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ORIGINAL ARTICLE

Characterization of the reproductive mode and life cycle of the whitish truffle *T. borchii*

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Abstract Truffles are the fruiting structures of ascomycetes in the genus *Tuber*. Because of their economic importance, truffles have been cultivated for many years using artificially inoculated host plants. Nevertheless, the life cycle and reproductive mode of *Tuber* spp. are still poorly understood. In filamentous ascomycetes, sexual reproduction is genetically controlled by the mating-type (*MAT*) locus. Among *Tuber* spp., the *MAT* locus has been recently characterized in the black truffles *Tuber melanosporum* and *Tuber indicum*. Here, by using sequence information derived from these species and from a *Tuber borchii* expressed sequence tag (EST) showing similarity to the *mat1* gene of *Alternaria brassicicola*, we embarked on a chromosome-walking procedure to sequence the complete MAT region of *T. borchii*. This fungus produces highly commercialized whitish truffles and represents a model species for addressing basic questions concerning the life cycle of *Tuber* spp. We show that *T. borchii* is heterothallic, as its *MAT* locus is organized into two idiomorphs, each harbored by different mycelial strains. The alignment of the *MAT* locus from black truffles and *T. borchii* reveals that extensive sequence rearrangements and inversions occurred between these species. Moreover, by coupling mating-type analyses to karyological observation, we show that mycelia isolated from ascocarps and mycorrhizae are formed by homokaryotic hyphae.

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Introduction

Tuber borchii Vittad. is an ectomycorrhizal ascomycete belonging to the order Pezizales that produces edible hypogeous fruit bodies. These are known as whitish truffles for the color of their peridium, and they are characterized by a pungent garlic-like odor. According to Montecchi and Sarasini (2000), this species belongs to the "*puberulum*" group, which comprises many European, Asiatic, and North American species (Jeandroz et al. 2008; Wang et al. 2007; Bonito et al. 2010, 2013). *T. borchii* is one of the most widespread *Tuber* species. It has a broad distribution in Europe, being found from southern Finland to Sicily and from Ireland to Hungary and Poland (Hall et al. 2007). It is well adapted to various environments (e.g., from coastal pine forests to hilly areas of the hinterland), it can grow associated with many different plant species (e.g., *Pinus* spp., *Corylus avellana*, *Ostrya carpiniofolia*, *Quercus* spp., *Cystus* spp.), and its truffle production is generally abundant. Although *T. borchii* truffles are widely commercialized in Italy and other European countries, their market value is low compared to other cultivable truffle species (e.g., *Tuber melanosporum* Vittad. and *Tuber aestivum* Vittad.). For all these reasons, there are only a few *T. borchii* plantations in Europe (Zambonelli et al. 2002).

Aside from the cultivation and commercial perspectives, *T. borchii* is of interest as, in contrast to other *Tuber* species, its mycelium is easy to isolate and cultivate in vitro (Sisti et al. 1998). Efficient inoculation procedures have also been developed to induce the synthesis of *T. borchii* mycorrhizae on a number of host species both in greenhouses and in vitro (Sisti et al. 1998; Zeppa et al. 2000; Giomaro et al. 2005; Iotti et al. 2012a). Due to these features, *T. borchii* has become a model

species among *Tuber* spp. to perform biochemical and genetic studies and gain a better understanding of the cross-talk that governs the mutual recognition and symbiotic relationship between *Tuber* spp. and their hosts (Zeppa et al. 2000; Giomaro et al. 2005; Ceccaroli et al. 1999, 2003; Zeppa et al. 2002; Lacourt et al. 2002; Ambra et al. 2004; Polidori et al. 2007).

Despite the interest in the biology, ecology, and cultivation of this *Tuber* species, only a few studies have been dedicated to understanding its life cycle and reproductive modes. In this regard, we note that recent years have witnessed a tremendous leap toward these insights in other truffle species. Early population genetics studies using co-dominant SSR markers revealed an apparent complete absence of heterozygosis in *T. melanosporum* ascocarps, which was explained by a closed mating system or even an exclusive selfing in this species (Bertault et al. 1998). These conclusions were based on the assumption that the *Tuber* spp. life cycle, including the symbiotic (ectomycorrhiza) and reproductive (ascocarp) stages, was sustained by a secondary (dikaryotic) mycelium, as the hyphae of mycelia isolated in vitro and of mycorrhizae frequently display paired nuclei (Fasolo-Bonfante and Brunel 1972; Lanfranco et al. 1995). However, these findings have more recently been superseded. It was in fact shown that in *Tuber magnatum* and *T. melanosporum*, not only mycorrhizae were haploid, but ascocarps were also formed by haploid hyphae (Rubini et al. 2005; Paolocci et al. 2006; Riccioni et al. 2008). Specifically, it was shown that these species can outcross and that the haploid hyphae forming the non-ascogenous tissues of the gleba originate from only one of the two partners involved in the fertilization process, the maternal one. Furthermore, in mature truffles, it has been shown that the paternal contribution is present only in the ascospores, and because only few of these structures are damaged during standard DNA isolation methods, paternal DNA can be detected only when either DNA is isolated from purified pools of ascospores or a high number of PCR cycles is used. From these considerations, it has been argued that the development of secondary (dikaryotic) mycelium is presumably limited to the first stage of fruiting body development and that the life cycle of *T. magnatum* and *T. melanosporum* is prevalently haploid (Rubini et al. 2005, 2011b, 2012; Paolocci et al. 2006; Riccioni et al. 2008).

Although these studies have provided more insight on the reproductive modes of *Tuber* spp., the fertilization process, a key step for entering in the sexual phase, remains elusive. The typical gametangia of ascomycetes (i.e., ascogonia and antheridia), in fact, have never been identified with certainty in any truffle species (Rubini et al. 2007). Indeed, a structure resembling an ascogonium has been observed only once in *T. melanosporum* (Callot 1999). Thus, whether fertilization in *Tuber* is mediated by the differentiation of gametangia or simply results from a somatogamic process has yet to be fully elucidated (Rubini et al. 2007, 2014; Le Tacon et al. 2015). Regardless of how it performs, according to the abovementioned life cycle model, the fertilization step should occur after the formation of the ectomycorrhizae and should be temporarily linked to the formation of fruit bodies (Rubini et al. 2012).

Whether a filamentous ascomycete (*Pezizomycotina*) reproduces by haploid-selfing or outcrossing is genetically controlled by the mating-type (*MAT*) locus (Turgeon and Yoder 2000). This locus contains the two master mating-type genes *MAT1-1-1* and *MAT1-2-1*, which encode transcription factors with an α1 domain and a high mobility group (MATA_HMG) domain, respectively (Debuchy et al. 2010). Heterothallic ascomycetes are characterized by the presence of two different versions of the *MAT* locus called idiomorphs, with each haploid strain containing only one *MAT* gene, either *MAT1-1-1* or *MAT1-2-1*. Cross-fertilization is an obligate step in these species. Conversely, in homothallic species, both *MAT* genes are present in each haploid strain. Thus, homothallic fungi do not have distinctive mating types and each haploid strain can self-fertilize or cross with any other (Billiard et al. 2012).

Although the *MAT* genes of several *Pezizomycotina* have been characterized (Debuchy et al. 2010), cloning of these regions in *Tuber* spp. has been hampered for a long time by their poor sequence conservation among fungi of different lineages (Rubini et al. 2007). Despite the fact that an expressed sequence tag (EST) sequence of *T. borchii* showing similarity to *mat1* of *Alternaria brassicicola* was previously reported (Zeppa et al. 2002), only the recent sequencing of *T. melanosporum* genome has made the identification of the *MAT* locus of a *Tuber* species possible (Martin et al. 2010a). More specifically, as the *MAT* locus in the sequenced genome contains the *MAT1-2-1* gene only, and the second *MAT* gene, *MAT1-1-1*, has been identified in a different strain, it has been concluded that *T. melanosporum* is heterothallic (Rubini et al. 2011b). The characterization of the *MAT* genes of *T. melanosporum* has also been instrumental to clone their orthologs in *T. indicum*, the *Tuber* species being phylogenetically closest to *T. melanosporum*, and to show that this species is also heterothallic (Belfiori et al. 2013). *MAT* sequences have also been cloned from other *Tuber* species, and commercial use of this information is covered by a patent application (Martin et al. 2012).

In this study, the *MAT* locus of *T. borchii* has been sequenced with the aims of (i) ascertaining the reproductive mode of this species; (ii) comparing the structure and sequences of *MAT* genes and idiomorphs of *T. borchii* with those of other *Tuber* species; and (iii) providing markers (e.g., PCR primers) for typing *T. borchii* strains according to their mating type. Additionally, to gain insight into the life cycle of this species, the growth rate, morphology, and distribution of nuclei in free-living mycelia isolated from ascocarps and mycorrhizae were investigated.

Overall, the definition of the *T. borchii* life cycle and the identification of its reproductive mode are of interest to address basic questions concerning the dynamics and biology of the fertilization step in *Tuber* spp. These studies are also of relevance for improving the cultivation of these fungi.

Materials and methods

Sample sources

A set of 26 *T. borchii* ascocarps collected between 2007 and 2013 in Central Italy at different producing sites and under different host species was used in this study (Table 1). The truffles from each site were collected with the help of truffle pickers. All the ascocarps were identified by a morphological examination according to Pegler et al. (1993) and Montecchi and Sarasini (2000) and by sequencing the PCR-amplified internal transcribed spacer

Table 1 List of *T. borchii* samples used in this study

Specimen	Type	Source	Provenance	Putative host plant	Mating type[a]
Tar014	A	–	Città di Castello (Central Italy)	na	*MAT1-2*
Tar042	A	–	Città di Castello (Central Italy)	q	*MAT1-2*
Tar325	A	–		q	*MAT1-2*
Tar328	A	–		q	*MAT1-2*
Tar415	A	–		q	*MAT1-2*
Tar136	A	–	Lamati (Città di Castello, Central Italy)	q	*MAT1-2*
Tar145	A	–	I Coppi (Città di Castello, Central Italy)	q	*MAT1-2*
Tar146	A	–	Perrubbio (Città di Castello, Central Italy)	o	*MAT1-1*
Tar147	A	–		o	*MAT1-1*
Tar157	A	–	Rapastello (Città di Castello, Central Italy)	q	*MAT1-1*
Tar158	A	–		q	*MAT1-1*
Tar165	A	–		q	*MAT1-1*
Tar178	A	–	Collevecchio (Città di Castello, Central Italy)	p	*MAT1-2*
Tar179	A	–		p	*MAT1-2*
Tar180	A	–		p	*MAT1-2*
Tar185	A	–		a	*MAT1-2*
Tar202	A	–	Barzotti (Città di Castello, Central Italy)	q	*MAT1-1*
Tar208	A	–	Collevecchio (Città di Castello, Central Italy)	a	*MAT1-2*
Tar209	A	–		a	*MAT1-1*
Tar210	A	–		a	*MAT1-2*
Tar282	A	–	Montemaggiore (Città di Castello, Central Italy)	q	*MAT1-2*
Tar283	A	–		q	*MAT1-2*
Tar284	A	–		q	*MAT1-2*
Tar285	A	–		q	*MAT1-2*
Tar240	A	–	Castrovillari (Cosenza, Southern Italy)	na	*MAT1-2*
Tar242	A	–		na	*MAT1-2*
tb6	M	ECM		p	*MAT1-1*
tb7	M	ECM		p	*MAT1-2*
tb15	M	ECM		p	*MAT1-2*
tb16	M	ECM		p	*MAT1-1*
tb136	M	A (Tar136)		–	*MAT1-2*
tb146	M	A (Tar146)		–	*MAT1-1*
tb147	M	A (Tar147)		–	*MAT1-1*
tb165	M	A (Tar165)		–	*MAT1-1*
tb202	M	A (Tar202)		–	*MAT1-1*
tb208	M	A (Tar208)		–	*MAT1-2*
tb209	M	A (Tar209)		–	*MAT1-1*
tb210	M	A (Tar210)		–	*MAT1-2*

A ascocarp, *M* mycelium isolated in vitro, *ECM* mycorrhiza, *p Pinus nigra*, *q Quercus pubescens*, *o Ostrya carpinifolia*, *a Abies cephalonica*, *na* not available

[a] The mating type of the ascocarps refers to the maternal tissue only, as detected with low number of PCR cycles

(ITS) region of the nuclear ribosomal genes (rDNA) according to Rubini et al. (1998).

The *T. borchii* ectomycorrhizae (ECMs) were collected under *Pinus nigra* trees in a natural truffle-producing site in central Italy. The ECMs were identified by morphotyping (Zambonelli et al. 1993; Giomaro et al. 2000) and PCR analysis with the species-specific ITS primer pair TB1-TB2 according to Amicucci et al. (1998).

Isolation and characterization of *T. borchii* mycelia

T. borchii mycelia were isolated from ascocarps and ECMs. Fresh ascocarps were carefully washed under running tap water and surface-sterilized by immersion in 95 % ethanol for 1 min. From each ascocarp, a small portion of the internal gleba was then removed with a scalpel under sterile environment and placed in an agarized medium in Petri dishes. Single ECMs, collected under a stereomicroscope, were maintained in water and placed for 3 min in a 0.2 % (*v*/*v*) NaClO solution. The ECMs were then washed three times in sterile water, dried in sterile filter paper, and placed in an agarized medium in Petri dishes. The modified Melin-Norkrans (MMN) medium (Marx 1969) supplemented with 6 g/l agar and 50 mg/l ampicillin (pH 6.6) was used. The Petri dishes were incubated at 22–24 °C in the dark and inspected daily under a stereomicroscope. The isolated mycelia were grown in agarized MMN medium without antibiotic. All the isolated mycelia (Table 1) were subcultured to fresh MMN plates every 3–4 months and maintained at 4 °C. Dual cultures of mycelia with the same or opposite mating types were performed according to Iotti et al. (2012b). The pairs with opposite mating types tested were tb15/tb16, tb7/tb6, tb15/tb6, and tb16/tb7. Control experiments were performed with the pairs tb15/tb7 and tb16/tb6 or by double inoculation of the same strain.

Morphological analyses were performed with stereo and light microscopes. The hyphae were stained with Tripan Blue (Sigma, Milan, Italy) according to Brundrett et al. (1995), and nuclei within the hyphae were stained with 4′,6-diamidino-2-phenylindole (DAPI) according to Rubini et al. (2011b). To evaluate the growth rate, Petri dishes (10-cm diameter) filled with 25 ml agarized MMN were inoculated with mycelium plugs (6-mm diameter) taken from the periphery of colonies grown for 20 days on the same medium. The plates were incubated at 24 °C in the dark, and the growth curve was determined by measuring the colony diameter in two perpendicular axes every 3–4 days and by averaging the measure of three replicates according to Mischiati and Fontana (1993).

DNA and RNA isolation, PCR amplification, and sequencing

DNA isolation was performed according to Paolocci et al. (1999) and PCR amplification of the ITS region was according to Rubini et al. (1998), using the ITS1-ITS4 primer pair (White et al. 1990). The isolation and sequencing of the mating-type region was performed with the primers reported in Supplemental Table S1 and Fig. S1. PCR amplifications were performed in a 50 μl mixture containing 10× PCR buffer (Euroclone, Milan, Italy), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 10 μM each primer, 1 U Taq polymerase (Euroclone, Milan, Italy), and 20 ng genomic DNA. PCR reactions were performed in a 9700 PCR system (Life Technologies, Carlsbad, CA, USA) with the following thermal profile: 2 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 50–65 °C for 20 s depending on the primers used, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Long-range PCR amplification of the MAT1-1 region (approximately 10 kb, see "Results") was performed with LA-Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) using the following two-step thermal profile: 30 s at 94 °C, 30–40 cycles of denaturation at 94 °C for 30 s and annealing/extension at 68 °C for 15 min, and a final extension of 7 min at 72 °C.

A partial sequence of *T. borchii MAT1-1* was obtained from GenBank, and a short fragment of the *MAT1-2-1* gene was isolated by PCR. These fragments were used as templates to sequence the complete *MAT* genes and to extend the sequences of both idiomorphs toward their 5′ and 3′ ends by genome walking. To this purpose, both the Universal GenomeWalker™ Kit (Clontech, Palo Alto, CA, USA) and the inverse PCR (IPCR) procedure (Ochman et al. 1988) were used. GenomeWalker libraries of adapter-ligated fragments were prepared according to the supplier's instructions from DNA isolated from tb15 and tb16 mycelia (Table 1). Multiple steps of genome walking were performed using either AP1 or AP2 adapter primers provided with the kit, coupled with specific primers. The IPCR was performed to extend the 5′ region of *MAT1-2* and the 3′ region of *MAT1-1* obtained with the GenomeWalker kit. For this purpose, 1 μg of genomic DNA from tb15 and tb16 were digested with the enzyme *Hind*III and were circularized by self-ligation. IPCR and nested IPCR on the tb15 library were performed with the b30/b31 and b29/b32 primer pairs, respectively. IPCR and nested IPCR on the tb16 library were performed with the b15/b17 and b16/b18 primer pairs, respectively. All primers used are reported in the Supplemental Table S1 and Fig. S1.

RNA isolation and rapid amplification of cDNA ends (RACE) analysis were performed according to Rubini et al. (2011b). RNA was isolated from a 20-day-old culture of tb15 strain. For MAT1-2-1 cDNA amplification, the gene-specific reverse primers b23 and the 3′ RACE adaptor primer were used.

The PCR amplicons were purified using the JetQuick PCR purification kit (Genomed, GmbH, Löhne, Germany) or cloned in *E. coli* using standard procedures (Sambrook et al. 1989). Sequencing was performed with the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Life Technologies,

Carlsbad, CA, USA) according to the supplier's instructions and run on an ABI 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Sequence visualization and assembly were performed using FinchTV (Geospiza, www.geospiza.com/finchtv) and Bioedit software (Hall 1999). Primers were designed with the help of PerlPrimer software v. 1.1.16 (Marshall 2004). All sequences were deposited in GenBank under the following accession nos.: KT165325-KT165350 (ITS from *T. borchii* ascocarps), KT165351-KT165362 (ITS from mycelial isolates), KM210558, and KM210559 (assembled MAT1-2 and MAT1-1 contigs).

Identification of *T. borchii MAT1-1-1* and *MAT1-2-1* genes

The *T. melanosporum* mating-type genes were used to search for *T. borchii* orthologous sequences deposited in public databases. By using the *T. melanosporum MAT1-1-1* sequence as query in BLASTn searches, the *T. borchii* EST AF487329 with 85 % sequence identity was retrieved. Conversely, neither BLASTn nor TBLASTn searches identified any *T. borchii* sequence with a significant similarity to the *T. melanosporum MAT1-2-1* nucleotide or amino acid sequences, respectively.

To test for the presence of the putative *MAT1-1-1* gene in the *T. borchii* ascocarps and mycelia under investigation, the AF487329 sequence was aligned with the corresponding *MAT1-1-1* gene of *T. melanosporum* (Supplemental Fig. S2a) and the *T. borchii MAT1-1-1*-specific primers b1 and b3 were designed. These primers produced the expected amplicon of approximately 530 bp from seven ascocarps and seven mycelia isolated in vitro (Table 1). All the remaining samples did not produce any amplicons, despite the fact that their DNA yielded the expected PCR product when amplified with ITS primers (Table 1 and data not shown). Because only some strains produced the expected *MAT1-1-1*-specific product, heterothallism in *T. borchii* was inferred and these samples were regarded as the putative MAT1-1 strains. Conversely, the samples that did not produce any PCR amplicon were considered as putative MAT1-2 strains and were used as targets to search for the *MAT1-2-1* gene by PCR with the primers P1 and P2, previously designed on the *T. melanosporum MAT1-2-1* gene (Rubini et al. 2011b). Because these primers did not produce detectable amplicons in any *T. borchii* sample, a new primer pair (b38/b39, Supplemental Table S1, Fig. S1) was designed on the HMG domain conserved between the *T. melanosporum* and *T. indicum MAT1-2-1* genes. When tested on *T. borchii* DNA using low-stringency PCR conditions consisting of an annealing temperature of 50 °C and high $MgCl_2$ concentration (4 mM), this primer pair produced an amplicon of approximately 1 kbp in those *T. borchii* ascocarps and mycelia that failed to produce the *MAT1-1-1*-specific amplicon (Table 1). The PCR amplicon from ascocarp Tar415 was cloned, sequenced, and aligned with the *T. melanosporum MAT1-2-1* gene (Supplemental Fig. S2b). BLASTX search against the *T. melanosporum* Gene Models database (http://mycor.nancy.inra.fr/IMGC/TuberGenome/blast.php) also confirmed the similarity (68 % amino acidic identity) of Tar415 fragment with the *T. melanosporum* MAT1-2-1 predicted protein (GSTUMT00001090001). The *MAT1-2-1*-specific primers b23 and b33 were then designed on the *T. borchii* HMG-box region to produce a DNA fragment of approximately 580 bp (Supplemental Table S1, Fig. S1). To test for the mating type of the *T. borchii* samples, a multiplex PCR procedure was set by combining the primer pairs b1-b3 and b23-b33

Isolation of *T. borchii* MAT idiomorphs

The two mycelial strains tb15 and tb16, harboring the *MAT1-2-1* and *MAT1-1-1* genes, respectively, were used as templates to isolate, by means of genome walking and IPCR, the two *T. borchii* MAT idiomorphs.

In tb16 the partial *MAT1-1-1* sequence (AF487329) was extended toward the 5′ and 3′ ends (Supplemental Fig. S1). In tb15, the genome walking approach, starting from the *MAT1-2* gene fragment obtained as reported above, allowed us to extend the MAT1-2 idiomorph by approximately 5 kbp toward its 5′ end only. The 3′ end of this idiomorph was then isolated using sequence information retrieved from tb16. Within the tb16 contig itself, a sequence corresponding to the *T. melanosporum* gene model GSTUMT00001092001 was found and named *TbOrf2* (Supplemental Fig. S1). Notably, this gene was present at the 3′ flanking regions of both *T. melanosporum* idiomorphs (Rubini et al. 2011b).

Under the hypothesis that this sequence was also flanking the 3′ end of the *T. borchii* MAT1-2 region, the primers b34 and b35 were designed to target the tb16 *TbOrf2* gene (Supplemental Table S1, Fig. S1) and were employed in PCR. These primers produced an amplicon of approximately 320 bp not only from tb16, as expected, but also from tb15. The sequence analysis confirmed that these amplicons showed a high sequence similarity to each other (approximately 90 % of sequence identity) and to the *T. melanosporum* gene model GSTUMT00001092001. Subsequently, to specifically amplify the 3′ end of the MAT1-2 idiomorph from tb15, the b36 primer was designed on *TbOrf2* and used in combination with the *MAT1-2-1* gene-specific primer b33 (Supplemental Table S1, Fig. S1). This primer pair, however, did not produce any PCR amplicon in tb15. Conversely, a 6870-bp-long amplicon was obtained when the primer b37, designed in inverse orientation with respect to b36, was used in combination with the primer b33.

Results

Characterization of *T. borchii* ascocarps and in vitro isolated mycelia

The ITS rDNA sequences from the 26 ascocarps identified as *T. borchii* by morphological observation (Table 1) were analyzed to confirm the species identity and assess genetic polymorphism. All the samples displayed an amplicon of approximately 600 bp, and both BLAST analysis and alignment with *T. borchii* ITS sequences retrieved from GenBank confirmed that all specimens belonged to *T. borchii*. The comparison of the ITS sequences did not show any remarkable polymorphism: all samples shared the same haplotype, with the only exception of Tar042, which differed from the other samples by a single nucleotide substitution. The two haplotypes identified in this study clustered within the *T. borchii* clade I described by Bonuso et al. (2010) (Supplemental Fig. S3).

Four mycelial strains from ECMs and eight from ascocarps were isolated (Table 1). The identity of all the isolates was confirmed by sequencing the ITS region. The mycelia growing in Petri dishes in MMN agar formed circular colonies with submerged and aerial hyphae (Fig. 1a). Microscopical observations showed that the aerial hyphae often carried a small condensation drop at the tip and frequently collapsed on the agar surface, forming coiled structures (Fig. 1b). Staining with DAPI revealed that the cells were multinucleated, with the number of nuclei ranging from 2 to 7. Nuclei were frequently paired, and several pairs of nuclei within single cells were observed (Fig. 1c, d).

The mycelial strains exhibited different growth rates (Supplemental Fig. S4). The strains tb136, tb7, and tb209 grew faster than the others, reaching the border of the plate in 27, 34, and 55 days, respectively. Also, the growth of tb147 and tb15 was fast initially, but when their colonies reached the diameter of about 5.5 and 7 cm after 34 days of cultivation, their growth increased slowly or not at all. Tb6 was the slowest; it stopped growing after 22 days, forming small colonies with a diameter of approximately 2.5 cm. All the other strains stopped growing after 55 days with colonies of 4, 5, and 6 cm in diameter. Dual cultures of mycelia with the same or opposite mating types did not show any relevant morphological changes: the two mycelial colonies grew normally, regardless of the mating type of the strains employed in the assay. When in contact, they continued to grow without showing any polarized growth or the formation of inhibition zones (data not shown).

Identification of *T. borchii* MAT genes and idiomorphs

The MAT genes of *T. borchii* were identified by different strategies. A EST sequence (AF487329) corresponding to a putative *T. borchii MAT1-1-1* gene was identified by performing BLAST searches based on the *T. melanosporum* MAT sequences. Conversely a PCR fragment belonging to a *MAT1-2-1* gene was isolated from *T. borchii* using primers derived from the *T. melanosporum* and *T. indicum MAT1-2-1* sequences. Using MAT-specific primers designed on these fragments in a multiplex PCR, we tested the mating type of the ascocarps and mycelia reported in Table 1. When a low number of PCR cycles (25) was performed, a single PCR fragment

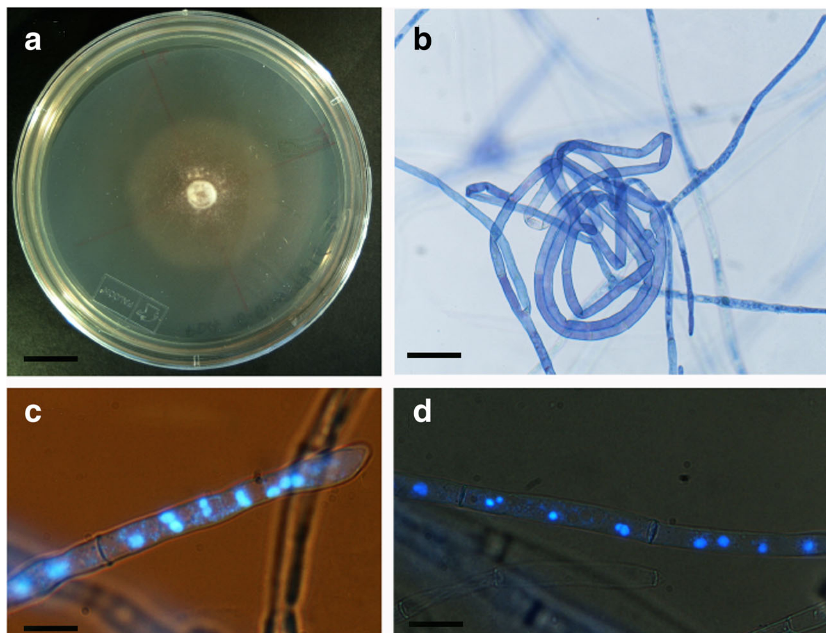

Fig. 1 Morphology of *T. borchii* mycelium grown in vitro. **a** Mycelium growing on solid MMN media, *scale bar* 13 mm; **b** details of hyphae on the agar surface and forming coiled structures (Trypan Blue staining), *scale bar* 30 μm; **c** apical cell; and **d** internal cell of a multinucleated hypha showing the presence of paired nuclei, *scale bars* 10 μm

was obtained from the ascocarps; however, when the PCR cycles were increased to 50, both the *MAT1-1* and *MAT1-2* fragments were produced in some samples (Fig. 2b). Conversely, a single PCR fragment, either *MAT1-2* or *MAT1-1*, was obtained from each mycelial strain irrespective of the number of PCR cycles performed (Fig. 2a, b, Table 1).

Starting from the above described partial *MAT* sequences, the two *T. borchii* idiomorphs were sequenced by adopting a strategy based on genome walking and IPCR and by using as template the two mycelial strains tb15 and tb16, harboring the *MAT1-2-1* and *MAT1-1-1* genes, respectively. As a result, 11.4- and 14.6-kbp-long contigs were assembled (Supplemental Fig. S1, Fig. 3).

Structure of T. borchii MAT idiomorphs and genes

The sequences within the *MAT* locus of *T. borchii* were compared to each other and with those of black truffle species (Fig. 3). The assembled contigs of the two *T. borchii* strains showed a nearly identical region of approximately 1000 bp, likely representing the common region flanking the 5′ end of the idiomorphs. In this region, an ORF (*TbOrf3*) was identified, although it was only partially sequenced (Fig. 3). This ORF shared high similarity (approximately 89 %) with *TiOrf3*, an ankyrin-containing gene previously identified within the *MAT1-2* idiomorph of *T. indicum* (Belfiori et al. 2013). In tb16, a sequence named *TbOrf2* was identified downstream the *MAT1-1-1* gene. A *TbOrf2*-like sequence was also present in the genomic region upstream of the *MAT1-2-1* gene in tb15 (Fig. 3). This sequence was indeed in an inverted orientation with respect to *TbOrf2* of strain tb16, and its putative coding sequence contained several stop codons. Because several attempts to extend downstream the partial *TbOrf2*-like sequence present in tb15 failed, the 3′ side of the idiomorphic region remains uncertain. Furthermore, in tb16, the putative 3′ region flanking the *MAT* locus showed a different gene composition with respect to *T. melanosporum*. More specifically, in tb16, another ORF was identified downstream of *TbOrf2*, which was named *TbOrf4* (Fig. 3). This ORF showed similarity with the origin recognition complex subunit 1 (ORC1) gene (e.g., XP_003710500; XP_003836193) of different fungal species, including the gene model GSTUMT00001242001 of *T. melanosporum*. This gene, however, is not linked to the *MAT* locus in *T. melanosporum* because in this species, it is located in a different genomic region.

A large portion of the non-coding regions of *T. borchii* idiomorphs also shared similar sequences, but in inverted orientation (Fig. 3, region A). Among these non-coding regions, some sequences also shared similarity with sequences present in the idiomorphs of the black truffle species. More specifically, next to the 5′ end of both *MAT* genes of all the truffle species considered, an 800-bp-long sequence was present (Fig. 3, region B).

Within the putative idiomorphic region of each strain, a single *MAT* gene, named *TborcMAT1-1-1* in tb15 and *TborcMAT1-2-1* in tb16, was identified. These two genes are in opposite orientation to each other and in inverted orientation with respect to those present in *T. melanosporum* and *T. indicum* (Fig. 3; Rubini et al. 2011b; Belfiori et al. 2013). However, the *MAT* genes of *T. borchii* showed a structure similar to those of *T. melanosporum* and *T. indicum*. The alignment of MAT1-2-1 cDNA and MAT1-1-1 EST sequences with those of the corresponding genomic DNA showed that the number and positions of introns were conserved: 2 and 3 in *TborcMAT1-1-1* and *TborcMAT1-2-1*, respectively. Only the first intron of both *T. borchii MAT* genes was longer than that of the corresponding one in the two black *Tuber* species (Supplemental Figs. S5a and S5b). The coding sequences of both *MAT* genes were also highly conserved among these *Tuber* spp. Compared with those of *T. melanosporum* and *T. indicum*, the TborcMAT1-1-1 and TborcMAT1-2-1 deduced proteins showed 86 and 80 %

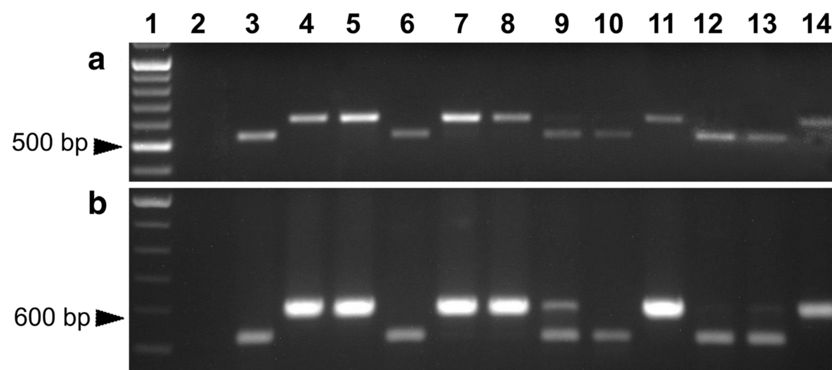

Fig. 2 Multiplex PCR amplification of DNA isolated from *T. borchii* mycelia and ascocarps. The b23, b33, b1, and b3 MAT-specific primers were used with either 25 (**a**) or 50 (**b**) PCR cycles, respectively. *Lane 1*, DNA ladder mix (Fermentas); *lane 2*, negative control (no DNA template); *lanes 3–6*, DNA from mycelium strains tb6, tb15, tb7, and tb16; *lanes 7–14*, DNA from ascocarps Tar325, Tar178, Tar146, Tar202, Tar210, Tar209, Tar165, and Tar240

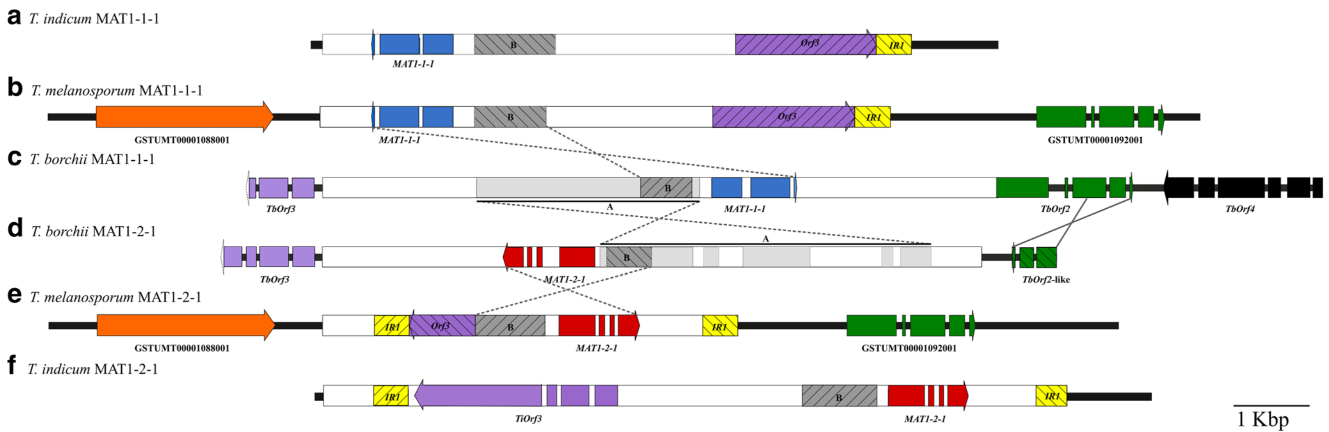

Fig. 3 Structure of *T. borchii MAT* locus compared with those of *T. indicum* and *T. melanosporum*. **a**, **f** MAT1-1 and MAT1-2 regions of *T. indicum* sample Ti_U986, respectively (Belfiori et al 2013). **b**, **e** MAT1-1 and MAT1-2 regions of *T. melanosporum*, respectively (Rubini et al 2011b). **c**, **d** MAT1-1 and MAT1-2 regions of *T. borchii*, respectively. The *white boxes* indicate the idiomorphic regions. The sequences (region A) shared between the *T. borchii* idiomorphs are indicated with light gray boxes. The *MAT1-1-1*, *MAT1-2-1*, *Orf2*, *Orf3*, *Orf4*, and GSTUMT00001088001 genes are indicated with *arrowed boxes*. The IR1 inverted repeat bordering the idiomorphs, the sequences (region B) conserved upstream the two *MAT* genes, and the degenerate ORFs sharing similarity to *Orf3* are indicated by *hatched boxes*. The *hatching pattern* and *dotted lines* indicate the inverse orientation of similar sequences

(85 % in the MATA_HMG region) sequence similarity, respectively. Most of the polymorphism was located at the C-terminal region in the MAT1-1-1 protein with the presence of only six amino acid changes within the α-domain (94–96 % of similarity, Fig. 4a) while amino acid changes were scattered all over the sequence in the MAT1-2-1 protein (Fig. 4b).

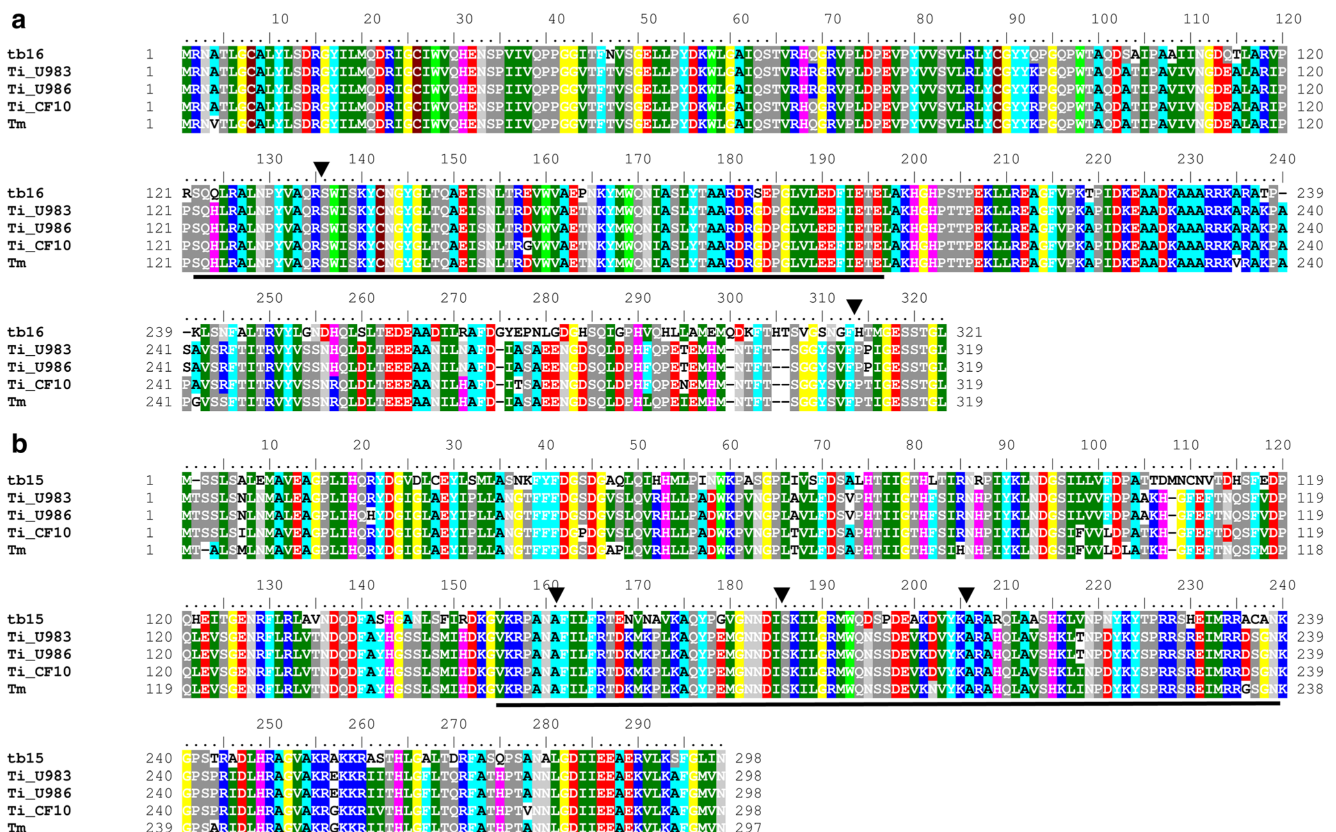

Fig. 4 Alignment of MAT1-1-1 (**a**) and MAT1-2-1 (**b**) encoded hypothetical proteins of *T. borchii* (tb16 and tb15), *T. melanosporum* (Tm) and *T. indicum* (Ti_U983, Ti_U986 and Ti_CF10). The *black triangles* indicate the conserved position of introns. The α1 and MATA_HMG domains are *underlined*

The two MAT proteins of *Tuber* also showed approximately 15 % of conserved residues distributed both within and outside the MATA_HMG and α1 domains (Fig. 5) and presence of evolutionarily conserved amino acids between the two functional domains when compared with other *Pezizomycotina* (Supplemental Fig. S6).

Discussion

In this study, we report the identification of the mating-type locus of *T. borchii*, a species belonging to the large group of whitish truffles. As this locus is organized into two idiomorphs harbored by different mycelial strains, with each idiomorph containing either *MAT1-1-1* or *MAT1-2-1*, it can be concluded that this species is heterothallic. Thus, *T. borchii* adds to the black truffles *T. melanosporum* and *T. indicum* among the *Tuber* spp. of economic relevance whose sexual reproductive mode involves obligate outcrossing. Additionally, by coupling the molecular analysis to the morphological and the karyological observations of mycelia isolated from ascocarps and mycorrhizas, we show that the life cycle of this species is prevalently haploid.

Our findings are important for improving the cultivation methods of *T. borchii.* The availability of *MAT1-1-1* and *MAT1-2-1* specific markers, for example, will enable us to evaluate the distribution of *T. borchii* strains with different mating type in the field. Similar researches recently performed for black truffle *T. melanosporum* revealed the importance of mating-type distribution for truffle production (Rubini et al. 2011a; Murat et al. 2013).

The identification of the *MAT* locus of *T. borchii* offers the possibility to approach unsolved questions in the reproductive biology of *Tuber* species using a model species alternative and/or complementary to *T. melanosporum.* Additionally, it will facilitate to unveil in the near future the mating type of other similar *Tuber* spp. and shed more light on species boundaries within the whitish truffle species complex.

***T. borchii* is heterothallic**

T. melanosporum was the first *Tuber* species where the *MAT* locus was characterized (Rubini et al. 2011b; Martin et al. 2010a) and represented the bedrock for successive identification of the homologous region in *T. indicum* (Belfiori et al. 2013). With the aim to identify the *MAT* locus of *T. borchii*, we started the present work by searching in public databases for *T. borchii* MAT sequences using as queries the *T. melanosporum MAT1-1-1* and *MAT1-2-1* genes. Whereas no putative orthologs of *MAT1-2-1* have been found, this in silico screening has allowed us to confirm the *T. borchii* EST AF487329 as a partial transcript of a *MAT* gene. This 718 bp EST was originally cloned by means of differential display between mRNA populations from ripe and unripe *T. borchii* fruit bodies in a study aimed at identifying genes involved in ascocarp development (Zeppa et al. 2002). Interestingly, these authors reported this EST to show 26 % of identity with respect to the mating-type protein mat1 of *A. brassicicola*, and to be neither expressed in free-living mycelia nor in ripe fruit bodies, but in unripe ascocarps only.

Thus, we designed specific primers on this putative *MAT1-1-1* gene to screen a set of *T. borchii* ascocarps. We observed that, using a low number of PCR cycles, the *MAT1-1-1*--specific band was produced only by a subset of the ascocarps analyzed. This result has been interpreted as a first indication

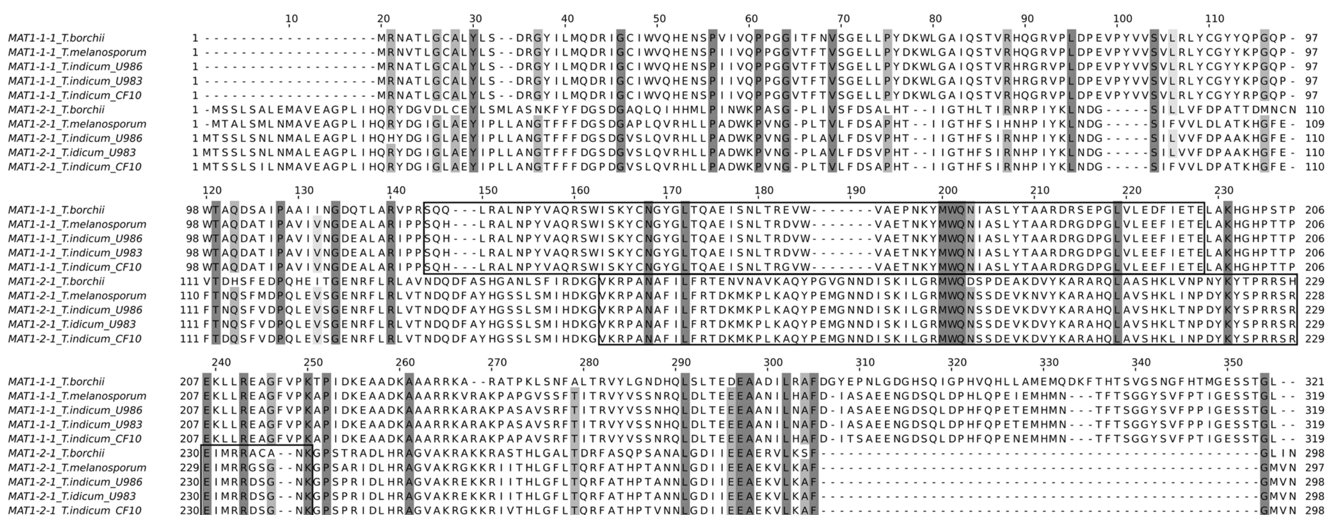

Fig. 5 Alignment of MAT1-1-1 and MAT1-2-1 deduced proteins of *Tuber* spp. The amino acid residues were shaded according to their % conserved identity. *Dark gray*, *gray*, and *light gray shading* indicate 100, 90, and 80 % identity threshold, respectively. The *boxes* indicate the α1 and MATA_HMG domains

that *T. borchii* is heterothallic and is consistent with our previous finding that the non-ascogenous tissue of the gleba in *Tuber* spp. is mainly formed by uniparental hyphae (Paolocci et al. 2006; Riccioni et al. 2008). To identify the mating type of *T. borchii*, we therefore pursued the same approach previously followed in *T. melanosporum* and *T. indicum* (Rubini et al. 2011b; Belfiori et al. 2013): the ascocarps that did not produce any PCR amplicon with primers specific for a given *MAT* gene were screened for the presence of the alternative one. Following this approach and by using sequence information from black truffles, we were eventually able to amplify a fragment of the putative *MAT1-2-1* gene.

The definitive evidence of heterothallism in *T. borchii* was obtained when we amplified DNA isolated from mycelia: regardless of the number of cycles performed, multiplex PCR with primers for the two putative *MAT* genes produced a single band, either *MAT1-2-1* or *MAT1-1-1*, in all 12 mycelial strains tested.

The MAT idiomorphs of *T. borchii* display extensive rearrangements with respect to those of black truffles

The *T. borchii* MAT region was isolated from two mycelial strains of opposite mating type named tb15 and tb16 by genome walking and inverse PCR. As results of these strategies, a 14.6- and 11.4-kbp-long contigs were assembled for tb15 and tb16, respectively. The sequencing of the MAT region from these two strains has confirmed an organization similar to that of *T. melanosporum* and *T. indicum*, with two idiomorphs (MAT1-1 and MAT1-2) each characterized by the presence of a single *MAT* gene, *TborcMAT1-1-1* and *TborcMAT1-2-1*, respectively. The *T. borchii MAT* genes show the same structure as those from *T. melanosporum* and *T. indicum*, and the corresponding deduced proteins share 80 to 86 % similarity with those of black truffles.

Because in tb15 the walking procedure did not allow us to clone the flanking region downstream of the *MAT* locus, the 3′ end of *T. borchii* idiomorphs remains to be defined. Notwithstanding, we observed some rearrangements and scarce gene synteny around the *MAT* locus of *T. borchii* compared with *T. melanosporum* and *T. indicum*. For example, *TbOrf4*, which is linked to *TbOrf2* and *MAT1-1-1* in tb16, is located elsewhere in the genome of *T. melanosporum*. The *Orf3* gene, located at the 5′ region of both *T. borchii* idiomorphs, is present within the MAT1-2 idiomorph in *T. indicum* (Belfiori et al. 2013), whereas only degenerate Orf3 sequences are present in the MAT1-1 idiomorph of *T. indicum* and in both idiomorphs of *T. melanosporum*. The *Orf2* gene, identified in both mating types of *T. melanosporum* downstream of the *MAT* locus (Rubini et al. 2011b) is located at the same position in *T. borchii.* However, in tb15, this sequence is present in inverse orientation with respect to tb16 and its putative coding sequence contains several stop codons. Thus, the Orf2-like gene detected in tb15 is not functional.

The MAT genes of *Tuber* provide insight in the evolution of the mating type in Pezizomycotina

The *MAT1-1-1* and *MAT1-2-1* genes are generally considered non-homologous; however, their evolutionary origin has been recently reconsidered (Martin et al. 2010b; Jackson et al. 2013). More specifically, the tertiary structure prediction and the presence of conserved amino acid residues suggested that the α1 and MATA_HMG domains may be related, and an evolutionary origin of α1 from an ancestral MATA_HMG domain has been proposed. The alignment of MAT1-1-1 and MAT1-2-1 proteins of *Tuber* spp. not only confirms the presence of these evolutionarily conserved residues between the MATA_HMG and α1 domains but also shows the presence of many conserved amino acid residues outside the two functional domains. Notably, the two *MAT* genes of *T. borchii* are in inverse orientation, and conserved sequences in inverse orientations between the two *T. borchii* idiomorphs are also present in non-coding regions. The presence of conserved sequences in inverted orientation within the *MAT* locus is a feature that characterizes the black truffles as well as other ascomycetes (Rubini et al. 2011b; Belfiori et al. 2013; Conde-Ferráez et al. 2007; Sun et al. 2012).

These observations reinforce the hypothesis that inversions and the resulting suppression of recombination may have driven the diversification of sex-specific alleles at the *MAT* locus in ascomycetes and other organisms (Idnurm 2011). Since *Tuber* spp. belongs to a basal clade within Pezizomycotina, it is likely that their MAT organization represents the ancestral form in this group of fungi.

Furthermore, in *T. borchii*, both *MAT* genes are inverted with respect to their homologs from the two black *Tuber* species. A possible evolutionary scenario may involve the presence of the short inverted repeats (IR) identified at or next to the borders of the idiomorphs of black truffles (Fig. 3, IR1 hatched boxes; Rubini et al. 2011b; Belfiori et al. 2013) but lacking from those of *T. borchii.* We speculate that the IR may have driven the inversion of the entire *MAT* locus of black truffles with respect to *T. borchii*, causing also the inclusion of some genes, such as *TiOrf3*, within the idiomorphic region of the former species. Mechanisms underlying the inversion of *MAT* loci and involving IR sequences have been described in yeasts and filamentous ascomycetes (Chitrampalam et al. 2013; Hanson et al. 2014) and might have concurred to rearrange and diversify the MAT regions of different *Tuber* lineages as well.

Finally, it is interesting to note that in addition to the *MAT* genes, a short region of approximately 800 bp linked at their 5′ ends is also shared among the *Tuber* species

here considered (Fig. 3, region B). The presence of this conserved sequence in the same position just upstream of each *MAT* gene suggests that it may be an important regulatory region. In turn, it can be speculated that the two *MAT* genes from different *Tuber* species are controlled by a putatively conserved regulatory mechanism. Specific analyses are needed, however, to evaluate this hypothesis.

T. borchii mycelia isolated from ascocarps and mycorrhizae are plurinucleate and homokaryotic and do not show morphological changes in dual cultures

The mycelial strains here isolated show a similar morphology when grown in Petri dishes, and although some differences in growth rate were detected, these are correlated neither with the isolation source (mycorrhiza or ascocarp) nor with the mating type. Neither was any relevant morphological change observed when tests of mating interaction were performed. Similar results were obtained in dual cultures of *T. melanosporum* strains (Iotti et al. 2012b). Frequently, in mycelial cultures 20–30 days old, coiled hyphae that may resemble sexual structures of ascomycetes (i.e., ascogonia) have been observed. However, these structures were formed in both dual- and single-mating cultures, and they most likely and simply derive from the collapse of the aerial hyphae on the agar surface. The occurrence of similar structures has been previously described in mycelial cultures of *T. maculatum* (Iotti et al. 2002). The lack of any detectable morphological change upon the interaction between free-living mycelia of opposite mating type suggests that physical contact is not sufficient, at least under in vitro conditions, to promote in sexually compatible strains the switch from the vegetative to the reproductive phase and that other environmental and/or biological factors are required. This is an expected result for a symbiotic species.

The karyological analysis of *T. borchii* mycelial strains isolated from both ascocarps and ECMs showed the presence of multinucleate cells containing a variable number of nuclei, which are frequently paired. The presence of paired nuclei in mycelia isolated in vitro, as well as in the hyphae emanating from the ECMs of different *Tuber* species (i.e., *T. melanosporum*), has been previously reported and was initially interpreted as a dikaryotic vegetative stage for these fungi (Lanfranco et al. 1995). However, analyses carried out using SSR markers have shown that gleba and ECMs in both *T. melanosporum* and *T. magnatum* are formed by haploid hyphae (Paolocci et al. 2006; Riccioni et al. 2008) and that individual *T. melanosporum* mycorrhizae and mycelia isolated in vitro have a single *MAT* gene (Rubini et al. 2011b; Iotti et al. 2012b). Likewise, the present study shows that the gleba of *T. borchii* ascocarps as well as mycelia isolated from both ascocarps and single ECM tips harbor either the *MAT1-2-1* or *MAT1-1-1* genes. Indeed, we never obtained isolates showing both mating types; thus, it is unlikely that the ECMs or the gleba are formed by heterokaryotic hyphae. Additionally, and in keeping with data from other *Tuber* spp. (Paolocci et al. 2006; Rubini et al. 2011a), the present observations allow us to argue that any *T. borchii* mycorrhizal root tip results from plant root colonization by a single mycelial strain. Thus, a plausible explanation for the presence of paired nuclei in *T. borchii* hyphae as well as in those of other *Tuber* spp. is the occurrence of synchronous mitosis or waves of parasynchronous mitosis that, starting from the apex, propagates to the neighboring cells. Such a phenomenon has been frequently observed in filamentous ascomycetes with multinucleated hyphae (Gladefelter 2006).

Overall, these observations corroborate our previous contentions that the *Tuber* spp. life cycle is prevalently haploid and, consequently, that fertilization and ascocarp formation are temporally linked in these symbiotic fungi (Riccioni et al. 2008).

Mating types and taxonomic implications

MAT genes are functional and rapidly evolving traits (Wik et al. 2008), which have proven useful to study the taxonomy of closely related fungal species (Turgeon 1998). In this regard, sequences of *MAT* genes and comparison of the idiomorphs' structure allowed us to discover the existence of cryptic species in *T. indicum* (Belfiori et al. 2013). *T. borchii* belongs to a group comprising many closely related species (Montecchi and Sarasini 2000; Jeandroz et al. 2008; Bonito et al. 2010, 2013), and the polymorphisms of the ITS and EF-1α sequences have recently suggested the presence of cryptic species within this taxon (Bonuso et al. 2010). Thus, *MAT* genes and idiomorphic sequences may represent ideal genomic regions for gaining further insights into the taxonomy and species boundaries within the whitish truffle species complex.

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Compliance with ethical standards

Conflict of interest The authors are named on a patent application entitled "Molecular method for the identification of mating-type genes of truffles species," serial number WO/2012/032098; PCT/EP2011/065501, filed on 07-09-2011 by CNR, Plant Genetics Institute and INRA.

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