sex evolution. Moreover, understanding the investment in sexual reproduction of edible fungi is a critical challenge for improving their cultivation. The Périgord black truffle (*Tuber melanosporum* Vittad.) is a heterothallic ascomycete associated with trees through ectomycorrhizas and forming highly prized ascocarps. The aim of this study was to unravel the sexual reproduction strategy (hermaphroditic versus male/female specialization) of *T. melanosporum* in a truffle orchard by disentangling the contribution of female and male genotypes to the formation of ascocarps in a five-year investigation. Few genotypes were hermaphrodites, co-occurring with numerous genotypes behaving only as female or male, revealing trioecy. The genetic diversity of the male genotypes was higher than female diversity, suggesting for male elements a higher recruitment from ascospores. Most of the female and male genotypes were transitory (present only one year), whereas some genotypes persisted for several years: female-fertile genotypes as mycorrhizas, and male-fertile genotypes as soil free-living mycelium. Contrary to other ascomycetes, a high number of female-fertile only genotypes was found. We hypothesized that the mycorrhizal life style favours female-fertile strains and therefore that the life strategy influences fungal sexual strategy.

Introduction

In fungi, sexual lifestyles are rich with species displaying typical mating between compatible strains (i.e. heterothallism) whereas others are able to reproduce sexually in the absence of a compatible partner (i.e. homothallism). The life cycle of the ascomycetes species is mainly haploid and heterothallic species form male elements (e.g. conidia, sexual spores or hyphae) (Glass and Kuldau, 1992; Leslie and Klein, 1996), and female elements (ascogonia) are usually much more complex. In theory, one strain of a heterothallic ascomycete is hermaphrodite or monoecious (Esser and Kuenen, 1967), meaning that it can form either male or female elements (Glass and Kuldau, 1992). A haploid (hence self-sterile) hermaphrodite propagating by asexually producing spores or reproducing sexually was proposed to be an idealized heterothallic ascomycete (Leslie and Klein, 1996). Hermaphroditism is not always maintained as demonstrated for *Hypomyces solani* (*Fusarium solani*) (Hansen and Snyder, 1943), and the occurrence of strains forming only male or female elements (i.e. dioecy) was observed in ascomycetes (Benjamin, 1950). Trioecy, the co-occurrence of hermaphrodite and dioecy, was observed for *Triceromyces* species (Benjamin, 1986) and *Fusarium fujikuroi* (Leslie, 1995). Few examples of trioecy have been described in fungi, although it is widespread in animals and plants (Weeks et al., 2006; Chaundhuri et al., 2011; Armova-Zvuloni et al., 2014; Joseph and Murthy, 2014; Mirski and Brzosko, 2015). Moreover, the relative abundance of hermaphrodite, male/female producing strains in the field for mycorrhizal (plant symbiotic) ascomycete species has never been investigated.

Beside the theoretical and evolutionary interest of investigating fungal sexual reproduction, it is also critical for overcoming the cultivation of species forming edible mushrooms. This is particularly true for the most famous edible fungi (such as Boletes, Chanterelles, and truffles), whose growth and sexual reproduction are strictly linked to host plants through a symbiotic

association called ectomycorrhiza (Murat et al., 2008). Cultivation of the black Perigord truffle (*Tuber melanosporum* Vittad.) started forty years ago thanks to inoculated seedlings (Chevalier and Grente, 1979); however, the control of its sexual reproduction cycle is not achieved (Le Tacon et al., 2015). *Tuber melanosporum* is a heterothallic ascomycete species (Martin et al., 2010; Rubini et al., 2011b) with the female mycelium found in the root system as ectomycorrhiza and forming the structure (i.e. peridium and gleba) of the ascocarps (Fig. 1). The origin of male elements is unknown, but it was suggested to be conidia, ascospores, or soil mycelium (Fig. 1; Le Tacon et al., 2015). The small-scale genetic structure of female genotypes was characterized (Rubini et al., 2011a; Murat et al., 2013), although male genotypes were never analysed under field conditions. Female genotypes were detected in the root system as this is where they tended to aggregate according to their mating type (Murat et al., 2013). The same result was found recently in one natural production site of *T. aestivum* in Germany (Molinier et al., 2016). Interestingly, a high density of ascocarps was not observed for either species in the contact zone of the opposite mating types suggesting a more complex system of sexual reproduction.

The aim of this study was to unravel the sexual reproduction strategy (trioecious versus dioecious or hermaphroditic) of *T. melanosporum* in the field. We used a five-year sampling of *T. melanosporum* ascocarps, ectomycorrhizas and soil in one orchard and established the genotypes of both female and male elements. The investigation of sex strategy in truffles provides new insights into the sex specialisation in fungi, but more importantly, it improves our understanding of sexual reproduction and its impact on the eukaryotic tree of life.

Results

Ascocarps and ectomycorrhiza samplings

A total of 241 ascocarps were harvested during five consecutive seasons of truffle production (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 found near the trunk, at 4 to 5 m from it, in the zone of extension of the roots, and between it (Fig. S1). The productive area under each tree extended slowly over five years.

A total of 475 ectomycorrhizal root tips were collected (205 in 41 root samples, with 5 *T. melanosporum* morphotype each, in 2011 and 270 in 45 root samples, with 6 *T. melanosporum* morphotype each, in November 2014) (Fig. S1). Only 37 ectomycorrhiza samples did not amplified with the *T. melanosporum* specific primers, and the presence of *T. melanosporum* ectomycorrhizas was not confirmed only in five root samples (two in 2011 and three in 2014). For genotyping, only one *T. melanosporum* ectomycorrhiza per root sample was selected.

Genotyping of female elements (gleba and ectomycorrhiza)

The genotypes of ascocarp female elements (i.e. gleba) were successfully obtained for 238 out of the 241 ascocarps (Table 1 and Table S1). A total of 73 female multilocus genotypes (called hereafter genotypes) with significant Psex value were found during the five years (Table 1, Tables S1 and Table S2). Twelve genotypes were present in more than one season (Table S1). These genotypes accounted for 26% to 94% of the ascocarps for seasons 3 and 1 respectively, for a total of 154 truffles (64 % of the total harvested ascocarps over the five seasons). One persistent genotype (R002) was found throughout the season under the same tree (F11, area 3), representing 15% of the total ascocarps harvested (Fig. S2 and Table S1). Another genotype (R021) fructified under the A11 tree (in area 1) for three seasons and represented 16 % of the total ascocarp harvested (Fig. 2 and Fig. S1). The maximum size (clonal subrange) of the female genotypes was 22.36 m, corresponding to the R002 genotype

found in areas 1 and 3 of the truffle orchard (Table 1 and Fig. S2). Most female genotypes were found in only one ascocarp (Table S1).

A total of 117 ectomycorrhizas were genotyped, representing 9 and 14 genotypes for 2010 and 2014 samplings, respectively (Table 1 and Table S1). Five genotypes (R001, R002, R003, 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. 2). Eighty-six percent of ectomycorrhiza genotypes were detected also as female elements in ascocarps, represented in 16 genotypes with significant Psex value (Table S1). Similarly, the female element of the ascocarps shared the same genotype with that of the nearby ectomycorrhizas.

When considering only the mating type locus, the aggregation index (Ac) varied from 0.42 to 0.57 for ascocarp female element (gleba) and from 0.40 to 0.96 for ectomycorrhizas (Table 1). In all cases, the Ac was significant (P-value < 0.05), indicating that genotype of the same mating type tend to aggregate. 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 (E10 and B11) or by two contiguous patches of opposite mating types (F10, F11, D11, A11, and A12). Tree D11 harboured maternal strains 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 strains (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).

140 *Zygote analysis and genotyping of male elements*

141 We successfully genotyped 206 zygotes out of the 241 ascocarps (86.5 %; Table S1). By
142 subtracting the female haploid contribution, we were able to reconstruct all male genotypes. A
143 total of 138 male genotypes were found (Table 1 and Table S1). A total of 9 zygotes (4%)
144 were homozygous for all microsatellite loci (i.e. only mating type locus is heterozygous). In
145 zygotes, a significant deficit of heterozygosity was observed with F_{IS} values of 0.02 and 0.28
146 for seasons 1 and 3, respectively (Table 1). The relationship of female and male genotypes in
147 zygotes was investigated by kinship coefficient calculation. The kinship coefficient varied
148 from -0.5 to 1.5 (Fig. S4).

149 Most of the male genotypes (75%) were transitory, i.e. found only in one ascocarp (Table S1).
150 Only 8 persistent male genotypes with significant Psex values were found. These persistent
151 genotypes produced 21 % to 37 % of the ascocarps (Table 1). Sixteen male genotypes were
152 found during several seasons and 5 (only one genotype with significant Psex) were found
153 under different trees (Table S1). One genotype (R102) was detected for four seasons under
154 A11 where it fertilized 8 % of the ascocarps (Fig. S5). The maximum male genotype size was
155 22.64 m for genotype R102 with detection of most of them in one single ascocarp (Table 1).

156

157 *Identification of hermaphrodite strains*

158 The hermaphrodite strains are those detected as both female and male elements. In our
159 dataset, 8 genotypes (3.9%) were detected as female and male (detected in the gleba and/or
160 ectomycorrhizal tissues and in the zygotes). However, only 3 genotypes (1.5 % of the total
161 number of female and male genotypes) were supported by significant Psex values and thus
162 considered true hermaphrodites (Table 1 and Fig. 2). The R021 genotype was the most
163 frequent female genotype (see above), and it has been also detected in 5 ascocarps as male
164 element and once as ectomycorrhiza (Fig. 2A). The R060 genotype was found 5 times as

165 female genotype, once as male strains and once as ECM (Fig. 2B). Finally, the R068 was
166 detected 3 times in female strains, once in male strains, and once as ECM (Fig. 2C).
167 Conversely, 135 genotypes were detected only as male-fertile (64%) and 70 genotypes were
168 only female-fertile (33%).
169
170 *Small-scale genetic structure for both female and male elements*
171 According to the season, the Simpson's diversity varied from 0.81 to 0.98, 0.98 to 0.99, and
172 0.79 to 0.99 for female, male and ectomycorrhizal samples, respectively (Table 1). The inter-
173 individual diversity varied from 0.52 to 0.91 and 0.83 to 0.97 for female and male elements,
174 respectively (Table 1). Both indices were similar but they were slightly higher for male
175 elements compared to female ones and ectomycorrhizas. The genetic structure was assessed
176 using isolation by distance (IBD), and the slope was used to investigate dissemination
177 capacities (Fig. 3). A significant genetic structure was detected for female and male elements
178 when all samples were used together or when using a culled dataset. When all samples were
179 considered, a higher slope was found for female versus male strains (0.125 versus 0.049),
180 indicating a higher dissemination capacity or higher density of male elements (Fig. 3). This
181 analysis, however, could be biased due to the repeated sampling of the same genotype. Using
182 the culled dataset the slope was even higher for female versus male strains but it was not
183 significant (Fig. 3).
184
185 *Quantity of mating types mycelium in soil samples*
186 In the 20 soil cores harvested in May 2015 (Fig. S1), both mating type mycelium were
187 detected in 16 soil samples (Table 2). In one soil sample, any of both mating types of
188 mycelium were detected while in 2 soil samples only *MAT1-1* was found, and in one soil
189 sample only *MAT1-2* was detected. The quantity of mycelium ranged from 0 to 7.4 mg of

190 mycelium per gram of soil and 0 to 40.49 mg of mycelium per grams of soil for *MAT1-1* and
191 *MAT1-2*, respectively. With two exceptions (squares E4 and E8 in area 1), the most frequent
192 mycelium corresponded to those of maternal strains and ectomycorrhiza.
193
194 **Discussion**
195 In this study, we characterized *in situ the* genotypes of female and male elements for *T.*
196 *melanosporum* by analysing both maternal and zygotic tissues of ascocarps. This involved
197 disentangling female and male contributions of all fruiting bodies collected in an orchard for
198 five years. We revealed a strong genetic structure for both elements and trioecy through the
199 coexistence in this population of hermaphrodite, female-fertile only and male-fertile only
200 strains. Moreover, equilibrium between vegetative propagation and sexual reproduction was
201 observed, but the factors conditioning the shift between both modes are still unknown. More
202 broadly, the maintenance of sexual reproduction in a natural population is an intriguing
203 question always discussed among evolutionists (Lehtonen and Kokko, 2014). Fungi are good
204 models to investigate sex evolution since they are particularly rich in their mode of sexual
205 reproduction. The recent progress in fungal genomics has allowed investigation of sexual
206 reproduction in species where it had not been previously described such as the cheese
207 ascomycete *Penicillium roqueforti* (Ropars et al., 2012). For mycorrhizal fungal species, the
208 mating type locus was described from genomic data for arbuscular endomycorrhiza
209 (*Rhizophagus irregularis*, Ropars et al., 2016), ectomycorrhizal basidiomycetes (*Laccaria*
210 *bicolor*, Niculita et al. 2008) and ascomycetes (*T. borchii*, Belfiori et al., 2015; *T. indicum*,
211 Belfiori et al., 2013 and *T. melanosporum*, Rubini et al., 2011b). However, the distribution of
212 mating type strains and sexual reproduction strategies (hermaphrodite versus dioecy) has not
213 yet been investigated, although mycorrhizal fungi play key roles in ecosystem functioning and
214 biogeochemical cycles (Smith and Read, 2008).

215 In *T. melanosporum*, the female elements are provided by the ectomycorrhizas that play the
216 role of gametophytes (Rubini et al., 2011a; Murat et al. 2013). Conversely, the origin of the
217 male elements was unknown (Le Tacon et al., 2015). For both female and male elements, we
218 observed similar features with the co-occurrence of small size genotypes, often detected
219 transitorily in only one ascocarp and large perennial genotypes. These results suggest that, for
220 both female and male elements, there is a mix of new genotype recruitment from ascospores
221 and perennial strains disseminated by vegetative propagation.
222 A strong genetic structure was found for both female and male elements, as evidenced by IBD
223 analysis. The IBD is influenced by spore dispersal. In fungi, two main strategies of dispersal
224 exist: 1) wind for epigeous and 2) animals for hypogeous species. This strong genetic
225 structure is characteristic of hypogeous fungi that are expected to have reduced spore
226 dispersal than epigeous (Kretzer et al., 2005). When considering culled data, similar slopes
227 for both male and female elements (0.048 and 0.051, respectively), suggest similar capacities
228 of dissemination for both sexes. Focusing on male elements, 16 perennial genotypes were
229 found. Among them, genotype R102 was found in 18 ascocarps (8.7%) over 4 years (Fig. S5).
230 Only the three male genotypes corresponding to the true hermaphrodite genotypes were
231 present on ectomycorrhizas. This can be due to a poor ability to form associations with the
232 host of male-fertile genotypes that probably survive as free-living mycelium in the soil or are
233 linked to non-ectomycorrhizal plants. Indeed, herbaceous plants can host truffle mycelium,
234 but the nature of this interaction (i.e. surface contact only, endophytism, endomycorrhiza) is
235 unknown (Gryndler et al., 2014). Moreover, we detected the presence of both mating types in
236 the soil mycelium in 16 out of 20 soil cores. This result demonstrated that mycelium of the
237 opposite mating type is present close to female strains in ectomycorrhiza. We can therefore
238 hypothesize that genotypes producing only the male elements (the so-called male strain)
239 survive in the soil through vegetative propagation. In many ascomycetes, conidia (i.e. asexual

240 spores) serve for vegetative propagation or as male elements (Nelson, 1996; Maheshwari,
241 1999). Urban *et al.* (2004) described the existence of anamorphous structures producing
242 mitotic conidia in soils where *T. borchii* and *T. oligospermum* ascocarps were present. Healy
243 *et al.* (2013) suggested that Pezizales mitospores, including *Tuber* mitospores, which failed to
244 form ectomycorrhizas, could act as spermatia. However, the question of whether conidia of
245 *Tuber* species act as spermatia remains unanswered and mitotic conidia have not yet been
246 observed in *T. melanosporum* orchards.
247
248 Male genotypes presented a higher genetic diversity and were less perennial than female ones,
249 suggesting that ascospore recruitment is more important for male than for female elements. It
250 is therefore tempting to hypothesize that most of the male elements originate from
251 germinating ascospores whose mycelium does not survive after sexual reproduction. Indeed,
252 sexual spores have been proposed as male elements for ascomycetes[2] and for basidiomycetes
253 (Nieuwenhuis et al., 2011). The inoculation of ascospores in order to improve the production
254 of ascocarps is a common practice (Olivier et al., 2012) but is totally empirical, as scientific
255 background was lacking. This practice is not recent since Ciccarello (1564), Bradley (1726)
256 and Buffon (1749) proposed methods to produce ascocarps by inoculating pieces of truffles
257 under mature trees. The role of ascospores was not directly demonstrated in this study, but the
258 possibility to trap truffle production in a few cm^2, as recently demonstrated (Bonneau, 2016),
259 open new perspectives to confirm the role of ascospores as male elements.
260
261 Hermaphroditism is the common rule in heterothallic ascomycetes ((Glass and Kuldau, 1992;
262 Nieuwenhuis and Aanen, 2012), and haploid self-sterile hermaphrodite has been proposed as
263 the optimal situation (Leslie and Klein, 1996). For *T. melanosporum* Rubini et al., 2011a;
264 Murat et al., 2013) and *T. aestivum* (Molinier et al., 2016) female strains are not randomly

265 distributed according to their mating types since they form large patches of different
266 genotypes in the root system, all sharing the same mating type. In our study, we confirmed
267 this spatial aggregation of genotypes of the same mating type in the root system. This
268 aggregation was stable over five years despite genotype turnover (Fig. S3). Interestingly, few
269 ascocarps were harvested in the contact zone of both mating types suggesting that 1) these
270 strains were not hermaphrodites; 2) all opposite mating type strains could not mate; or 3) they
271 had very limited dispersal capacities. We found three genotypes as true hermaphrodites since
272 they were identified as both female and male elements. On the contrary, most genotypes were
273 identified as female-fertile or male-fertile only, suggesting a specialisation of the partners in
274 one sex. In the literature, the terms male-sterile and female-sterile are commonly used, but in
275 our study based on field observations, we cannot definitively rule out that the female-fertile
276 and male-fertile strains are sterile for the opposite sex. Indeed, two hypotheses can be
277 proposed: 1) the capacity to produce male or female elements is lost due to mutations or 2)
278 genetic or epigenetic regulation impedes forming male or female elements. If mutations drive
279 the sex specialisation in *Tuber*, female-fertile and male-fertile strains are effectively sterile for
280 the opposite sex. In case of a regulation mechanism, sex specialization may be reversible.
281 In our population, female-fertile strains were relatively frequent (33% versus 64% of male-
282 fertile strains). This finding contradicts the model developed by Leslie and Klein[2] for
283 pathogenic and saprotrophic fungi. They hypothesised that male-sterile (i.e. female-fertile)
284 strains have reduced fitness for vegetative propagation, and therefore, they are not frequent. In
285 *T. melanosporum* the female-fertile strains were found in ectomycorrhizas and 17 % of these
286 were persistent, suggesting they dispersed by vegetative propagation. The female structures
287 are more complex than male structures and they require more energy. The necessity of
288 female-fertile strains to form ectomycorrhiza for carbon acquisition from the host tree could
289 favour their fitness in vegetative propagation explaining the difference with the Leslie and

Klein (1996) model. We may attempt to propose that the fungal life cycle (pathogen/ symbiotic/ saprotrophic) could influence the reproduction strategy.

To summarize, in our population a mix of few hermaphrodites with a majority of female-fertile and male-fertile strains were identified suggesting trioecy. Trioecy is known in plants (Joseph and Murthy, 2014; Mirski and Brzosko, 2015) and animals ((Weeks et al., 2006; Chaudhuri et al., 2011). In fungi, trioecy was reported for the ascomycete Triceromyces (Benjamin, 1986), and it can exist for *F. fujikuroi* (Leslie, 1995), but it doesn't seem to be a widespread situation. In animals, trioecy can occur when environmental conditions change or when a species occupies a new habitat, leading to a transition from hermaphroditism to dioecy or *vice versa* (Weeks et al., 2006). Trioecy is therefore a transitory status and in *Caenorhabditis elegans* it seems evolutionarily unstable (Chaudhuri et al., 2011). In heterothallic ascomycetes, hermaphroditism could be the ancestral status since it is expected for most of the species (Glass and Kuldau, 1992). It is therefore tempting to hypothesize that hermaphroditism has been lost in our *T. melanosporum* population in order to favour female-fertile and male-fertile strains. In a theoretical analysis, Nauta and Hoekstra (1992) suggested that in heterothallic populations, hermaphroditism could only be stable if the fitness of ascospore formation is variable. For ascomycetes, 8 spores are expected in each asci but for *T. melanosporum* the number of spores per asci varied from 1 to 4 (Chevalier et al., 2001). In *Neurospora crassa* mutations leads to spore number reduction and some of these reduced the female or male fertility (Leslie and Raju, 1985). There were no differences in the number of spores per asci between hermaphrodite and female strains (data not shown) and currently it is not possible to conclude that spore fitness influences the sexual reproduction strategy in truffle. But the investigation of sexual reproduction of truffle species with 8 spores such as *T. panniferum* or *T. regianum* (Chevalier et al., 2001) can allow addressing this hypothesis.

315 To conclude, trioecy with a low rate of hermaphrodite was observed in a *T. melanosporum*
316 population. Sexual dimorphism could therefore be more frequent in fungi than expected, and
317 progress in genome sequencing could allow its investigation. Indeed, in contrast to animals
318 and plants, dioecious fungi often are morphologically similar, and sexual dimorphism can be
319 detected only at genome level or at gene regulation level (Samils et al., 2013). Moreover, the
320 life strategy could influence the sexual reproduction mode, which has been poorly
321 investigated to date. The understanding of sexual reproduction of economically interesting
322 fungal species such as those producing edible mushrooms is a major issue for a better control
323 of their life cycle and cultivation.
324
325 **Experimental procedures**
326 *Truffle orchard and sampling*
327 Ascocarps and ectomycorrhizas were sampled at a long-term experimental site located at
328 Rollainville in north-eastern France. The study site is described in a study by Murat *et al.*
329 (2013). Samples and trees were identified by a letter and number and mapped on a grid of 1 m
330 x 1 m squares set up with camping pickets (Fig. S1). Three different grids were made to
331 identify 3 areas (areas 1, 2 and 3) that cover all the productive zones of the plantation (Fig.
332 S1).
333
334 As described in Murat *et al.* (2013), the sampling started under trees F10, F11 and E10 (area
335 3) in 2010-2011 season and under the trees A11, A12, B11 (area 1) and D11 (area 2) in 2011-
336 2012 season (Fig. S1). The mature truffles were systematically harvested during the
337 production season with help from a well-trained dog, and at the time of harvest, they were
338 precisely mapped on the grid with 5 cm precision. The ascocarps were then washed to remove
339 soil particles and stored at -20°C for molecular analysis.

340 During this five-year interval, two ectomycorrhizal samplings were done. The first one was
341 done under F10/F11/E10 trees (area 3 in Fig. S1) in spring 2011, and results on that sampling
342 were published by Murat *et al.* (2013). The second one was done in November 2014, 45 root
343 samples were sampled all over the truffle orchard (Fig. S1). Root samples were harvested
344 from the first 10 cm of soil and mapped on the same grid used for the positioning of the
345 ascocarps. The ectomycorrhizas were carefully retrieved from the soil and washed in water
346 under a dissecting microscope. From each root sample, the *T. melanosporum* ectomycorrhizas
347 were identified as described by Zambonelli *et al.* (1993) and Rauscher *et al*. (1995), and
348 stored individually in microcentrifuge tubes at -20°C for molecular analyses.
349 In order to investigate the distribution and abundance of both mating types in the soil, 20 soil
350 cores were harvested in May 2015 (Fig. S1) at 10-15 cm depth. All plant debris, stones and
351 roots were discarded from the soil samples, and samples were kept at -20°C for DNA
352 extractions.
353
354 *DNA extractions*
355 Genomic DNA from the gleba (i.e. inner part of the ascocarps) and ectomycorrhizal tips were
356 extracted by using the DNeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France), following
357 the manufacturer's instructions.
358 From each ascocarp, a mixture of spores was isolated and their DNA was extracted as
359 described below. Thin slides of each ascocarp were put onto a layer of water in a Petri dish to
360 let spores be released into the water in order to isolate the pool of spores. The liquid was
361 collected in a 1.5 mL tube and centrifuged at 14,000 rpm for 5 minutes. The supernatant was
362 then discarded in order to obtain a mixture of asci and spores. DNA from the isolated mixture
363 of spores from each ascocarp was extracted as described in Rubini *et al.* (1999) with some
364 modifications. First, to each pool of spores, we added 300 mL of NTE buffer (Tris-HCl 200

365 mM, NaCl 250 mM EDTA 25 mM), two tungsten beads, and then disrupted the spores with a
366 Tissue Lyser (Qiagen) for 10 minutes at 30 J. The tubes were then centrifuged at 14,000 rpm
367 for 10 minutes, and the recovered supernatant was added to a new tube. Thirty microliters of
368 NaAc (3M) and 330 µL isopropanol were added, and those tubes were mixed and centrifuged
369 again at 14,000 rpm for 10 minutes. After discarding the supernatant and cleaning the pellet
370 with 200 µL of ethanol (70%), we recovered the pellet (DNA) in 50 µL of TE. DNA extracts
371 were stored at -20°C.
372 Total DNA from soil samples was extracted using the Power Soil® DNA extraction Kit
373 (MoBio, Laboratories, Carlsbad, CA) following manufacturer's protocol and stored at -20°C
374 for molecular analysis.
375
376 *Molecular genotyping*
377 All extracted DNA samples were amplified using species-specific *T. melanosporum* primers[48]
378 in order to check the species and DNA quality.
379 All of the samples in which *T. melanosporum* identity was confirmed were genotyped by
380 using primer pairs corresponding to ten microsatellite markers (Tm16_ATA12,
381 Tm241_TAA17, Tm2_TAT15, Tm98_TAT15, Tm112_TAT19, Tm9_ATCA12,
382 Tm1_ATTG18, Tm75_GAAA14, Tm22_CCTCAT17 and Tm269_TGTTGC15) as described
383 by Murat *et al*. (2011; 2013). The genotyping was done by the INRA plateform Gentyane
384 (Clermont-Ferrand). The *T. melanosporum* mating types of all the samples were analysed
385 using specific primers for either the *MAT1-1-1* or the *MAT1-2-1* genes using the PCR
386 conditions described by Rubini *et al.* (2011b). Hereafter, according to Rubini *et al.* (2011a),
387 the mating types are termed *MAT1-1* and *MAT1-2.*
388 From the mix of DNA spores, we obtained zygotic genotypes from which the male genotype
389 was deduced by subtraction of the corresponding female genotype.

390 The soil mycelium of both mating types was quantified by quantitative real time PCR (qPCR)
391 using a protocol developed from the international patent (EP2426215 A1) in a confidential
392 research program with ALCINA sarl (Montpellier, France). qPCR reactions were carried out
393 with a StepOne Plus^{TM} Real-Time PCR System machine provided with the StepOne software
394 v. 2.3 (Life Technologies, Carlsbad, CA). Two standard curves (R^2=0.99; Eff = 96.99% and
395 R^2=0.99; Eff = 99.67% for *MAT 1-1* and *MAT 1-2,* respectively) were obtained, as described
396 in Parladé *et al.* (2013), by mixing a known amount of soil harvested in a cereal field close to
397 the truffle orchard (in which the absence of *T. melanosporum* was confirmed) with a known
398 amount of fresh immature ascocarp of *T. melanosporum* belonging to one or the other mating
399 type. DNA from the mixture was extracted as all the other soil samples and serial tenfold
400 dilutions were done to obtain a standard curve for each mating type. Absolute quantification
401 of mating types in soil samples was obtained by interpolating their threshold cycle (Ct) values
402 on the corresponding standard curve.
403
404 *Data analyses*
405 All the samples with null alleles were discarded from our analyses. The multilocus genotype
406 identification was achieved using the software MLGsim 2.0 (2003). For genotypes sampled
407 more than once, the probability of independent occurrence (Pgen) and the PSex probability of
408 arising by chance (Psex) were calculated. The P-value for testing the significance of the PSex
409 for each genotypes was estimated using 1000 simulations. When the PSex values fell below
410 the P-value (0.05), it was concluded that identical genotypes originated from the same genet
411 (clonal multiplication).
412
413 The clonal diversity (R), the Simpson's diversity index modified for finite sample sizes (D),
414 the clonal subrange, and the aggregation index (Ac) for mating types were estimated using the

RClone package (2016) for R software, based on the GenClone software package v2.0 (2007). The inter-individual diversity (He=1-Qinter) and isolation by distance (IBD) analysis were obtained with GenePop software v4.2 online (1995; 2008). The significance of IBD was tested using Mantel test with 10,000 permutations. All values were calculated for all the data and also separately for female, male, and ECM data. A culled dataset was constructed to reduce the bias due to sampling the same genotype several times. For each season, the isobarycentre was considered for samples sharing the same genotype and having significant Psex. If two samples with the same genotypes were found in different areas of the truffle orchard (i.e. areas 1, 2 or 3), they were separated. Similarly, we did not calculate the isobarycentre with samples sharing the same genotype in different years.

All the maps were obtained with a dedicated python program developed for this study. The mapping program can be downloaded using a Unix terminal and the following command: git clone https://git.igzor.net/inra/iam_mapping.git

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Tables and Figure legends

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

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 maternal mating type in the sampling square. For square without ascocarps the most frequent mating type of maternal tissue in the surrounding square is indicated (identified by an asterisk).

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

Fig. 2. Map of distribution of the hermaphrodite genotypes R021, R060 and R068 in the Rollainville orchard. Representation of the distribution of the hermaphrodite genotypes along the different seasons and in the different areas of the orchard. a. Distribution of genotype R021. b. Distribution of genotype R060. c. Distribution of genotype R068. Dots represent female ascocarp genotypes, crosses represent ectomycorrhiza genotypes and triangles represent paternal genotypes. Each season is represented by a different colour.

Fig. 3. Isolation by distance analysis (IBD) representing genetic *vs* geographic distance for female and male genotypes.

Isolation by distance analyses obtained with female (a and c) and male (b and d) genotypes. a and b were obtained using all the samples while c and d were obtained using culled data to avoid oversampling of the same genotype. The slope with 95 % interval confidence is indicated for each graph.

607 Table 1. Sampling details, genotypic and genetic diversity for female, male elements and ectomycorrhiza for five years in the Rollainville

608 orchard.

609

	2010-2011	2011-2012	2012-2013	2013-2014	2014-2015	All seasons
Number of sampled ascocarps	17	42	30	101	51	238
Female elements						
Number of samples genotyped	17	42	30	98	51	238
Number of genotypes (no. 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 genotype found in a single ascocarp	2	11	15	12	3	42
Number of persistent genotype s (found in 2; 3; 4 or 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 diversity index (D)	0.88	0.94	0.98	0.92	0.81	0.99
Inter-individual diversity (1-Qinter = He)	0.52	0.84	0.91	0.90	0.77	0.88
Aggregation index (Ac) (p-value)	0.46 (0.002)	0.57 (0)	0.28 (0)	0.54 (0)	0.43 (0)	0.46 (0)
Male elements						
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 Fis	0.02	0.18	0.28	0.18	0.15	0.21
Number genotypes (no. 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 found in 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)

Ectomycorrhiza (ECM) samples						
Number of genotyped ECM	81				36	117
Number of genotypes (no. 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 strains						16/3/3
Aggregation index (Ac) - ECM and female (p-value)	0.96 (0)				0.40 (0)	0.77 (0)
Number of hermaphrodite* genotypes (no. genotypes with Psex < 0.05)	1 (1)	2 (2)	2 (1)	6 (2)	4 (2)	8 (3)

**Hermaphrodites are genotypes found as female and male elements*

612 Table 2. Quantity of soil mycelium of both mating types in mg of mycelium/g of soil.
613 The position in the truffle orchard is indicated as well as the maternal mating type in the
614 sampling square. For square without ascocarps the most frequent mating type of
615 maternal tissue in the surrounding square is indicated (identified by an asterisk).
616

Area	**Square**	**Maternal 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

617
618

Fig. 1

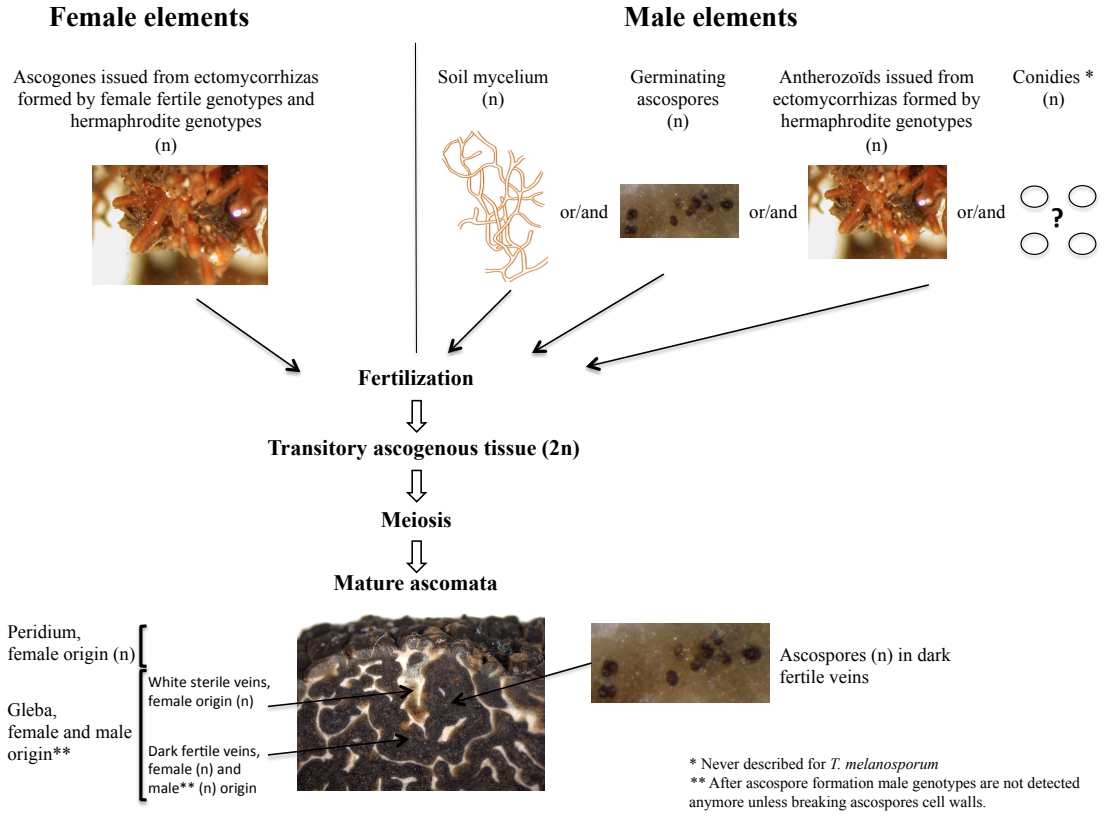

Female elements
Male elements
Ascogones issued from ectomycorrhizas formed by female fertile genotypes and hermaphrodite genotypes (n)
Soil mycelium (n)
Germinating ascospores (n)
Antherozoïds issued from ectomycorrhizas formed by hermaphrodite genotypes (n)
Conidies * (n)
or/and
or/and
or/and
?
Fertilization
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

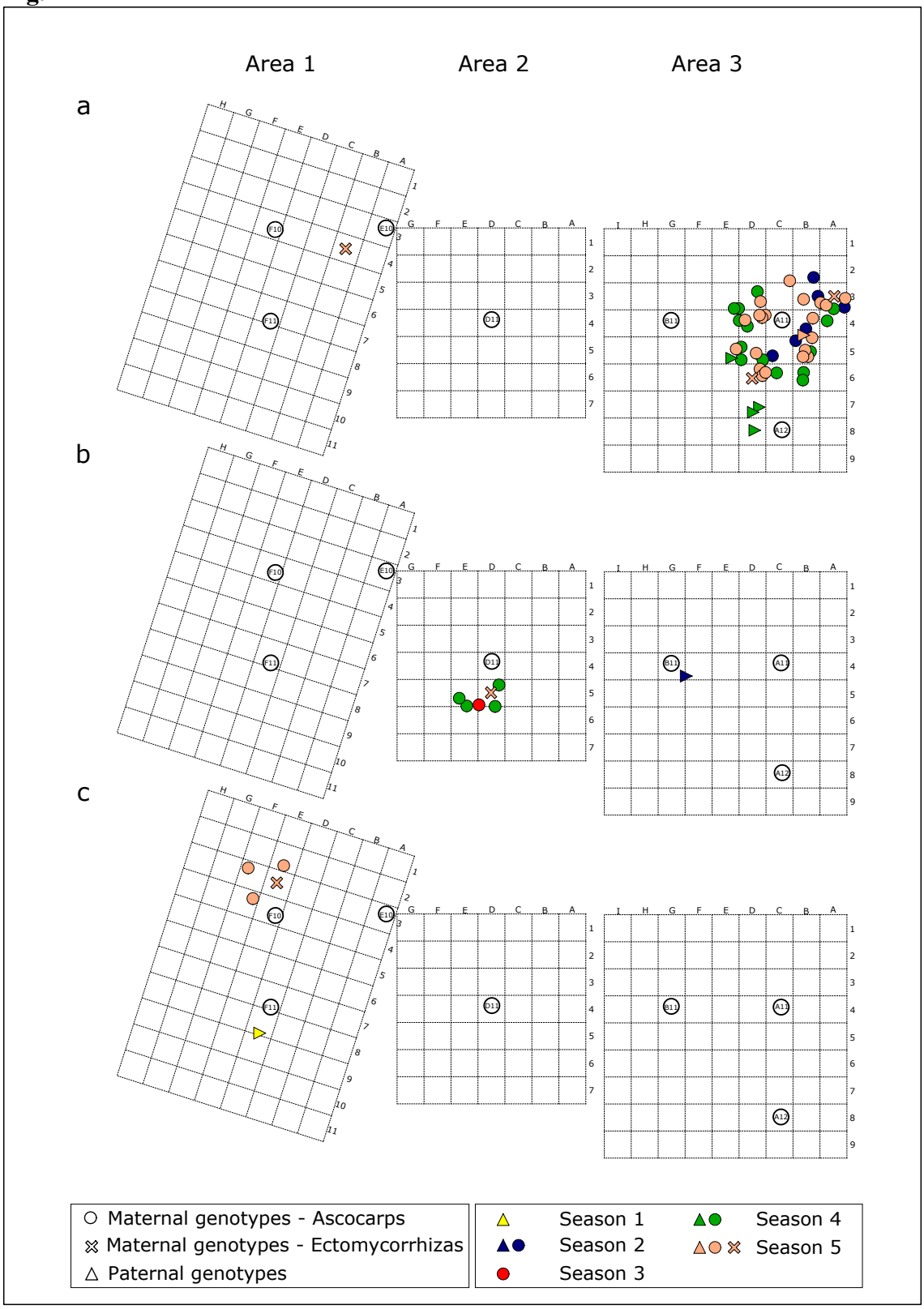
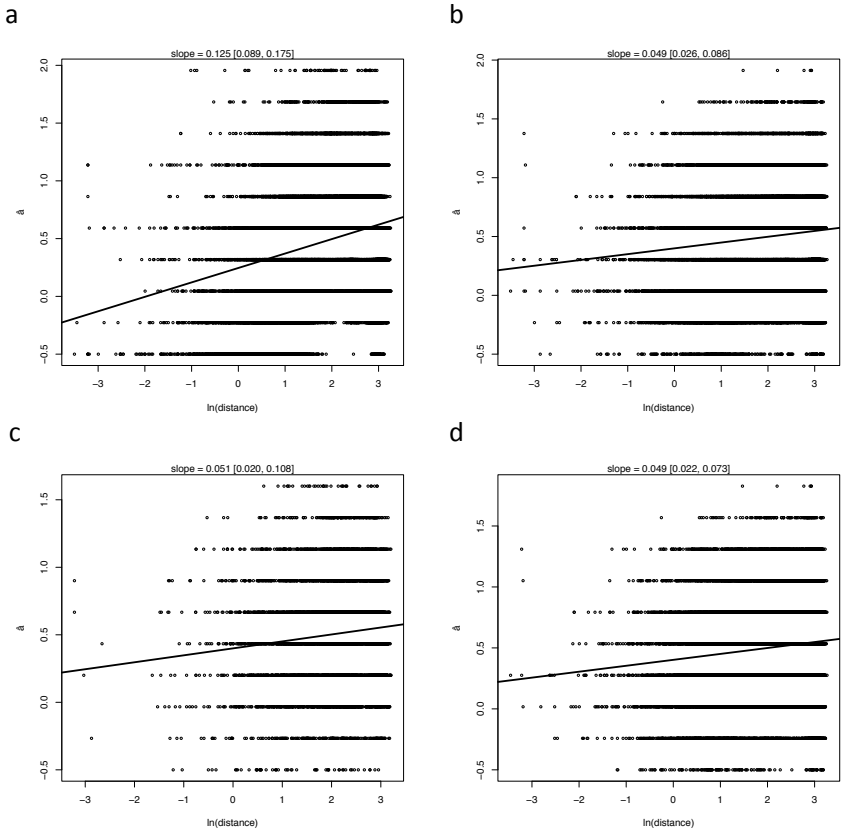

Area 1
Area 2
Area 3
a
b
c
Maternal genotypes - Ascocarps
Maternal genotypes - Ectomycorrhizas
Paternal genotypes
Season 1
Season 2
Season 3
Season 4
Season 5

Fig. 3


a
slope = 0.125 [0.089, 0.175]
b
slope = 0.049 [0.026, 0.086]
c
slope = 0.051 [0.020, 0.108]
d
slope = 0.049 [0.022, 0.073]
ln(distance)