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1 **Ascoma genotyping and mating type analyses of mycorrhizas and soil mycelia of**

2 ***Tuber borchii* in a truffle orchard established by mycelial inoculated plants**

3 **Leonardi P.[1], Murat C.[2], Puliga F.[1], Iotti M.[3], Zambonelli A.[1*]**

4

5 [1] Department of Agricultural and Food Sciences, University of Bologna, viale Fanin 44, 40127

6 Bologna, Italy

7 [2] Université de Lorraine, INRA, UMR IAM, 54000 Nancy, France

8 [3]Department of Life, Health and Environmental Science, University of L'Aquila, via Vetoio, 67100

9 Coppito, L'Aquila, Italy

10

11 **Author for correspondence: email: alessandr.zambonelli@unibo.it**

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13 Running Title: Mating strategies in a *T. borchii* orchard

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15 **Significance/originality statement**

16 The maternal and paternal genotypes in the first truffle orchard realized with plants inoculated with
17 the mycelia of the heterothallic fungus *T. borchii* were analyzed. The inoculated mycelia persisted in
18 soil 9 years after plantation and the paternal genotypes were mostly recombinants of the inoculated
19 mycelia, corresponding to their offspring. The study confirms that meiospores are the most likely
20 paternal candidate for truffle fertilization and improves our knowledge of sexual truffle reproduction.

21 **Abstract**

22 *Tuber borchii* (the Bianchetto truffle) is a heterothallic Ascomycete living in symbiotic association
23 with trees and shrubs. Maternal and paternal genotype dynamics have already been studied for the
24 black truffles *Tuber melanosporum* and *Tuber aestivum* but not yet for *T. borchii*. In this study we
25 analyzed maternal and paternal genotypes in the first truffle orchard realized with plants inoculated
26 with five different *T. borchii* mycelia. Our aims were to test the persistence of the inoculated mycelia,
27 if maternal and/or paternal genotypes correspond to inoculated mycelia and to assess the
28 hermaphroditism of *T. borchii*. The mating type of each isolate as well as those of mycorrhizas,
29 ascomata and extraradical soil mycelia was determined. Moreover, simple sequence repeat (SSR)
30 profiles of maternal and paternal genotypes were assessed in 18 fruiting bodies to investigate the
31 sexual behavior of this truffle.

32 The maternal genotypes of the fruiting bodies corresponded to those of the inoculated mycelia with
33 only two exceptions. This confirmed that the inoculated mycelia persisted 9 years after plantation. As
34 regards paternal partner, only two had the same genotype as those of the inoculated mycelia,
35 suggesting hermaphroditism. Most of the new paternal genotypes originated from a recombination of
36 those of inoculated mycelia.

37 **Key words:** mating type, SSR, truffle reproduction, meiospores, mycelial cultures

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39 **Introduction**

40 Ectomycorrhizal fungi assist plants in their growth, therefore playing key roles in forest ecosystem
41 functioning. In addition, some of them produce edible fructifications representing income
42 opportunities for farmers and foresters. True truffles are hypogeous fungi belonging to the genus
43 *Tuber,* which live in ectomycorrhizal association with a wide range of shrubs and trees (Zambonelli
44 *et al.,* 2016). The genus *Tuber* comprises around 200 species but only a few of them have a
45 considerable value, such as the European species *Tuber magnatum* Picco (Italian white truffle), *Tuber*
46 *melanosporum* Vittad. (black truffle), *Tuber aestivum* Vittad. (summer truffle) and *Tuber borchii*
47 Vittad. (bianchetto truffle) (Bonito *et al.,* 2010). In recent years, important milestones have been
48 reached, allowing a better understanding of the truffle life cycle. In 2006, Paolocci and colleagues
49 found that *T. magnatum* is heterothallic and its ectomycorrhizas are formed by primary
50 (homokaryotic) mycelia. This condition was also confirmed in *T. melanosporum* (Riccioni *et al.,*
51 2008; Rubini *et al.,* 2011a) and *T. borchii* (Belfiori *et al.,* 2016), indicating that heterothallic life style
52 is common within the *Tuber* genus and fruiting body production depends on the mating between
53 mycelia harboring different mating types. Mycelia of both mating types can act as maternal partner,
54 indicating hermaphroditism (Selosse *et al.*, 2017). The haploid maternal genotype of truffles forms
55 the sterile tissues of the fruiting body, and it is fed from the surrounding mycorhizas throughout the
56 maturation time (Rubini *et al.,* 2011a; Murat *et al.,* 2013; Le Tacon *et al.,* 2013). On the contrary, the
57 paternal genes are confined to the asci and, indeed, the paternal genotypes were rarely found to form
58 mycorrhizas (Taschen *et al.,* 2016; De la Varga *et al.,* 2017).

59 In addition to the characterization of mating type idiomorphs, the sequencing of the *T. melanosporum*
60 genome (Martin *et al.,* 2010) has allowed large-scale screening of SSR markers which are being
61 implemented on a small scale in field trials in order to unravel the interaction between maternal and
62 paternal genotypes. In the field, *T. melanosporum* displays a strong genetic isolation with strains of
63 opposite mating types spatially confined in separate patches in which a few dominant genotypes can

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64 persist throughout the years (Rubini *et al.,* 2011b; Taschen *et al.,* 2016; De la Varga *et al.,* 2017).
65 Due to the important turnover and small spatial size of the paternal genotypes, it has been suggested
66 that in most cases germinating ascospores could act as paternal partner (Selosse *et al.,* 2013; Le Tacon
67 *et al.,* 2016; Taschen *et al.,* 2016; De la Varga *et al.,* 2017; Selosse *et al.,* 2017). However, this
68 hypothesis remains to be demonstrated and the nature of the paternal genotype is still unclear.

69 *Tuber borchii* was long used as a model species within the *Tuber* genus for transcriptomic and
70 functional analyses. *Tuber borchii*, together with *T. aestivum*, is the species with the widest diffusion
71 in Europe, adapting to different climatic and soil conditions (Hall *et al.,* 2007; Zambonelli *et al.,*
72 2002; Gardin, 2005; Lancellotti *et al.,* 2016). For its gastronomic value and adaptability to different
73 environmental conditions, *T. borchii* cultivation was introduced in European Mediterranean countries
74 (Italy, Portugal and Spain) and in non-European countries (Hall *et al.,* 2017; Zambonelli *et al.,* 2015).
75 The cultivation of *T. borchii*, similarly to that of other truffles, is achieved by synthetizing
76 mycorrhizal plants in specialized nurseries by spore inoculum and planting them in suitable places
77 (Chevalier and Grente, 1978; Hall *et al.,* 2007). Mycelial inoculum was successfully tested in the
78 second half of the last century to obtain *Tuber* mycorrhizal plants (Palenzona *et al.,* 1972; Chevalier,
79 1973) but, later, it was applied only for experimental purposes due to the difficulties of obtaining
80 large quantities of mycelium for large-scale inoculation purposes (Iotti *et al.,* 2002; Giomaro *et al.,*
81 2005; Iotti *et al.,* 2012a). Also, once reliable methods for growing *T. borchii* mycelium had been
82 perfected, mycelial inoculation was no longer used because it was commonly thought that the
83 obtained plants would not have been able to produce fruiting bodies due to heterothallic sexual
84 reproduction of truffles (Zambonelli *et al.,* 2008).

85

86 Recently, the first truffle orchard realized with plants inoculated by *T. borchii* mycelia began to
87 produce (Iotti *et al.,* 2016). This truffle orchard was established in Cadriano (Bologna) inside an
88 intensive agricultural area where *T. borchii* had not been found for at least 30 years (Zambonelli and

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89 Morara, 1984; Morara *et al*., 2009). Moreover, the presence of large mycophagus mammals like wild
90 boars, which could introduce truffle spores in the orchard from far sites (Piattoni *et al.,* 2014), was
91 excluded. The plants were inoculated with five different strains singly and in mixture. Truffle
92 production was first assessed in February 2016, 9 years after planting. We hypothesized that the
93 strains used for inoculation had different mating types and that fertilization had occurred between
94 them (Iotti *et al.,* 2016). This plantation represents a unique opportunity to investigate *T. borchii*
95 sexual reproduction.

96 The aims of this study were to address the following questions: 1) were mycelium genotypes able to
97 be perennial for 9 years in the root system? 2) did maternal genotypes correspond to mycelium used
98 for plant inoculation? 3) did paternal genotypes correspond to offspring of original genotypes or was
99 hermaphroditism more frequent in *T. borchii* than *T. melanosporum*? In order to address these
100 questions, polymorphic simple sequence repeat (SSR) markers were identified in the *T. borchii*
101 genome (Murat *et al.,* 2018). These SSRs were applied to genotype the inoculated strains used for
102 plant colonization, as well as maternal and paternal genotypes of harvested ascomata. Mating type
103 distribution in the orchard was also determined for inoculated mycelia, mycorrhizas, ascomata and
104 soil samples.

105 **Results**

106 *SSR characterization*

107 More than one thousand SSRs (1,111 in total) were found in the *T. borchii* genome. Among the 31
108 SSR primer pairs selected *in silico* analysis, 14 were used in subsequent analyses since produced a
109 single band per sample and showed polymorphisms. They were used to analyze the polymorphism of
110 50 ascomata harvested in Italy and Hungary as well as the samples from the Cadriano truffle orchards.
111 MLGsim analysis showed that all the 50 ascomata from Italy and Hungary were different from each
112 other, resulting in 50 multilocus genotypes (MLGs) (genotypic diversity = 1; Supplementary Table
113 S1). The number of alleles ranged from 3 to 10 and the He from 0.208 to 0.739 (Table 1 and

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114 Supplementary Table S1). Most of the SSRs are localized in intergenic regions with the exception of
115 Tb151, Tb293, and Tb46 that are localized in gene models.
116 Table 1
117 *Genotyping of the inoculated mycelia*
118 The SSRs and mating type analyses made it possible to genotype the five *T. borchii* strains used to
119 inoculate truffle seedlings. The strain 2364 was assigned to C-MLG_13, the strain Tb98 to C-MLG_8,
120 the strain 1Bo to C-MLG_6, the strain 2292 to C-MLG_18 and the strain 2352 to the C-MLG_14.
121 Two strains (2292 and 1Bo) have the MAT 1-2-1 gene coding for the HMG-domain protein and the
122 other three strains (Tb98, 2352 and 2364) have the MAT 1-1-1 gene coding for the alpha-domain
123 protein (Table 2). Although the arrangement of the groups of plants inoculated with the same strain(s)
124 in four rows along the plantation was casual at the time of plantation, the distribution of the two
125 mating types proved to be spatially alternated across the truffle ground (Fig. 1).
126 Figs 1 and 2
127 *Genotyping of the maternal tissue (gleba) and mating characterization of ECM and soil mycelium*
128 The maternal tissue of 18 ascomata out of 33 collected in the truffle orchard was successfully
129 genotyped using both SSR and mating type primers (Table 2 and 3). At least two ascomata for each
130 plant group inoculated with the same strain were genotyped. The other 15 ascomata were only mating
131 typed and not considered for SSR analyses because the respective paternal genotypes failed to be
132 characterized (Table 2).
133 Table 2 and 3
134 Amplifications of the maternal tissue with both mating type and SSR primers gave single and specific
135 amplicons. The 18 ascomata were grouped into 6 different genotypes (C-MLGs): 4 with MAT 1-1-1
136 (C-MLG_8, C-MLG_13, C-MLG_10, C-MLG_14) and 2 with MAT 1-2-1 (C-MLG_3, C-MLG_18)
137 (Table 2 and Supplementary Table S2). Most of the analyzed ascomata (15/18) have the same C-
138 MLG of the strains used to inoculate the plants where they were collected (C-MLG_8, C-MLG_13,
139 C-MLG_18, C-MLG_14). Three female genotypes (C-MLG_8, C-MLG_13, C-MLG_14) showed a

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140 significant P sex value that supports the hypothesis that the ascomata of each of these genotypes are
141 part of the same clone (Supplementary Table S3). One ascoma (4658), found in the group of plants
142 inoculated with the strain 1Bo (C-MLG_6) had the same genotype (C-MLG_14) of the neighboring
143 strain 2352 (about 5 m far) (Figs. 1 and 2). Only two ascomata (4601, 4633) had female genotypes
144 (C-MLG_3 and C-MLG_10) different from those used to inoculate the plants (Table 2). In particular,
145 C-MLG_10 showed a SSR profile which differs from that of the inoculated strain 2364 (C-MLG_13)
146 for the presence at locus Tb1 of a different allele which is present in the strains Tb98 and 2292. On
147 the contrary, C-MLG_3 had two loci (Tb151 and Tb46) with alleles not present in any inoculated
148 strain (Supplementary Table S1). The genotype C-MLG_6 corresponding to the inoculated strain 1Bo
149 was never found as maternal genotype in the truffle ground.
150 The ascomata found in the plants inoculated with a mixture of strains showed the maternal genotypes
151 C-MLG_8 and C-MLG_14, corresponding to the strains Tb98 and 2352, respectively.
152 The persistence of the inoculated strains in the areas where the plants were originally planted was
153 also confirmed by mating type analyses of the other 15 ascomata. All these ascomata had the same
154 mating type as the strain used to inoculate the plants where they were collected (Table 2). Nine
155 samples were identified as MAT 1-1-1 and 6 as MAT 1-2-1. In the case of plants inoculated with the
156 mixture of strains, PCRs revealed the presence of both mating types although spatially isolated in
157 different plants: MAT 1-2-1 under the plants 15 and 17 (ascomata 4639 and 4660) and MAT 1-1-1
158 under the plants 51, 52 (ascomata 4640, 4657, 4664) and 13 (ascoma 4675) (Fig. 1, Table 2).
159 A total of 23 root samples taken under 23 of the collected ascomata were processed to characterize
160 mating types of ectomycorrhizas (ECMs) (Fig. 1, Table 2). Molecular analyses with *T. borchii*
161 species-specific primers confirmed the identity of the ECMs morphotyped in each root sample. In
162 total, 115 ECMs identified as *T. borchii* (5 for each collection point) were analyzed.
163 Remarkably, each ECM amplified a single and specific mating type amplicon. All ECMs had the
164 same mating type as the inoculated strain and as the ascoma under which they were collected (Table
165 2).

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166 Soil analysis also confirmed the persistence of the inoculated mating types. In almost all soil samples,
167 the mating type corresponded to that of the inoculated strain and of the ascoma maternal tissue and
168 ECMs collected in the same position, with only two exceptions: soils collected under ascomata 4677
169 and 4642. They were found in the same group of plants where the genotypes of the two ascomata
170 (4601 and 4633) differed from the one used to inoculate the plants (Table 2, Fig. 2).
171
172 *Genotyping of paternal tissue (spores)*
173 The SSRs analysis performed on the spores was successful only for 18 of the 33 analyzed ascomata.
174 Sixteen paternal genotypes were found on the 18 ascomata characterized by SSRs (Table 2 and 4).
175 The percentage of success of spore DNA extraction was only 55% probably due to the difficulties in
176 breaking *T. borchii* spores or the too low quantity of extracted DNA.
177 Table 4
178 In zygotes, the F_{IS} was 0.058 with a p-value of 0.11, indicating a non-significant departure from 0;
179 consequently, even if the F_{IS} is positive there is no heterozygote deficit.
180 Most of the paternal C-MLGs were different from the female C-MLGs and were characterized by the
181 recombination of the alleles of the inoculated strains (Fig. 3). Four paternal MLGs (C-MLG_1; C-
182 MLG_2; C-MLG_4, C-MLG 16, ascomata 4600, 4652-4599, 4651 and 4635 respectively) showed
183 new alleles (loci Tb151 and/or Tb46) never detected in the inoculated mycelia, although they were
184 detected in the maternal tissue of ascoma 4601 (Supplementary Table S1). Two paternal C-MLGs
185 had new alleles not present in any maternal genotype in the loci Tb17 (C-MLG_9) and Tb293 (C-
186 MLG_9 and C-MLG_16) (Fig. 3, Supplementary Table S2). Only two MLGs (C-MLG_14 and C-
187 MLG_18) were detected as both maternal and paternal genotypes but only one (C-MLG_14)
188 according to the P-Sex value can be considered as a true hermaphroditic strain (Table 4 and
189 Supplementary Table S3). This hermaphroditic C-MLG_14 was found as male and female in adjacent
190 areas of the experimental field (Fig. 2). Only one C-MLG found only as male (C-MLG_2) for two

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191 samples was supported by a significant P sex value, indicating that this genotype is a clone
192 (Supplementary Table S3).
193 Fig. 3
194 **Discussion**
195 In this study, truffles harvested in a *T. borchii* orchard were investigated by using mating type and
196 SSR markers for the first time. Mating type primers were recently designed and tested only on *T.*
197 *borchii* pure cultures and not applied to environmental samples (Belfiori *et al*., 2016). On the other
198 hand, SSR analyses were carried out on other truffle species (*T. aestivum* and *T. melanosporum*) but
199 never on *T. borchii* (Rubini *et al*., 2005; Riccioni *et al*., 2008; Murat *et al.,* 2013; Molinier *et al.,*
200 2015, 2016a, 2016b; Taschen, *et al.,* 2016; De la Varga *et al.,* 2017; Schneider-Maunory *et al.*, 2018).
201 In this study, *T. borchii*-specific SSR loci were selected, tested for their polymorphisms on 50
202 ascomata of different origin and used to assess the genetic structure of *T. borchii* population in the
203 studied truffle orchard. The level of polymorphism of the developed SSR is highly variable (He
204 ranges from 0.208 to 0.739) as already observed for other *Tuber* spp. (Murat *et al.,* 2011; Molinier *et*
205 *al.,* 2013).

206 Most of the studies on the genetic structure of truffle population have been conducted in human-made
207 or natural truffle grounds where the identity of the strains colonizing roots was not known (Murat *et*
208 *al.,* 2013; Taschen, *et al.,* 2016; Molinier *et al.,* 2016b; De la Varga *et al.,* 2017). On the contrary, the
209 *T. borchii* plantation under investigation was established with plants inoculated with five different
210 mycelial strains without knowing their mating type. As supposed by Iotti *et al.* (2016), the inoculated
211 strains were found to belong to different mating types which is the necessary condition for fruiting in
212 a heterotallic fungus like *T. borchii* (Belfiori *et al.,* 2016).

213

214 *Clonal structure suggests the persistence of the inoculated strains*

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215 Ascoma SSR analysis demonstrated that the maternal C-MLGs of the fruiting bodies was the same
216 as the corresponding inoculating strains, as also supported by the significant Psex value of three
217 female genotypes corresponding to the inoculated strains. Several works demonstrated that in *Tuber*
218 species the maternal tissue of ascomata and surrounding ECMs have the same genotype (Rubini *et*
219 *al.*, 2011b, Murat *et al.,* 2013; Molinier *et al.,* 2016b; Taschen et al. 2016; De la Varga *et al.,* 2017).
220 Moreover, a physical and nutritional link between these fungal structures has been proved (Le Tacon
221 *et al.*, 2013; Deveau *et al.*, 2019). For these reasons, our results suggest the persistence of the
222 inoculated mycelia 9 years after plantation on the root systems. A similar result was already found
223 for seedlings inoculated with the basidiomycete *Laccaria bicolor* for which the introduced strain was
224 still present in the inoculated plots 10 years after plantation (Selosse *et al.,* 1998).

225 The maternal genotypes of ascomata 4601 and 4633 (C-MLG_3 and C-MLG_10, respectively) found
226 in adjacent groups of plants at the southern edge of the truffle orchard differed from those of the
227 inoculated mycelia. Ascomata 4601 had two alleles not present in the inoculated strains (loci Tb151
228 and Tb46) and this suggests an introgression of a foreign genotype on the host roots inoculated with
229 1Bo. Ascoma 4633 showed a different combination of the alleles found in the inoculated strains and
230 thus could originate from ECMs colonized by a germinating spore corresponding to an offspring of
231 inoculated mycelia.

232 Mating type of ECMs and almost all ascomata and soil mycelia was the same as the inoculated strain,
233 confirming the results obtained by SSR analyses. The only exception was found in the group of plants
234 inoculated with the strain 1Bo where a different mating type (MAT 1-1-1) was found in the soil (Fig.
235 2). Since the strain 2352 located in the adjacent group of plants has MAT 1-1-1, its mycelium was
236 probably able to migrate towards the plants inoculated with strain 1Bo. This migration could also
237 have been favored by the death of one uninoculated hazel separating the group of plants inoculated
238 with the strains 1Bo and 2352. Moreover, the strain 1Bo did probably not establish itself in the truffle
239 ground because of the low level of root colonization at the planting time (data not shown). Indeed

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240 1Bo genotype was not found as maternal genotype in these or other plants and its specific alleles of
241 the loci Tb155 and Tb46 were also never found in any paternal genotype. The 1Bo mycelium was, in
242 fact, isolated in 1997 and repeated subcultures are known to affect the viability and infectivity of a
243 *Tuber* strain (Piattoni *et al.,* 2017). Both the adjacent strain 2352 and the foreign genotype C-MLG_3
244 found as female in the ascomata 4658 and 4601, respectively, could have taken advantage of the
245 scarce colonization of strain 1Bo (Fig. 2). However, we cannot exclude the possibility that the
246 mycelium and ECMs of strain 1Bo were still present in the soil and the production of the ascoma of
247 this strain could have occurred.

248

249 The plants inoculated with the mixture of strains showed that MAT 1-1-1 and MAT 1-2-1 dominated
250 in separate areas either considering ECMs, soil mycelium or maternal tissue of ascomata. In order to
251 better understand the dynamics of the mating types, we analyzed another 29 ascomata, and the
252 respective ECMs and soils collected in the following year (February-March 2017). These additional
253 analyses confirmed that, the ascomata, mycorrhizas and soil mycelium had the same mating type of
254 the inoculated strain (Table and Fig. S5). As in 2016, the only exceptions were the two sample sets
255 collected under plants inoculated with the strain 1Bo. Fourteen of these 29 ascomata were collected
256 under the plants inoculated with the mixture of strains and only MAT 1-1-1 was found in these new
257 samples (Table and Fig. S5). This result confirms that also in *T. borchii* a single mating type tends to
258 dominate in a single soil patch, as found for *T. melanosporum* and *T. aestivum* in natural and
259 cultivated truffle ground, and confirms that in field conditions only one strain or multiple strains of
260 the same mating type tend to colonize one single plant (Rubini *et al.,* 2011b; Linde and Selmes, 2012;
261 Zampieri *et al.*, 2012; Murat et al., 2013; Rubini *et al.,* 2014; Molinier *et al.,* 2016b; De la Varga *et*
262 *al.*, 2017). This can be explained by considering that a vegetative incompatibility (VI) system exists
263 in *T. borchii* which prevents hyphal interactions between hyphae of different strains (Sbrana *et al.,*
264 2007). Although the sets of genes which regulated VI in filamentous ascomycetes was not found in

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265 *T. melanosporum* genome (Iotti *et al.,* 2012b), other molecular mechanisms controlling self/nonself
266 recognition are involved in segregation between strains of different mating types in the field (Rubini
267 *et al.,* 2011b; Selosse *et al.,* 2013) and prevent hyphal fusion in axenic conditions (Iotti *et al.,* 2016).
268

269 *Meiospores are the most important origin of male genotype*

270 Most of the male C-MLGs are new genotypes and seem to be originated from spores, confirming the
271 hypothesis made for *T. melanosporum* that the locally dispersed spores are the major source of male
272 genotypes (Selosse *et al*., 2013; Taschen *et al*., 2016; Le Tacon *et al*., 2016; De la Varga *et al*., 2017).
273 In fact, the male C-MLGs showed an allele recombination of the alleles present in the maternal
274 genotypes which are mostly (5 out of 6) represented by the inoculated strains. The numbers of
275 generations to account for the observed diversity of paternal recombinant genotypes should be at least
276 two. In fact, for example, C-MLG 15 could derive from a first mating between C-MLG_8 and C-
277 MLG_18 and then the mycelium/conidia originated from the spores of the formed ascoma could have
278 fertilized strain 2352 or 2364. Thus the ascoma production started before our first survey with trained
279 dogs in the truffle ground (Iotti *et al*., 2016). Only three male genotypes presented new alleles and
280 perhaps we could suppose that they originated from spores coming from other sites. Although it is
281 not possible to exclude the arrival of spores from the natural *T. borchii* grounds which are located
282 several kilometers away (Iotti *et al.,* 2016), likely they could also have come from cultivated *T.*
283 *borchii* orchards in the area. *Tuber borchii* cultivation has become very popular in Italy in the last
284 few years after the first results obtained by spore inoculation (Zambonelli *et al.,* 2000).

285 The role of spores in fertilization could explain the increase in truffle production obtained by
286 inoculating spores in the field (Murat *et al.,* 2016) and the decrease in production attributed to ascoma
287 overharvesting in natural truffle grounds. Moreover, it stresses the importance of animals in spore
288 dispersal (Piattoni *et al*., 2014; Zambonelli et al., 2017; Ori *et al*., 2018) not only to promote truffle
289 colonization of new areas but also to favor truffle fertilization in non-productive plants.

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291 Only one strain (strain 2352; MAT 1-1-1; C-MLG_14) displayed actual evidence for
292 hermaphroditism. This strain was found as male in one ascoma (4601) collected in the adjacent plants
293 inoculated with another strain (1Bo, MAT 1-2-1). This ascoma was located under the same group of
294 plants where both mating types were found in the soil and the C-MLG_14 was also found as female
295 in one ascoma (4658). Although not statistically supported, another C-MLG (C-MLG_18) was found
296 in adjacent groups of plants as male and female, supporting the possibility that fertilization can occur
297 also between mycelia. These results suggest that hermaphroditism is not frequent in *T. borchii,* as
298 already reported for *T. melanosporum* (De la Varga *et al.,* 2017).

299

300 *Consideration on the truffle life cycle*

301 Although this study gives new important insights into truffle biology, it was not able to completely
302 resolve the mystery of the reproduction strategy in truffles. How are the ascospores able to fertilize
303 the mycelium of different mating types? We can suppose that the ascospores germinate and originate
304 a mycelium that, directly or by conidia formation, fertilizes other compatible mycelia in the soil or
305 on the roots. As suggested for *T. melanosporum,* competition and/or vegetative incompatibility events
306 can prevent the growth of this new mycelium in the root system extensively colonized with the
307 mycelium of the opposite mating type (De la Varga *et al*., 2017). However, when host roots are not
308 colonized by other truffle strains, the new genotype could be able to establish on them and function
309 as a new female (maternal) genotype. *T. melanosporum* and *T. magnatum* presented a significant
310 heterozygote deficit with high levels of inbreeding (Paolocci *et al*., 2006; Riccioni *et al*., 2008;
311 Taschen *et al*. 2016; De la Varga *et al*., 2017). In *T. borchii* we did not find such inbreeding since Fis
312 was not significantly different from 0, suggesting no departure from panmixia in the Cadriano
313 population. This means that all opposite genotypes have the same probability to breed. Could it be
314 explained by the possibility of *T. borchii* to form conidia? Indeed, the conidia formation in *T.*

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315 *melanosporum* and *T. magnatum* was never observed in contrast to *T. borchii* and other species in the
316 Puberulum clade (Urban *et al.*, 2004; Healy *et al.*, 2012; Ian Hall personal communication).
317 Additional studies will be necessary to clarify these aspects of truffle life cycle, focusing on field
318 experiments to detect conidia in the Cadriano population as well as in the laboratory to induce conidia
319 production and ascospore germination.

320

321 **Experimental Procedures**

322 ***Plantation and ascoma sampling***

323 The study was carried out in an experimental truffle orchard in Cadriano (Bologna, Italy) established
324 in autumn 2007-2008, planting seedlings of *Pinus pinea* L., *Quercus pubescens* Willd., *Quercus*
325 *robur* L. and *Corylus avellana* L. The seedlings were inoculated with 5 different *T. borchii* pure
326 cultures (strains Tb98, 2352, 2292, 1Bo, 2364) separately and together as described by Iotti *et al.*
327 (2016). Seedlings inoculated with the same strain(s) were grouped along four rows and separated by
328 non-inoculated guard plants of *C. avellana* (Fig. 1). Ascoma production was firstly verified in 2016
329 when 99 ascomata (total weight 722.2 g) were collected through February and March (Iotti *et al.,*
330 2016). Each ascoma was weighed and fragments were either freeze-dried at -65 °C for three days in
331 a Virtis Benchtop 2 K lyophilizer (SP Industries) and then stored at -20 °C (gleba) or fixed in FAA
332 (gleba and peridium) for molecular and morphological analyses, respectively. The remaining portion
333 of each ascoma was dried and deposited in the Mycological Herbarium of Hypogeous Fungi of the
334 Bologna University (CMI-UNIBO). Fruiting position and metadata of 33 truffles processed in this
335 study are reported in Fig. 1 and Table 3.

336

337 ***Mycelial strains***

338 The 5 strains of *T. borchii* used for the seedling inoculation were preserved at 4 °C in 15 ml tubes

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339 containing 6 ml of Potato Dextrose Agar half strength (hsPDA) (Difco) in the culture collection of
340 the Mycological Center of Bologna University (CMI-UNIBO). The cultures were renewed every year
341 on fresh hsPDA.

342 For their genetic characterization, the cultures were transferred on fresh modified woody plant
343 medium (mWPM) (Iotti *et al.,* 2005) without agar addition and incubated in the dark at 23 ± 1 °C for
344 60 days.

345

346 ***Soil and root sampling***

347 Soil and ECMs were sampled under 23 ascomata collected in February 2016 (Table 2) during truffle
348 surveys. A 20-cm-long soil core was taken under each ascoma by using a 6-cm-diameter soil corer.
349 ECMs were carefully separated from soil and washed in sterile water while any root fragment, stone
350 or organic debris was removed under a stereomicroscope (× 12) from the remaining soil.

351 ECMs were examined under a stereomicroscope (× 40) and those of *T. borchii* were identified based
352 on their morphological features (Zambonelli *et al.,* 1993). *Tuber borchii* ECMs were vortexed in a
353 1.5 ml tube for 30 s, spun for 2 min at 17,000 g to remove soil particles from the mantle and then
354 stored in sterile water at -80 °C pending further molecular characterization.

355 Soil samples were freeze-dried at - 65 °C for three days and then pulverized and homogenized by
356 mortar and pestle. Three 15 ml tubes containing 5 g of soil were prepared for each sample and then
357 stored at - 20°C until DNA extraction.

358

359 ***Molecular assays***

360 *DNA extraction*

361 The complexity of the genetic analyses carried out in this study and, in particular, the necessity to
362 differentially target the maternal and paternal tissues of ascomata have implicated the selection of

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363 different strategies to isolate and/or amplify DNAs.

364 A direct PCR strategy avoiding DNA isolation (Iotti and Zambonelli, 2006) was applied to confirm
365 the identity of ECMs and to characterize the mating type of mycelia, ascomata and ECMs.

366 Soil DNA was extracted using the CTAB-based protocol described by Iotti *et al.* (2012c) adapted for
367 1 g of soil. Crude DNA solutions were then purified using the Nucleospin Plant II kit (Macherey-
368 Nagel, Düren, Germany) following the manufacturer's instructions. Total DNAs were quantified by
369 a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) and their quality evaluated with
370 optical density (OD) 260/280 nm and 260/230.

371 DNA extraction for analyses of SSR polymorphisms was performed using three different protocols:
372 1) DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) was used to isolate DNA from 50 mg of
373 lyophilized mycelia by applying the manufacturer's instructions; 2) Nucleospin Plant II kit
374 (Macherey-Nagel, Düren, Germany) was used to isolate DNA from 50 mg of frozen gleba
375 (corresponding to the female tissue) by applying the manufacturer's instructions; 3) spore DNA was
376 isolated by placing thin slices of lyophilized gleba in 1 ml of sterile water within a petri plate; after
377 precipitation from the floating gleba slice to the bottom of the plate, about 200 spores were transferred
378 in a 1.5 ml tube and their DNA isolated according to De la Varga *et al.* (2017). DNA extraction from
379 each spore sample was repeated up to three times if the amplification of paternal genotypes failed.

380 DNA extracts were stored at -20°C until processed.

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381 Tuber borchii *identification*

382 The species-specific primer pair TboI-TboII (Amicucci *et al.,* 1998) were used to confirm the identity
383 of the morphotyped *T. borchii* ECMs by direct PCR and to detect the presence of *T. borchii* extra-
384 radical mycelium in DNA soil extracts. For PCRs we used 1 µl of a 1:10 dilution of DNA (10–50 ng
385 DNA) in a reaction volume of 10 µl. The REDTaq DNA polymerase and REDTaq PCR reaction
386 buffer (1.1 mM $MgCl_2$ final concentration) were used according to the protocol of SIGMA, with 200

387 μM of each dNTP, 0.2 μM of each primer, and 75 μg BSA (only for ECMs). PCR reactions were
388 performed in a BioRad thermalcycler with the following conditions: 6 min at 94 °C followed by 34
389 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and
390 a final extension at 72 °C for 5 min. PCR products were run on 2% agarose gel and visualized by
391 staining with ethidium bromide.

392

393 *Mating type identification*

394 Mating type identification of pure cultures, ascomata, ECMs (five from each soil sample), and extra-
395 radical soil mycelium was performed by applying a multiplex PCR, using both the specific primer
396 pairs B1-B3 and B23-B33 (Belfiori *et al.,* 2016) designed to identify the *T. borchii* MAT 1-1-1 and
397 MAT 1-2-1 genes, respectively.

398 Multiplex PCRs of mating type genes were performed in a total volume of 25 μl consisting of 1× Ex-
399 Taq Buffer (TaKaRa), 400 nM for each dNTP, 40 μg of bovine serum albumin, 400 nM for each
400 primer, 0.75 U of Ex Taq® DNA polymerase (TaKaRa). Few aerial hyphae, or small portions of
401 gleba (sterile veins) and ECM mantle were transferred directly to the PCR tubes in place of the
402 extracted DNA. The direct PCRs were performed with the following conditions: 6 min at 94 °C
403 followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72
404 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were run on 2% agarose gel. The
405 same conditions were applied to identify the mating type of extra-radical soil mycelium by adding 30
406 ng of soil DNA extracts to the PCR mixtures and avoiding BSA addition.

407

408 *Selection of polymorphic SSR primers*

409 MISA program (http://pgrc. Ipk gatersleben.de/misa/download/misa.pl) was used to search for SSR
410 markers in the recently released *T. borchii* genome (Murat *et al.,* 2018). Tri-, tetra-, penta-, and
411 hexanucleotide satellites with at least 6 repetitions and excluding composite SSRs were selected as

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412 search parameter. Subsequently, primer pairs were designed based on the sequences flanking the
413 selected SSRs using Primer 3 software (Rozen and Skaletsky, 2000). The functionality of the
414 designed primers was tested using AmplifX software (http://crn2m.univ-mrs.fr/pub/amplifx-dist).
415 After *in silico* analyses, a total of 31 primer pairs were designed and tested in PCR and visualized in
416 4% agarose gel. Among then 14 were retained since they produced only one band per sample and
417 showed polymorphisms. They were used to search for polymorphisms on 50 ascomata from Italy and
418 Hungary (Supplementary Tables S1 and S4). The genotyping was realized in the Gentyane platform
419 (INRA, Clermont-Ferrand, France). The mating type of these ascomata was also determined as
420 described above.

421

422 *SSR identification*

423 The extracted DNA from mycelia, gleba and spores was amplified using a set of primer pairs
424 amplifying the 14 polymorphic SSR loci (Table 1 and Supplementary Table S4). The amplification
425 of maternal genotypes (gleba) was carried out only for the ascomata on which the amplification of
426 paternal genotypes (spores) was successful.

427 PCRs were performed in a total volume of 10 µl consisting of 1× REDTaq Buffer (1.1 mM $MgCl_2$
428 final concentration), 200 µM for each dNTP, 0.2 µM of each primer and 1U DNA polymerase (RED
429 Taq Sigma-Aldrich) and 1 µl template DNA diluted 10 times (10–50 ng DNA).

430 The PCR reactions were performed with the following conditions: 4 min at 94 °C followed by 34
431 cycles of denaturation at 94 °C for 30 s, annealing at 55-60-65°C for 30 s depending on the primer,
432 extension at 72 °C for 30s, and a final extension at 72 °C for 5 min. PCR products were run on 4%
433 agarose gel.

434 Genomic DNA was analyzed using an ABI 3730XL sequencer (Applied Biosystems, Foster City,
435 California, USA) from "Plateforme de Génotypage GENTYANE" (Clermont-Ferrand, France). The
436 size of the alleles was analyzed with the Peak scanner software v.1.0.

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437 SSR analyses were carried out only on ascomata because, when the genome sequencing of *T. borchii*
438 (Murat *et al.,* 2018) made possible to select the SSR markers, the mycorrhizas were already analyzed
439 for mating type characterization by using direct PCR and successively disrupted (see above).

440 ***Data analyses***

441 Gene diversity or expected heterozygosity (He) was calculated using GenAlex v6.51.b2 (Peakall and
442 Smouse, 2006) in Excel 2011. The He is a measure of the probability that two copies of the same
443 gene chosen randomly in a population will have different alleles (Nei, 1973). This estimator is based
444 on the allele frequency and can be calculated as: $He = 1\sum Rpi^2$. The genotypic diversity was calculated
445 using Multilocus 1.3 (Agapow and Burt, 2001). It corresponds to the probability that two individuals
446 taken at random have different genotypes. In other words, this value is 0 if every individual has the
447 same genotype, and 1 if every individual has a different genotype.

448 The fixation index F_{IS} was calculated for zygote (maternal+paternal genotypes) in the Cadriano
449 population using GenAlex v6.51.b2. In this analysis only the nine polymorphic SSR in this population
450 were used. The significant departure from $F_{IS} = 0$ was estimated using the R script developed by
451 Taschen *et al*. (2016); script available at http://dx.doi.org/10.5061/dryad.vm11r.

452 MLG analysis of the inoculated mycelia, and the maternal (gleba) and paternal (spores) ascoma
453 tissues harvested in Cadriano was realized using the 14 polymorphic SSR markers combined with the
454 mating type gene. MLGsim 2.0 (Stenberg *et al.,* 2003) was used for MLG identification and the
455 calculation of the likelihood (PSex) that copies of MLGs result from sexual reproduction or clonal
456 spread. The threshold value (< 0.05) for testing the significance of the PSex for each genotype was
457 estimated using 1000 simulations. When the PSex values fell below the threshold value, it was
458 concluded that identical genotypes originated from clonal multiplication.

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467 **References**

468 Agapow, P.M., and Burt A. (2001) Indices of multilocus linkage disequilibrium. Mol Ecol
469 Notes 1:101–102.

470 Amicucci, A., Zambonelli, A., Giomaro, G., Potenza, L., and Stocchi V. (1998) Identification
471 of ectomycorrhizal fungi of the genus *Tuber* by species-specific ITS primers. Mol Ecol 7: 273-277.

472 Belfiori, B., Riccioni, C., Paolocci, F., and Rubini, A. (2016) Characterization of the
473 reproductive mode and life cycle of the whitish truffle *Tuber borchii*. Mycorrhiza 26: 515–527.

474 Bonito, G.M., Gryganskyi, A.P., Trappe, J.M., and Vilgalys, R. (2010) A global meta-analysis
475 of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Mol
476 Ecol 19: 4994-5008.

477 Chevalier, G. (1973) Synthese axenique des mycorhizes de *Tuber brumale* Vitt. a partir de
478 cultures puresdu champignon. Ann Phytopathol 5: 163-182.

479 Chevalier, G., and Grente, J. (1978) Application practique de la symbiose ectomycorrhizienne:
480 production a grande echelle de plants mycorrhizes par la truffe (*Tuber melanosporum* Vitt.).
481 Mushroom Science 10: 483– 505.

482 De la Varga, H., Le Tacon, F., Lagoguet, M., Todesco, F., Varga, T., Miquel, I., *et al*. (2017)
483 Five years investigation of female and male genotypes in Périgord black truffle (*Tuber melanosporum*
484 Vittad.) revealed contrasted reproduction strategies. Environ Microbiol 19: 2604-2615.

For Peer Review Only

485 Deveau, A., Clowez, P., Petit, F., Maurice, J.-P., Todesco, F., Murat, C., *et al.* (2019). New
486 insights into black truffle biology: discovery of the potential connecting structure between a *Tuber*
487 *aestivum* ascocarp and its host root. Mycorrhiza 29: 219–226.

488 Gardin, L. (2005) I tartufi minori in Toscana. Gli ambienti di crescita dei tartufi marzuolo e
489 scorzone. Firenze: Quaderno ARSIA [WWW document] URL
490 http://www.provincia.pisa.it/uploads/2012_08_28_17_46_12.pdf

491 Giomaro, G., Sisti, D., and Zambonelli, A. (2005) Cultivation of edible ectomycorrhizal fungi
492 by in vitro mycorrhizal synthesis. In In vitro Culture of Mycorrhizas. Declerck, S., Strullu, D.G., and
493 Fortin, J.A. (eds). Soil Biology series, vol.4. Berlin Heidelberg: Springer-Verlag, pp. 253–267.

494 Hall, I., Brown, G., and Zambonelli, A. (2007) Taming the Truffle. The History, Lore, and
495 Science of the Ultimate Mushroom. Portland: Timber Press.

496 Hall, I., Fitzpatrick, N., Miros, P., and Zambonelli, A. (2017) Counter-season cultivation of
497 truffles in the Southern Hemisphere: an update. Italian Journal of Mycology 46: 21-36.

498 Healy, R.A., Smith, M.E., Bonito, G.M., Pfister, D.H., Ge Z.W., Guevara, G.G., *et al.,* (2012)
499 High diversity and widespread occurrence of mitotic spore mats in ectomycorrhizal Pezizales. Mol
500 Ecol 22: 1717–1732.

501 Iotti, M., Amicucci, A., Stocchi, V., Zambonelli, A. (2002) Morphological and molecular
502 characterization of mycelia of some *Tuber* species in pure culture. New Phytol 155: 499-505.

503 Iotti, M., Barbieri, E., Stocchi, V., and Zambonelli, A. (2005) Morphological and molecular
504 characterization of mycelia of ectomycorrhizal fungi in pure culture. Fungal Divers 19: 51-68.

505 Iotti, M., and Zambonelli, A. (2006) A quick and precise technique for identifying
506 ectomycorrhizas by PCR. Mycol Res 110: 60 – 65.

507 Iotti, M., Piattoni, F., and Zambonelli, A. (2012a) Techniques for host plant inoculation with
508 truffles and other edible ectomycorrhizal mushrooms. In Edible ectomycorrhizal mushrooms, current

For Peer Review Only

509 knowledge and future prospects. Zambonelli, A., and Bonito, G.M. (eds). Soil Biology series, vol.34.
510 Berlin Heidelberg: Springer-Verlag, pp. 145 – 161.

511 Iotti, M., Rubini, A., Tisserant, E., Kholer, A., Paolocci, F., and Zambonelli, A. (2012b)
512 Self/nonself recognition in *Tuber melanosporum* is not mediated by a heterokaryon incompatibility
513 system. Fungal Biol 116: 261-275.

514 Iotti, M., Leonardi, M., Oddis, M., Salerni, E., Baraldi, E., and Zambonelli A. (2012c)
515 Development and validation of a real-time PCR assay for detection and quantification of *Tuber*
516 *magnatum* in soil. BMC Microbiol 12: 93.

517 Iotti, M., Piattoni, F., Leonardi, P., Hall, I.R., and Zambonelli, A. (2016) First evidence for
518 truffle production from plants inoculated with mycelial pure cultures. Mycorrhiza 26: 793–798.

519 Lancellotti, E., Iotti, M., Zambonelli, A., and Franceschini, A. (2016) The Puberulum group
520 sensul lato (whitish truffles). In True truffle (*Tuber* spp.) in the world. Zambonelli, A., Murat, C., and
521 Iotti, M. (eds). Soil Biology, vol. 47. Berlin Heidelberg: Springer-Verlag, pp. 105-124.

522 Le Tacon, F., Zeller, B., Plain, C., Hossann, C., Bréchet, C., and Robin, C. (2013) Carbon
523 transfer from the host to *Tuber melanosporum* mycorrhizas and ascocarps followed using a 13C
524 pulse-labeling technique. PLoS One 8: e64626.

525 Le Tacon, F., Rubini, A., Murat, C., Riccioni, C., Robin, C., Belfiori, B., *et al.* (2016)
526 Certainties and uncertainties about the life cycle of the Périgord black truffle (*Tuber melanosporum*
527 Vittad.) Ann For Sci 73: 105–117.

528 Linde, C.C., and Selmes, H. (2012) Genetic diversity and mating type distribution of *Tuber*
529 *melanosporum* and their significance to truffle cultivation in artificially planted truffieres in Australia.
530 Appl Environ Microbiol 78: 6534–6539.

For Peer Review Only

531 Martin, F., Kohler, A., Murat, C., Balestrini, R., Coutinho, P.M., Jaillon, O., *et al.* (2010)
532 Périgord black truffle genome uncovers evolutionary origins and mechanisms of symbiosis. Nature
533 464: 1033 – 1038.

534 Molinier, V., Murat, C., Morin, E., Gollotte, A., Wipf, D., and Martin F (2013) First
535 Identification of polymorphic microsatellite markers in the burgundy truffle, *Tuber aestivum*
536 (Tuberaceae). Appl Plant Sci 1: 1200220

537 Molinier, V., Murat, C., Peter, M., Gollotte, A., De la Varga, H., Meier, B., *et al.* (2015) SSR-
538 based identification of genetic groups within European populations of *Tuber aestivum* Vittad.
539 Mycorrhiza. 26: 99-110.

540 Molinier, V., Murat, C., Peter, M., Gollotte, A., De la Varga, H., Meier, B., *et al.* (2016a) SSR-
541 based identification of genetic groups within European populations of *Tuber aestivum* Vittad.
542 Mycorrhiza 26: 99–110.

543 Molinier, V., Murat, C., Baltensweiler, A., Büntgen, U., Martin, F., Meier, B., *et al.* (2016b)
544 Fine-scale genetic structure of natural *Tuber aestivum* sites in southern Germany. Mycorrhiza 26:
545 895–907.

546 Morara, M., Saitta, A., Venturella, G., Gargano, M.L., and Zambonelli, A. (2009) I funghi
547 ipogei dell'Emilia Romagna e della Sicilia a confronto. In: Annales Confederationis Europaeae
548 Mycologiae Mediterraneensis. Venturella, G. and Gargano M.L. (eds) XVII Giornate Micologiche
549 della CEMM, 14–21 Novembre 2009, Cefalù, Italia, pp 53–68

550 Murat, C., Riccioni, C., Belfiori, B., Cichocki, N., Labbé, J., Morin, E. *et al.* (2011) Mining
551 microsatellites in the Perigord black truffle genome and identification of new molecular markers.
552 Fungal Genet Biol 48: 592–601.

For Peer Review Only

553 Murat, C., Rubini, A., Riccioni, C., De la Varga, H., Akroume, E., Belfiori, B., *et al.* (2013)
554 Fine-scale spatial genetic structure of the black truffle (*Tuber melanosporum*) investigated with
555 neutral microsatellites and functional mating type genes. New Phytol 199: 176-187.

556 Murat, C., Bonneau, L., De la Varga, H., Olivier, J.M., Sandrine, F. and Le Tacon, F. (2016)
557 Trapping truffle production in holes: a promising technique for improving production and unravelling
558 truffle life cycle. Italian Journal of Mycology 45: 47-53.

559 Murat, C., Kuo, A., Barry, W.W., Clum, A., Dockter, R.B., Fauchery, L., *et al.* (2018) Draft
560 genome sequence of *Tuber borchii* Vittad., a whitish edible truffle. Genome Announc 6: e00537-18.

561 Nei, M. (1973) Analysis of gene diversity in subdivided populations. PNAS 70: 3321–3323.

562 Ori, F., Trappe, J., Leonardi, M., Iotti, M., and Pacioni, G. (2018) Crested purcopines (*Hystrix*
563 *cristata*): mycophagist spore disperser of the ectomycorrhizal truffle *Tuber aestivum.* Mycorrhiza 28:
564 561-565.

565 Palenzona, M., Chevalier, G., and Fontana, A. (1972) Sintesi micorrizica tra i miceli in coltura
566 pura di *Tuber brumale*, *T. melanosporum*, *T. rufum* e semenzali di conifere e latifoglie. Allionia 18:
567 41–52.

568 Paolocci, F., Rubini, A., Riccioni, C., and Arcioni, S. (2006) Reevaluation of the life cycle of
569 *Tuber magnatum*. Appl Environ Microbiol 72: 2390–2393.

570 Peakall, R., and Smouse, P.E. (2006) GenAlEx 6: genetic analysis in Excel. Population genetic
571 software for teaching and research. Mol Ecol Notes 6: 288–295.

572 Piattoni, F., Amicucci, A., Iotti, M., Ori, F., Stocchi, V., and Zambonelli, A. (2014) Viability
573 and morphology of *Tuber aestivum* spores after passage through the gut of *Sus scrofa*. Fungal Ecol
574 9: 52–60.

575 Piattoni, F., Leonardi, P., Boutahir, S., Iotti, M., and Zambonelli, A. (2017) Viability and
576 infectivity of *Tuber borchii* after cryopreservation. CryoLetters 38: 58-64.

For Peer Review Only

577 Riccioni, C., Belfiori, B., Rubini, A., Passeri, V., Arcioni, S., and Paolocci, F. (2008) *Tuber*
578 *melanosporum* outcrosses: analysis of the genetic diversity within and among its natural populations
579 under this new scenario. New Phytol 180: 466–478.

580 Rozen, S., and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist
581 programmers. In Bioinformatics Methods and Protocols. Misener, S., and Krawetz, S.A. (eds).
582 Methods in Molecular Biology™, vol 132. Totowa, NJ: Humana Press, pp. 365–386.

583 Rubini, A., Paolocci, F., Riccioni, C., Vendramin, G.G., and Arcioni, S. (2005) Genetic and
584 phylogeographic structures of the symbiotic fungus *Tuber magnatum*. Appl Environ Microbiol 71:
585 6584-6589.

586 Rubini, A., Belfiori, B., Riccioni, C., Tisserant, E., Arcioni, S., Martin, F., and Paolocci, F.
587 (2011a) Isolation and characterization of MAT genes in the symbiotic ascomycete *Tuber*
588 *melanosporum*. New Phytol 189: 710-722.

589 Rubini, A., Belfiori, B., Riccioni, C., Arcioni, S., Martin, F., and Paolocci, F. (2011b) *Tuber*
590 *melanosporum*: mating type distribution in a natural plantation and dynamics of strains of different
591 mating types on the roots of nursery-inoculated host plants. New Phytol 189: 723-735.

592 Rubini, A., Riccioni, C., Belfiori, B., and Paolocci, F. (2014) Impact of the competition between
593 mating types on the cultivation of *Tuber melanosporum*: Romeo and Juliet and the matter of space
594 and time. Mycorrhiza 24:19–27.

595 Sbrana, C., Nuti, M.P., and Giovanetti, M. (2007) Self-anastomosing ability and vegetative
596 incompatibility of *Tuber borchii* isolates. Mycorrhiza 17: 667–675.

597 Schneider-Maunoury, L., Clément, C., Coves, H., Lambourdière, J., Leclercq, S., Richard, F.,
598 Selosse, M.A., and Taschen, E. (2018) Is *Tuber melanosporum* colonizing the roots of herbaceous,
599 non-ectomycorrhizal plants? Fungal Ecol 31: 59-68.

For Peer Review Only

600 Selosse, M.A., Jacquot, D., Bouchard, D., Martin, F., and Le Tacon, F. (1998) Temporal
601 persistence and spatial distribution of an American inoculant strain of the ectomycorrhizal
602 basidiomycete *Laccaria bicolor* in a French forest plantation. Mol Ecol 7: 561– 573.

603 Selosse, M.A., Taschen, E., and Giraud, T. (2013) Do black truffles avoid sexual harassment
604 by linking mating type and vegetative incompatibility? New Phytol 199: 10–13.

605 Selosse, M.A., Schneider-Maunoury, L., Taschen, E., Rousset, F., and Richard, F. (2017) Black
606 truffle, a hermaphrodite with forced unisexual behavior. Trends Microbiol 25: 784-787:

607 Stenberg, P., Lundmark, M., and Saura, A. (2003) MLGsim: a program for detecting clones
608 using a simulation approach. Mol Ecol Notes 3: 329–331.

609 Taschen, E., Rousset, F., Sauve, M., Benoit, L., Dubois, M., Richard, F., and Selosse, M. (2016)
610 How the truffle got its mate: insights from genetic structure in spontaneous and planted Mediterranean
611 populations of *Tuber melanosporum*. Mol Ecol 25: 5611-5627.

612 Urban, A., Neuner-Plattner, I., Krisai-Greilhuber, I., Haselwandter, K. (2004) Molecular
613 studies on terricolous microfungi reveal novel anamorphs of two *Tuber* species. Mycol Res 108: 749–
614 758.

615 Zambonelli, A., Salomoni, S., and Pisi, A. (1993) Caratterizzazione anatomo-morfologica e
616 micromorfologica delle micorrize di *Tuber* spp. su *Quercus pubescens* Willd. Micol Ital 3: 73-90.

617 Zambonelli, A., Iotti, M., Rossi, I., and Hall, I. (2000) Interaction between *Tuber borchii* and
618 other ectomycorrhizal fungi in a field plantation. Mycol Res 104: 698-702.

619 Zambonelli, A., Iotti, M., Giomaro, G., Hall, I., and Stocchi, V. (2002) *T. borchii* cultivation:
620 an interesting perspective. In Edible mycorrhizal mushrooms. Proceedings of 2nd international
621 workshop on edible ectomycorrhizal mushrooms. Hall, I., Wang, Y., Danell, E., and Zambonelli, A.
622 (eds). Christchurch: New Zealand Institute for Crop and Food Research, CD ROM.

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623 Zambonelli, A., Iotti, M., and Piattoni, F. (2008) Problems and perspectives in the production
624 of *Tuber* infected plants. In Mushroom biology and mushroom products. Proceeding of the sixth
625 International conference on Mushroom Biology and Mushroom products. Lelley, J.I., and Buswell,
626 J.A. (eds). Krefeld: GAMU GmbH, pp. 263 – 271.

627 Zambonelli, A., Iotti, M., and Hall, I. (2015) Current status of truffle cultivation: recent results
628 and future perspectives. Italian Journal of Mycology 44: 31–40.

629 Zambonelli, A., and Morara, M. (1984) Le specie di Tuberales dell'Emilia Romagna, ecologia
630 e distribuzione. Natura e Montagna 4:9–32.

631 Zambonelli, A., Murat, C., and Iotti, M. (2016) True truffle (*Tuber* spp.) in the world. Soil
632 Biology, vol. 47. Berlin Heidelberg: Springer-Verlag.

633 Zambonelli, A., Ori, F., and Hall, I. (2017) Mycophagy and spore dispersal by vertebrates. In
634 The fungal community. Its organization and role in ecosystems, 4th edn. Dighton, J., and White, J.F.
635 (eds). Boca Raton, FL: CRC Press Taylor and Francis Group, pp. 347–358.

636 Zampieri, E., Rizzello, R., Bonfante, P., Mello, A. (2012) The detection of mating type genes
637 of *Tuber melanosporum* in productive and non productive soils. Applied soil Ecology 57:9-15.

638

639 **Legends of tables**

640 Table 1 – Characteristics of the 14 SSRs used to characterize the *Tuber borchii* clones involved in
641 this study.

642 Table 2 – Genotyping of the inoculated strain and of the ascoma, ectomycorrhizas and mycelium at
643 the points where the samples were collected.

644 Table 3 – Metadata of the *T. borchii* ascomata of Cadriano truffle ground used in this study.

645 Table 4 - Genetic diversity for maternal, paternal genotypes.

646 **Figure legends**

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647 Fig. 1 - Scheme of the experimental plantation realized with mycelial inoculated seedlings. The
648 circles show the codes of inoculated strains or their mixture (Mix). Different circle types indicate
649 different host plants. Triangles and asterisks indicate the position of samples and types of analyses
650 applied to them. The dotted rectangle indicates the area of the plantation enlarged in Fig. 2.
651 Fig. 2- Area of the plantation where a true hermaphrodite strain was found (C-MLG_14). This
652 strain was found as female in ascomata 4651, 4652 and 4658 and as male in ascoma 4601 located in
653 the adjacent plants. This ascoma presents a new female C-MLG (C-MLG_3).
654 Fig. 3 - Characteristics of the maternal (a) and paternal (b) genotypes of the 18 analyzed ascomata.
655 **Supplemental material**
656 Table S1 – Multilocus genotypes (MLG) found in the 50 ascomata analyzed from different
657 populations.
658 Table S2 –Multilocus genotypes (C-MLG) found in the Cadriano plantation.
659 Table S3- Genotype description, probability of genotype occurrence resulting from distinct sexual
660 events (PSex) for all samples harvested in the plantations.
661 Table S4- Tested microsatellite primers. In bold the selected polymorphic microsatellites.
662 Table S4- Tested microsatellite primers. In bold the selected polymorphic microsatellites.
663 Table and Fig. S5- Mating type analysis of the ascomata, ectomycorrhizas and soil mycelia of the
664 samples collected in 2017. In the figure triangles indicate the position of samples analyzed.

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SSR name	Repeat motif	Genome localization[a]	Na[b]	He[c]
Tb244	$(GAGGGA)_6$	Intergenic	5	0.562
Tb1	$(TATTTT)_{10}$	Intergenic	3	0.626
Tb83	$(GACT)_8$	Intergenic	3	0.263
Tb11	$(AGGC)_8$	Intergenic	4	0.319
Tb151	$(AAC)_8$	Gene model (UTR)	8	0.472
Tb155	$(GGA)_{12}$	Intergenic	7	0.649
Tb156	$(GAG)_8$	Intergenic	5	0.432
Tb17	$(TTTAGA)_5$	Intergenic	3	0.266
Tb206	$(CCTT)_8$	Intergenic	3	0.617
Tb293	$(AGAAGG)_5$	Gene model (intron)	10	0.739
Tb43	$(CTTTT)_5$	Intergenic	4	0.255
Tb704	$(AAAG)_8$	Intergenic	6	0.595
Tb43bis	$(TACC)_8$	Intergenic	4	0.208
Tb46	$(AGA)_9$	Gene model (CDS)	6	0.456

[a]UTR = untraslated region, CDS = coding sequence

[b]Na = Number of allele observed

[c]He = expected heterozygosity

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plant n.	sample n.	inoculated strain n.	mating type* strain	ascoma	ECM	soil	strain genotype	ascoma genotypes maternal	paternal
1	4597	2292	2	2	2	2	C-MLG_18	nd	nd
46	4598	Tb 98	1	1	nd	nd	C-MLG_8	C-MLG_8	C-MLG_7
10	4599	Tb 98	1	1	nd	nd	C-MLG_8	C-MLG_8	C-MLG_2
69	4600	2364	1	1	nd	nd	C-MLG_13	C-MLG_13	C-MLG_1
63	4601	1Bo ←	2	2	nd	nd	C-MLG_6	**C-MLG_3**	C-MLG_14
47	4602	Tb 98	1	1	nd	nd	C-MLG_8	C-MLG_8	C-MLG_18
33	4633	2364 ←	1	1	1	1	C-MLG_13	**C-MLG_10**	C-MLG_20
39	4634	2292	2	2	nd	nd	C-MLG_18	C-MLG_18	C-MLG_5
68	4635	2364	1	1	nd	nd	C-MLG_13	C-MLG_13	C-MLG_16
15	4639	MIX	na	2	2	2	na	nd	nd
51	4640	MIX	na	1	1	1	na	nd	nd
44	4641	Tb 98	1	1	1	1	C-MLG_8	nd	nd
65	4642	1Bo	2	2	2	**1**	C-MLG_6	nd	nd
6	4644	Tb 98	1	1	1	1	C-MLG_8	nd	nd
47	4647	Tb 98	1	1	1	1	C-MLG_8	nd	nd
10	4648	Tb 98	1	1	nd	nd	C-MLG_8	C-MLG_8	C-MLG_15

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44	4649	Tb 98	1	1	nd	nd	C-MLG_8	C-MLG_8	C-MLG_19
23	4650	2352	1	1	1	1	C-MLG_14	nd	nd
59	4651	2352	1	1	1	1	C-MLG_14	C-MLG_14	C-MLG_4
61	4652	2352	1	1	1	1	C-MLG_14	C-MLG_14	C-MLG_2
68	4653	2364	1	1	1	1	C-MLG_13	C-MLG_13	C-MLG_9
45	4654	TB98	1	1	1	1	C-MLG_8	nd	nd
41	4655	2292	2	2	2	2	C-MLG_18	nd	nd
44	4656	TB98	1	1	1	1	C-MLG_8	C-MLG_8	C-MLG_7
52	4657	MIX	na	1	1	1	na	C-MLG_14	C-MLG_17
65	4658	1 Bo ←	2	**1**	nd	nd	C-MLG_6	**C-MLG_14**†	C-MLG_11
6	4659	TB98	1	1	1	1	C-MLG_8	nd	nd
17	4660	MIX	na	2	2	2	na	nd	nd
27	4662	2352	1	1	1	1	C-MLG_14	nd	nd
52	4664	MIX	na	1	1	1	na	C-MLG_8	C-MLG_21
13	4675	MIX	na	1	1	1	na	C-MLG_8	C-MLG_12
65	4677	1Bo	2	2	2	**1**	C-MLG_6	nd	nd
13	4699	MIX	na	1	1	1	na	nd	nd

***1 correspond to the mating type MAT 1-1-1, 2 correspond to MAT 1-2-1.**
† C-MLG of the strain 2352
The arrow indicate the samples having a maternal C-MLG (in bold) different from the inoculated strain
nd = not determined
na = not applicable

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ascoma	sampling date	weight (g)	plant	tree species	inoculated strain
4597	08/02/2016	35	1	*Pinus pinea*	2292
4598	08/02/2016	34	46	*Pinus pinea*	Tb98
4599	08/02/2016	13.9	10	*Quercus pubescens*	Tb98
4600	08/02/2016	54.7	69	*Corylus avellana*	2364
4601	08/02/2016	20.9	63	*Quercus pubescens*	1Bo
4602	08/02/2016	2	47	*Pinus pinea*	Tb98
4633	12/02/2016	0.59	33	*Quercus robur*	2364
4634	12/02/2016	1	39	*Quercus pubescens*	2292
4635	12/02/2016	2.28	68	*Quercus robur*	2364
4639	19/02/2016	5.53	15	*Pinus pinea*	MIX
4640	19/02/2016	9.73	51	*Quercus pubescens*	MIX
4641	19/02/2016	6.84	44	*Quercus pubescens*	Tb98
4642	19/02/2016	3.42	65	*Pinus pinea*	1BO
4644	19/02/2016	0.27	6	*Quercus pubescens*	TB98
4647	19/02/2016	27.73	47	*Pinus pinea*	TB98
4648	25/02/2016	9.94	10	*Quercus pubescens*	Tb98
4649	25/02/2016	0.53	44	*Quercus pubescens*	Tb98
4650	25/02/2016	6.18	23	*Pinus pinea*	2352
4651	25/02/2016	0.38	59	*Pinus pinea*	2352
4652	25/02/2016	17.23	61	*Pinus pinea*	2352
4653	25/02/2016	7.39	68	*Quercus robur*	2364
4654	25/02/2016	2.73	45	*Quercus pubescens*	TB98
4655	25/02/2016	5.17	41	*Quercus pubescens*	2292
4656	25/02/2016	1.19	44	*Quercus pubescens*	Tb98
4657	25/02/2016	12.79	52	*Quercus pubescens*	MIX
4658	25/02/2016	6.66	65	*Pinus pinea*	1Bo
4659	02/03/2016	4.67	6	*Quercus pubescens*	Tb98
4660	02/03/2016	6.33	17	*Pinus pinea*	MIX
4662	02/03/2016	19.24	27	*Quercus pubescens*	2352
4664	02/03/2016	6.96	52	*Quercus pubescens*	MIX
4675	18/03/2016	11.24	13	*Pinus pinea*	MIX
4677	18/03/2016	2.06	65	*Pinus pinea*	1Bo
4699	06/04/2016	5.87	13	*Pinus pinea*	MIX

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Number of sampled ascocarps	18
Number of inocula genotyped	5
Maternal genotypes	
Number of samples genotyped	18
Number of genotypes	6
Number of genotypes with Psex < 0.05	3
Maximum number of ascocarps per genotype	8
Number of genotype represented by a single ascocarp	3
Paternal genotypes	
number genotyped zigotes	18
Number of homozygosus zygotes	0
Zigotes Fis	1
Number of genotypes	16
Number of genotypes with Psex < 0.05	1
Maximum number of ascocarps per genotype	2
Number of male genotypes represented by a single ascocarp	12
Hermaphodite genotypes	
Total number (number of genotypes whit Psex < 0.05)	1

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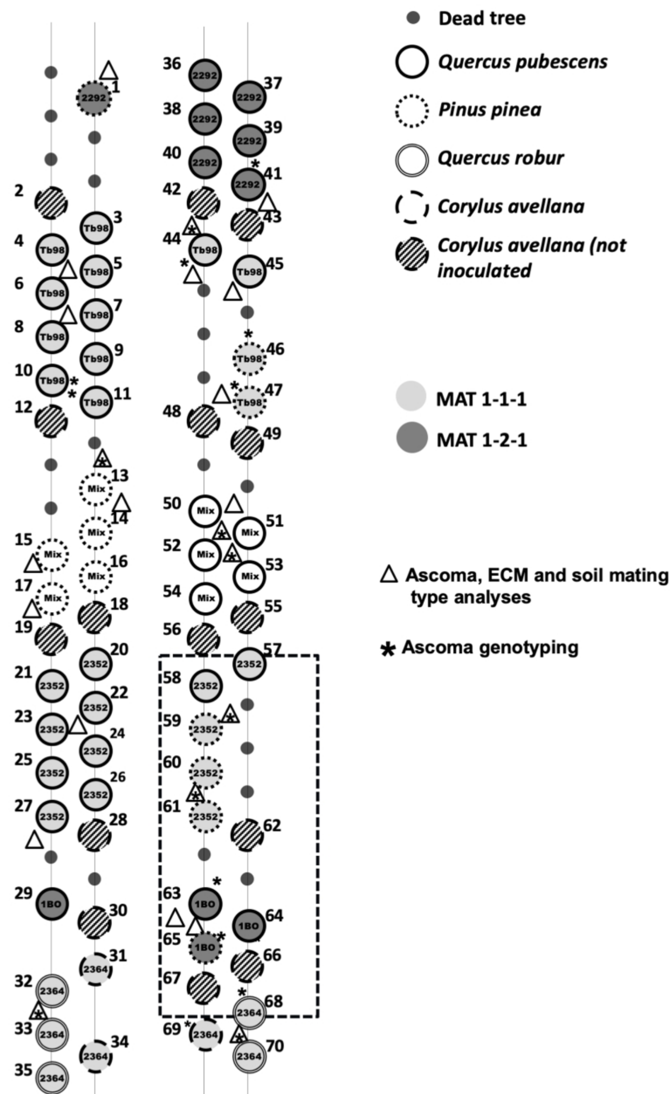

Fig. 1 - Scheme of the experimental plantation realized with mycelial inoculated seedlings. The circles show the codes of inoculated strains or their mixture (Mix). Different circle types indicate different host plants. Triangles and asterisks indicate the position of samples and types of analyses applied to them. The dotted rectangle indicates the area of the plantation enlarged in Fig. 2.

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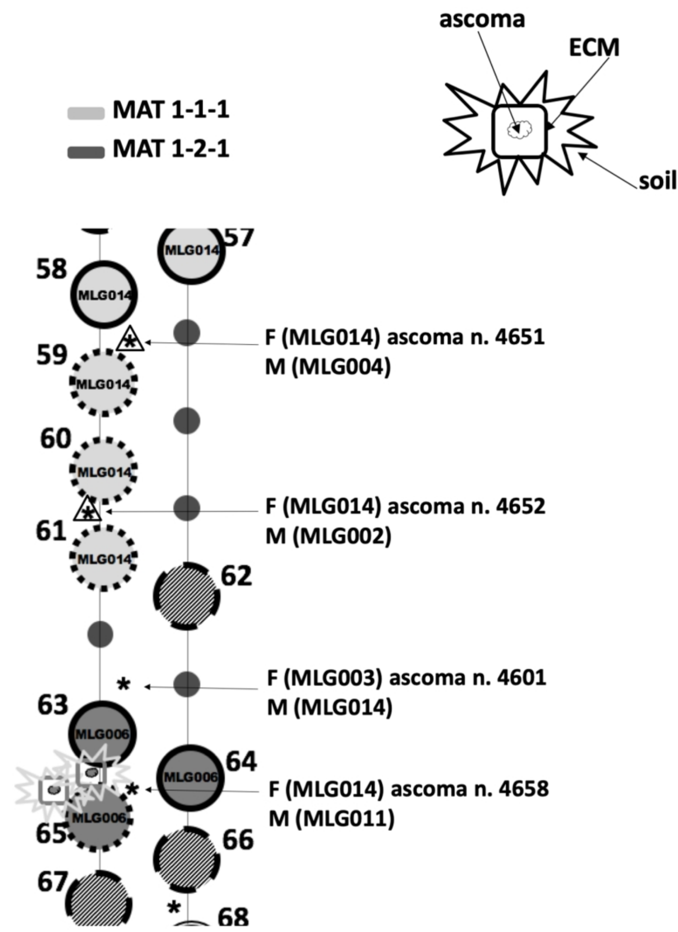

Fig. 2- Area of the plantation where a true hermaphrodite strain was found (C-MLG_14). This strain was found as female in ascomata 4651, 4652 and 4658 and as male in ascoma 4601 located in the adjacent plot This ascoma presents a new female C-MLG (C-MLG_3).

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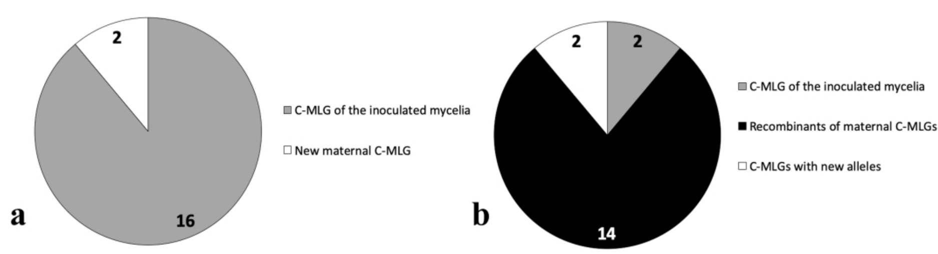

Fig. 3 - Characteristics of the maternal (a) and paternal (b) genotypes of the 18 analyzed ascomata.