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FEMS Microbiology Letters 235 (2004) 109–115

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Tuber aestivum and *Tuber uncinatum*: two morphotypes or two species?

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Received 22 January 2004; received in revised form 12 March 2004; accepted 8 April 2004

First published online 27 April 2004

Abstract

Tuber spp. are ectomycorrhizal fungi that establish symbioses with shrubs and trees. Because of their different smell and taste, *Tuber uncinatum* and *Tuber aestivum* are two truffle morphotypes with a different market value, but whether or not *T. uncinatum* and *T. aestivum* are different *taxa* is still an open debate among mycologists. In order to identify molecular keys characterizing both *T. aestivum* and *T. uncinatum* morphotypes, ITS/RFLPs analyses were carried out on a large collection of samples from all over Italy and from other European countries, followed by a study of the phylogenesis of ITS, β-tubulin and EF 1-α genes, on representative samples. The present study provides compelling evidence that: (i) *T. uncinatum* and *T. aestivum* belong to the same species, (ii) neither morphotype presents a specific molecular fingerprint, but they may even share identical alleles at any of the loci analysed; (iii) *T. aestivum* is most likely under a selfing reproductive mode. Our findings suggest that ecological, rather than genetic causes may account for differences in sporal morphology, taste and smell between *T. aestivum* and *T. uncinatum* truffles.

Keywords: *Tuber* spp.; Gene genealogy; ITS; ITS/RFLP; β-Tubulin; EF 1-α

1. Introduction

Tuber is a major genus of ectomycorrhizal fungi within *Pezizales* producing hypogeous fruit bodies, known as truffles. Some species belonging to this *genus* are economically important because they produce edible truffles with a distinctive flavour. Ascocarps of *Tuber* species are generally distinguished by their phenotypic characteristics, mainly based on morphological features of asci and spores. Fruit bodies belonging to *Tuber aestivum* Vittad., *Tuber uncinatum* Chatin or to the black Asian truffle species *Tuber indicum* Cooke and Massee and *Tuber himalayense* Zhang and Minter, generally display a large set of sporal configurations associated with quite pronounced molecular variability [1–5]. The presence in these *Tuber* spp., of strains exhibiting a *continuum* of morphological traits between two *taxa*, often makes it difficult to differentiate between varieties and species. Therefore, whether *T. uncinatum* is a *bona fide* species or only a subspecies of *T. aestivum* is still an open debate among mycologists [1,6–9]. Species differentiation is not merely a taxonomical issue, but has profound ecological and economical implications, such as marketing of both fruit bodies and nursery-inoculated host plants, which are largely used in reforestation programs for growing truffle species.

The present study focuses on extensive molecular analyses of fruit bodies which, on the basis of preliminary macro- and microscopic observations, had been ascribed to either *T. aestivum* or *T. uncinatum*. The aim was to verify whether phylogenetically important markers can allow species/strain discrimination between these two morphotypes and test *T. aestivum*/*T. uncinatum* samples for the presence of clonal or recombining individuals.

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0378-1097/$22.00
doi:10.1016/j.femsle.2004.04.029

2. Material and methods

2.1. Sample sources and DNA isolation

Morphological analyses were performed on fresh fruit bodies, by using distilled water as mounting media for microscopic analysis. *T. aestivum* and *T. uncinatum* characterization was based on a number of parameters previously reported [10,11]. In Table 1 are listed all the truffles examined, their collection sites and time. Genomic DNA was isolated from ascocarps basically according to Paolocci et al. [12].

2.2. PCR amplification, RFLP and sequence analysis of ITS

The universal ITS1/ITS4 primers were derived from White et al. [13]. PCR amplification was carried out as reported by Paolocci et al. [14], but with the addition in the PCR mixture of 10% (v/v) of DMSO. ITS was amplified from a total of 61 *T. aestivum*/*T. uncinatum* ascocarps.

About 30 ng of each PCR product was restricted using the endonucleases *Msp*I, *Mse*I and *Taq*I (Biolabs) according to the suppliers' instructions. The restricted fragments were separated on a 2% agarose gel. Double-strand sequence analyses were carried out as previously described [12].

2.3. Amplification and sequencing of the β-tubulin and EF 1-α genes

The forward primer Bt2a, derived from Glass and Donaldson [15] and the reverse primer Btspect (5′-GTCGGGGAACTCTTCACGGATCTTRGAG-3′) designed by aligning truffle β-tubulin genes cloned in our laboratory (unpublished results), were used to amplify a homologous β-tubulin gene fragment on the *T. aestivum*/*T. uncinatum* samples.

The primers EFtFw: 5′-TACAA(A/G)TGCGGTG-GTAT(T/C)GACAAGCG-3′ and EFtBw 5′-GACGC-ATGTCACGGACGGCGAAACG-3′ were designed on the conserved regions of the EF 1-α gene in *Ascomycetes*.

PCR amplification was carried out in GeneAmp PCR system 9700 using 10–20 ng of target DNA. The final aqueous volume of 50 μl contained 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer and 2.5 U Taq Polymerase (Amersham Pharmacia Biotech). The cycling protocol was as follows: an initial denaturation at 94 °C for 90 s, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 45 s (β-tubulin) or for 1 min (EF 1-α) for each cycle; the last cycle was followed by a final extension at 72 °C for 7 min. The β-tubulin and EF 1-α genes were amplified from 18 fruit bodies considered in the ITS sequencing analysis. In addition, for phylogenetic purposes, the same genes were also amplified from *Tuber mesentericum*, *Tuber melanosporum* and *Tuber magnatum*.

2.4. Sequence alignments and statistical analyses

The ITS, β-tubulin and EF 1-α sequence alignments were obtained using ClustalX software [16].

Sequence statistics, nucleotide diversity and distance-based analyses were performed using MEGA version 2.1 [17]. Phylogenetic trees were obtained using the Neighbor-joining method based on the two-parameter distance model of Kimura [18]. All gaps were excluded from phylogenetic analyses by selecting the ''complete deletion'' option in MEGA. Bootstrap tests were performed using 1000 replicates.

Congruence for the gene genealogies was inferred from maximum parsimony analysis (MP) by applying the permutation tail probability test (PTP) [19] and the incongruence length difference/partition homogeneity test (ILD/PHT) [20] as implemented in PAUP 4.0b10 [21]. Only parsimony informative characters were considered, and gaps were treated as new states. To give each locus equal weight, the informative characters (4, 2, 22 for β-tubulin, EF 1-α and ITS, respectively) were weighted reciprocally as reported by Kroken and Taylor [22].

3. Results

3.1. Morphological analysis of ascocarps

Differentiation between *T. aestivum* and *T. uncinatum* was achieved by morphological observation [10,11]. In agreement with Gross [11], those of the *T. aestivum*/*T. uncinatum* samples displaying an average 2 μm high sporal *reticulum* were ascribed to the *T. aestivum* morphotype, whereas those with a sporal *reticulum* averaging about 4 μm in height were ascribed to *T. uncinatum* (Table 1). However, several fruit bodies showed an intermediate sporal *reticulum* height. They are marked in Table 1 with ([b]) to indicate their uncertain classification.

3.2. Amplification and RFLP analysis of the ITS

Positive and highly reproducible PCR amplification of DNA from all the specimens examined was achieved by adding 10% (v/v) DMSO to the reaction mixture, suggesting the presence of a G/C rich region in the ITS of, at least, some truffles. Length polymorphism was detected between *T. aestivum*/*T. uncinatum* and *T. mesentericum* ITS fragments, which were about 700 and 685 bp long, respectively.

Table 1
ITS analysis on *T. aestivum*/*T. uncinatum* samples

Sample name	RFLP pattern			Haplotype	Collection site	Country	Collection date	Morphotype
	*Msp*I	*Taq*I	*Mse*I					
E1[a]	1	1	1	A	Abruzzo	Italy	26/5/96	*aestivum*
E7	1	1	1	A	Abruzzo	Italy	6/12/96	*aestivum*[b]
E17[a]	1	1	1	A	Abruzzo	Italy	6/12/96	*uncinatum*
E27	1	1	1	A	Abruzzo	Italy	20/03/97	*uncinatum*[b]
E28	1	1	1	A	Abruzzo	Italy	20/3/97	*aestivum*
E29[a]	1	1	1	A	Abruzzo	Italy	20/3/97	*aestivum*[b]
E31	1	1	1	A	Abruzzo	Italy	20/3/97	[c]
E37	1	1	1	A	Abruzzo-Molise	Italy	20/3/97	[c]
E60[a]	1	1	1	A	Umbria	Italy	1/8/97	*aestivum*
E4	2	1	1	B	Molise	Italy	26/5/96	*uncinatum*[b]
E5[a]	2	1	1	B	Molise	Italy	26/5/96	*uncinatum*
E15	2	1	1	B	Emilia Romagna	Italy	6/12/96	*uncinatum*
E38	2	1	1	B	Abruzzo	Italy	20/3/97	*uncinatum*
E18[a]	2	1	1	B	Abruzzo	Italy	6/12/96	*uncinatum*
E24[a]	2	1	1	B	Emilia Romagna	Italy	6/12/96	*uncinatum*
E75	2	1	1	B	Abruzzo	Italy	20/10/99	*aestivum*
E2[a]	2	1	2	C	Abruzzo	Italy	26/5/96	*aestivum*
E34	2	1	2	C	Abruzzo	Italy	20/3/97	*uncinatum*
E47	2	1	2	C	Umbria	Italy	1/8/97	*aestivum*
E3	2	2	2	D	Molise	Italy	26/5/96	*uncinatum*
E21	2	2	2	D	Emilia Romagna	Italy	6/12/96	*uncinatum*
E22	2	2	2	D	Emilia Romagna	Italy	6/12/96	*uncinatum*
E23	2	2	2	D	Emilia Romagna	Italy	6/12/96	*uncinatum*
E6	2	2	2	D	Abruzzo	Italy	6/12/96	[c]
E8	2	2	2	D	Abruzzo	Italy	6/12/96	*aestivum*
E26[a]	2	2	2	D	Abruzzo	Italy	20/3/97	*uncinatum*
E30	2	2	2	D	Abruzzo	Italy	20/03/97	*uncinatum*[b]
E32[a]	2	2	2	D	Abruzzo	Italy	20/3/97	*uncinatum*
E40[a]	2	2	2	D	Abruzzo	Italy	20/3/97	*uncinatum*
EP8[a]	2	2	2	D	Piemonte	Italy	16/11/98	*aestivum*
E61[a]	2	2	2	D	Umbria	Italy	1/8/97	*aestivum*
E58[a]	2	2	2	D	Umbria	Italy	1/8/97	*aestivum*
EP7[a]	2	2	2	D	Piemonte	Italy	16/11/98	*aestivum*
E50[a]	2	2	2	D	Umbria	Italy	1/8/97	*aestivum*[b]
E9	2	2	2	D	Abruzzo	Italy	6/12/96	*aestivum*[b]
E88	2	2	2	D	Istria	Slovenia	24/10/99	*aestivum*
E89	2	2	2	D	Istria	Slovenia	24/10/99	*uncinatum*[b]
E85	2	2	2	D	Istria	Slovenia	24/10/99	*aestivum*
E86	2	2	2	D	Istria	Slovenia	24/10/99	*aestivum*[b]
E113	2	2	2	D	Istria	Croatia	24/10/99	*aestivum*
E114	2	2	2	D	Istria	Croatia	24/10/99	*aestivum*
E119	2	2	2	D	Istria	Croatia	24/10/99	*aestivum*
E123	2	2	2	D	Istria	Croatia	24/10/99	*aestivum*
E72	2	2	2	D	Abruzzo	Italy	20/10/99	*aestivum*
E73	2	2	2	D	Abruzzo	Italy	20/10/99	*aestivum*
E74	2	2	2	D	Abruzzo	Italy	20/10/99	*aestivum*
E82	2	2	2	D	Abruzzo-Molise	Italy	22/10/99	*aestivum*
E129	2	2	2	D	Liguria	Italy	19/11/99	*aestivum*
E130	2	2	2	D	Liguria	Italy	19/11/99	*aestivum*
E131	2	2	2	D	Liguria	Italy	19/11/99	*aestivum*
E132	2	2	2	D	Liguria	Italy	19/11/99	*aestivum*[b]
E133	2	2	2	D	Liguria	Italy	19/11/99	*aestivum*[b]
E134	2	2	2	D	Lombardia	Italy	22/11/99	*aestivum*
E135	2	2	2	D	Lombardia	Italy	22/11/99	*aestivum*[b]
E136	2	2	2	D	Lombardia	Italy	22/11/99	*uncinatum*[b]
E137	2	2	2	D	Lombardia	Italy	22/11/99	*aestivum*
E138	2	2	2	D	Lombardia	Italy	22/11/99	*aestivum*
E139	2	2	2	D	Lombardia	Italy	22/11/99	*aestivum*
E140	2	2	2	D	Lombardia	Italy	22/11/99	*aestivum*
E141[a]	2	2	2	D	Lombardia	Italy	22/11/99	*uncinatum*
E142[a]	2	2	2	D	Unknown	France	20/12/99	*uncinatum*

Table 1 (*continued*)

The GenBank Accession Nos. are: ITS (AF516779, AF516781–AF516792, AF516798, AY226037–AY226039, AY226041, AY226042); β-tubulin and EF 1-α (AF516801–AF516807, AF516809–AF516813, AF516817, AF516819, AY170361, AY170365, AY226029, AY226030, AY226032, AY226033, AY226035, AF516769–AF516774, AY170348–AY170357, AY226023, AY226025–AY226028).

The same samples are deposited at Herbarium Dipartimento di Scienze ambientali, Università dell'Aquila (code AQUI265–AQUI282).

[a] Indicates samples on which sequence analysis of ITS, β-tubulin and EF 1-α genes was performed.

[b] Indicates fruit bodies showing an ambiguous sporal ornamentation pattern.

[c] Indicates samples having empty *asci*.

RFLP analysis of the ITS was initially performed using the enzyme *Msp*I, by which Guillemaud et al. [3] and Mello et al. [9] were able to identify three different RFLPs classes within their collections. Conversely, within our 61 samples this endonuclease revealed only two of the three *Msp*I patterns reported in the literature (Table 1, Fig 1(a)). The majority (85.2%) of *T. aestivum*/*T. uncinatum* ascocarps showed pattern 2 (*Msp*I-2), whereas pattern 1 (*Msp*I-1) was observed in the remaining 14.8%, most of which collected from the same region. Moreover, truffles showing the *Msp*I-2 pattern displayed a further polymorphism when RFLP analysis was performed with the enzymes *Taq*I and *Mse*I (Figs. 1(b) and (c)). The *Taq*I restriction enzyme revealed two different patterns in the *T. aestivum*/*T. uncinatum* samples, accounting for 31.1% and 68.9%, respectively; similarly, *Mse*I RFLP analysis revealed two patterns in the ratio 26.2–73.8%. The combination of three restriction enzymes therefore allowed us to discover more ITS/RFLP classes than had been identified previously [3,9]. Overall, this analysis revealed the presence of at least four different ITS classes, or haplotypes, named A, B, C and D, within our *T. aestivum*/*T. uncinatum* samples (Table 1 and Fig. 1), with D being the most frequent ITS haplotype (68.9%). Considering the additional *Msp*I pattern identified in previous studies [3,9], all the ITS *T. aestivum*/*T. uncinatum* genetic variability exhibited by truffles from different European collection sites can be grouped into five RFLP classes. Relationships between the RFLP classes and either morphological classification or truffle harvesting time and collection site did not emerge.

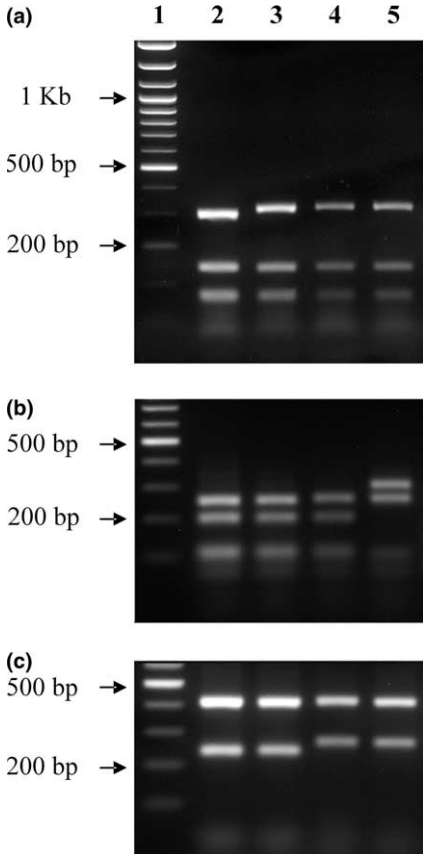

Fig. 1. RFLP analysis of the ITS region amplified with ITS1/ITS4 primers from *T. aestivum*/*T. uncinatum* ascocarps. (a) *Msp*I RFLP patterns. Lane 1: Gene Ruler DNA Ladder mix (Fermentas); lane 2: *Msp*I-1 on E1; lanes 3–5: *Msp*I-2 on E18, E2 and E58. (b) *Taq*I RFLP patterns. Lane 1: Gene Ruler DNA Ladder mix (Fermentas); lanes 2–4: *Taq*I-1 on E1, E18 and E2; lane 5: *Taq*I-2 on E58. (c) *Mse*I RFLP patterns. Lane 1: Gene Ruler DNA Ladder mix (Fermentas); lanes 2 and 3: *Mse*I-1 on E1 and E18; lanes 4 and 5: *Mse*I-2 on E2 and E58.

3.3. ITS sequence analysis

ITS sequencing was carried out on truffles selected from within the four RFLP classes according to the following criteria: morphotype, harvest time and collection sites (Table 1). For example, sequences were performed on two truffles, E61 and E32, which showed the same RFLP pattern, but were ascribed one to *T. aestivum* and the other to *T. uncinatum* based on morphological criteria and ripening times.

ITS sequences obtained from *T. aestivum*/*T. uncinatum* were aligned with those of *T. mesentericum*, *T. magnatum*, *T. melanosporum* and *T. indicum*, selected as reference species for phylogenetic analyses. The Neighbor-joining tree (Fig. 2(a)) showed that all the brown truffles [23], grouping *T. aestivum*/*T. uncinatum* and *T. mesentericum* fruit bodies, were well separated from the black truffles *T. melanosporum* and *T. indicum*, but had the most similarity with *T. magnatum*. Within the *T. aestivum*, *T. uncinatum* and *T. mesentericum* spp., the phylogenetic tree showed two major clusters corresponding to the *T. aestivum*/*T. uncinatum* and *T. mesentericum* complexes, respectively. Despite the high number of samples, the *T. aestivum* cluster was quite uniform (average nucleotide diversity = 0.75%). The ITS tree topology supported the classification of all the ITS

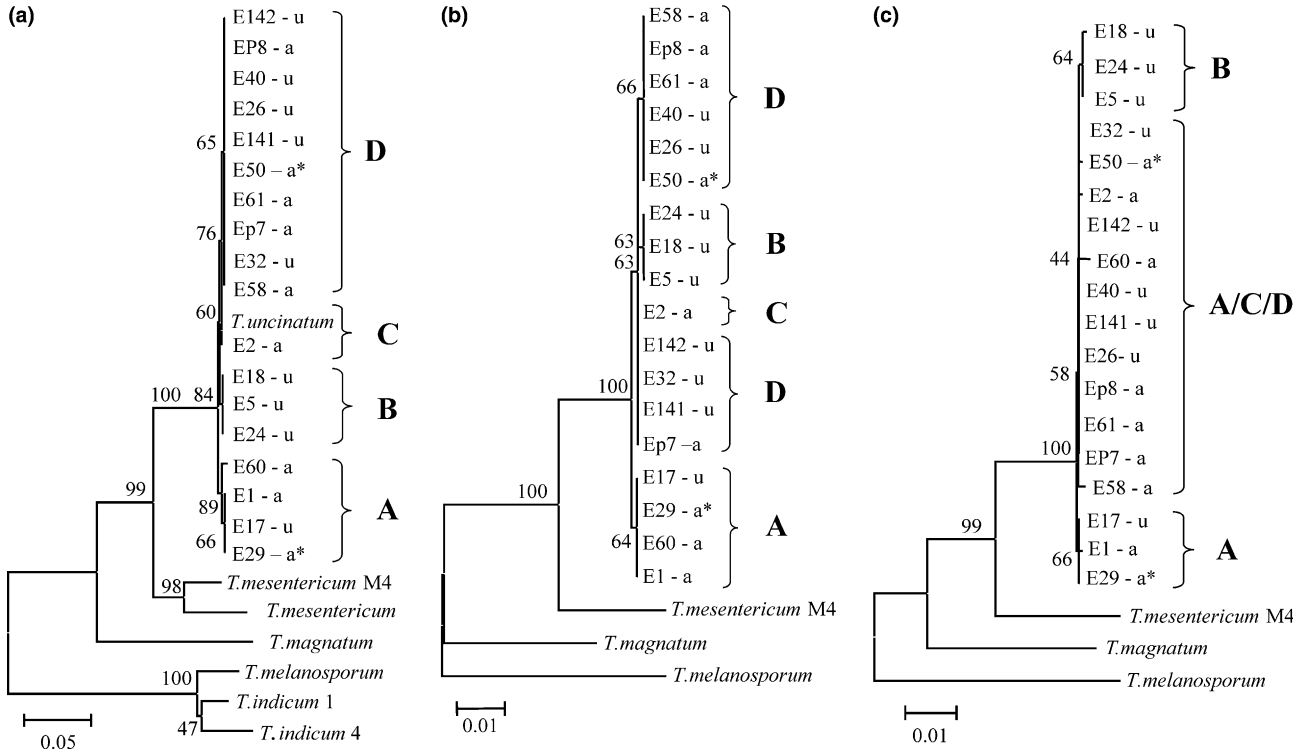

Fig. 2. Neighbor-joining trees obtained from: (a) ITS1-5.8s-ITS2, (b) β-tubulin and (c) EF 1-α sequences. *T. aestivum* (a), *T. uncinatum* (u) and ambiguous morphotype (*). Numbers near the branches represent the bootstrap values (%) from 1000 replicates. All the trees are rooted using *T. melanosporum* as outgroup. ITS sequences retrieved from GenBank are the following: *T. uncinatum* (AF132509); *T. mesentericum* (AF132508); *T. magnatum* (AF106888); *T. melanosporum* (U89359); *T. indicum*-1 (U89362); *T. indicum*-4 (AF106882). Letters from A to D indicate the different ITS alleles identified through RFLP analyses of the ITS region.

haplotypes revealed by RFLP analysis, while confirming the impossibility of splitting the *T. aestivum* and *T. uncinatum* samples into two separate groups.

3.4. Amplification and sequence analysis of the β-tubulin and EF 1-α genes

In order to better assess the degree of heterogeneity among and within the *Tuber* spp. considered in this study, a genomic fragment relative to β-tubulin and EF 1-α genes was PCR amplified on the 18 samples previously selected for ITS sequence analysis as well as on *T. mesentericum*, *T. magnatum* and *T. melanosporum*. Direct sequence analysis confirmed that only one amplicon per individual was obtained without sequence ambiguities. The identity of amplicons was confirmed by database search. The alignment of truffle β-tubulin sequences showed four different β-tubulin alleles within *T. aestivum*/*T. uncinatum*. Fig. 2(b) shows the Neighbor-joining tree derived from the alignment of the β-tubulin sequences considering both coding and non-coding regions. Overall, the *T. aestivum*/*T. uncinatum* cluster is quite homogeneous (average nucleotide diversity = 0.27%). The high degree of nucleotide identity of the coding region of the EF 1-α genes from different filamentous *Ascomycetes* allowed us to design the primer pair EFtFw/EFtBw and successfully amplify a fragment of this target gene in truffle species. All samples showed a 1.3-kbp long amplicon, with a protein-coding region split into four exons, as evidenced by the alignment with genomic and cDNA sequences of different fungal species. In truffle species, introns are conserved with respect to both number and position. Conversely, *Tuber* spp. showed a different intron organization from other filamentous fungi (data not shown). The *T. aestivum*/*T. uncinatum* sequences showed an extremely high conservation rate (average nucleotide diversity = 0.17%) and provided us with only two parsimony informative characters for phylogenetic concordance analysis (Fig. 2(c)).

In sum, all the trees relative to the loci analysed showed that: (1) brown truffles are phylogenetically closer to the white truffle *T. magnatum* than to the black truffle *T. melanosporum*; (2) *T. mesentericum* is the most likely *T. aestivum* sister *taxon* and (3) identical alleles are shared by samples exhibiting the *T. aestivum* or the *T. uncinatum* morphotype.

3.5. Gene genealogies

The sequencing of the three loci considered in this study on 18 *T. aestivum* samples provides us with a number of parsimony informative characters to test for congruence of gene genealogies.

We assessed congruence of gene genealogies using PTP and ILD/PHT tests. MP analysis of the 10,000 randomized data sets produced by the PTP test resulted in trees ranging from 268 to 360 steps. The observed tree length (132 steps) fell outside the range of tree lengths for the randomized data sets ($P = 0.0001$), allowing us to reject the null hypothesis of panmixia. On the other hand, all the trees resulting from MP analysis of the 10,000 randomized data sets produced by the ILD/PHT, were 132 step long like the sum of the original tree length for each locus, allowing us to accept the null hypothesis of clonality ($P = 1.00$).

The results of both statistical tests and the finding that no individual was found to be heterozygous at the loci analysed seem to corroborate the hypothesis of a strict self-fertilizing propagation system in *T. aestivum*, as previously reported on the basis of allozyme analyses [2,7]. The present data are however not sufficient to completely rule out the hypotheses of a long-term strict inbreeding and pseudohomothallism, since both reproductive modes could not generate random associations of alleles within and between loci [24].

4. Discussion

The complexity of the truffle life cycle combined with the difficulties to grow these fungi and the impossibility to mate them in controlled conditions, have been major obstacles to the understanding of the mating and of the propagation system of these hypogeous fungi. Hence, the species concepts are overwhelmingly phenotypic, but caution must be used when grouping truffles into species or strains/varieties based only on fruit body morphology alone, as most of the widely distributed truffle species include ascocarps with a high degree of polymorphism and divergent morphological traits. Only molecular-assisted screening of a large fruit body collection for each species can provide evidence about the reproductive system and species delimitation in these fungi. In the present study, therefore, capillary morphological and molecular analyses were carried out on *T. aestivum* and *T. uncinatum*, two widely distributed truffle morphotypes, to assess their genetic relationship. This is the first report on *Tuber* spp., where the sequences of the rDNA spacer regions have been contrasted with other genes, specifically the β-tubulin and EF 1-α genes, to identify population variation at the DNA sequence level. Recently, Mello et al. [9], differently from other authors [1,6–8], claimed successful differentiation of *T. uncinatum* from *T. aestivum* by means of ITS and ISSR analyses. For a deeper understanding of the genetic variability within these *taxa*, more than 60 samples from Italy, Istria and France, morphologically ascribed to either *T. aestivum* or *T. uncinatum*, were initially analysed by means of ITS/RFLPs. Although the RFLPs are co-dominant markers, they showed only one ITS allele on each of the truffles analysed, in accordance with Guillemaud et al. [3]. None of the ITS classes correlates either with the phenotypic ascocarp traits (e.g., the sporal *reticulum* height) or with the truffle ripening time. Also, no correlation between ITS polymorphism and ascocarp origin was found. Taken together, these data lend further support to the thesis that molecular-assisted analyses do not corroborate species distinction between *T. aestivum* and *T. uncinatum*. Differently from Mello et al. [9], our large-scale sample screening allowed us to detect more than a single ITS haplotype within *T. uncinatum* morphotypes, and, most importantly, *T. aestivum* samples sharing the same ITS haplotypes with *T. uncinatum* (Table 1). Therefore, the ITS locus is unlikely to be a diagnostic marker for differentiating *T. aestivum* from *T. uncinatum*. In addition, the overall genetic dissimilarity existing within the ITS of *T. aestivum*/*T. uncinatum* samples, as per this study, can be confidently confined within intraspecific variability when comparing the sequences of *T. aestivum* and *T. mesentericum*. Despite the fact that the latter species is a *T. aestivum* sister species, and some authors [25] even regard it as a *T. aestivum* variety, two clearly divergent clusters split the *T. aestivum*/*T. uncinatum* and the *T. mesentericum* complex apart. When the recently published *T. uncinatum* ITS sequences [9] were added to our ITS phylogenetic tree, they were found to be dispersed among the *T. aestivum* B, C and D classes (data not shown).

The same conclusion can be drawn by analysing the β-tubulin and EF 1-α phylogenetic trees (Fig. 2). Likewise, these analyses not only showed that *T. uncinatum* and *T. aestivum* are monophyletic, but also that they can share identical β-tubulin and 1-α elongation factor alleles.

Although multiple alleles for each of the three loci considered were revealed, no individual was found to be heterozygous at any locus. Furthermore, PTP and ILD/MH statistical analyses proved congruence among ITS, β-tubulin and EF 1-α gene genealogies. Molecular approaches different from gene genealogies, such as allozyme in *T. aestivum* [2,7] and microsatellites in *T. melanosporum* [26] prompted the authors to reject the hypothesis of heterothallism and put forward a homothallic or even an exclusive selfing propagation system in truffles. All the data herein reported point to a very close mating system in *T. aestivum*. Yet, more data and analyses are needed to assess whether no random associations of alleles within and between loci result indeed from primary homothallism, pseudohomothallism or even from a strict inbreeding on a long-term scale [24,27].

In conclusion, the data obtained with the three co-dominant markers used in this study strongly argue against the thesis that *T. uncinatum* and *T. aestivum* cluster into two separate groups [9]. On the contrary, these data lead us to suggest that specific, though still unclear, soil and climatic conditions may induce

T. aestivum to fruit under different conditions, which, in turn, could affect the flavour and aroma of the truffle and the sporal morphology of ascocarps, thereby giving rise to the *T. aestivum* and *T. uncinatum* morphotypes. Should this thesis be proved correct, there would be no point in continuing to use distinct marketing approaches for *T. aestivum* and *T. uncinatum* inoculated plants. This study paves the way for innovative and more ecology-oriented research approaches to determine whether environmental conditions alone may be sufficient to induce a given mycelial strain to fruit as either *T. aestivum* or *T. uncinatum*, since single truffle grounds or even single host plants producing both morphotypes were reported [7].

The cloning of more genomic loci and their analysis using a larger sample from all over Europe and specimens from truffle grounds producing both *T. aestivum* and *T. uncinatum* morphotypes, are now in progress in our laboratory. The expected results might further strengthen the hypotheses of a strict selfing propagation system in *T. aestivum*, and of an ecological pressure as the leading force that shapes *T. aestivum* plasticity.

Acknowledgements

The research was funded by the Italian National Research Council, Progetto Strategico ''Biotecnologie dei funghi ectomicorrizici: dalle applicazioni agro-forestali a quelle agro-alimentari''. Contribution no. 45 from the Institute of Plant Genetics, Division of Perugia.

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