Org Divers Evol (2013) 13:503–512
DOI 10.1007/s13127-013-0146-2

ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

A multigene phylogeny demonstrates that *Tuber aestivum* and *Tuber uncinatum* are conspecific

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Received: 4 February 2013 / Accepted: 6 July 2013 / Published online: 10 August 2013

Abstract For almost 2 centuries it has been disputed whether *Tuber aestivum* and *Tuber uncinatum* constitute two different species of truffles. Molecular markers have been applied previously to contribute to resolving this question, coming to different conclusions. In this study, we address this question by analyzing the genetic structure of truffles assigned to either of the two putative species from a geographically broad sampling across Europe. We used an approach involving multigene phylogenies and coalescent analyses of nine regions from five genes. All tests conducted supported the conspecificity of *Tuber aestivum* and *Tuber uncinatum*.

Keywords Phylogenetic analyses · Multi-marker study · *Tuber uncinatum* · *Tuber aestivum*

Introduction

The family *Tuberaceae*, described for the first time in 1822 (Du Mortier 1822), comprises seven genera, among them *Tuber* P. Micheli ex F.H. Wigg (Bonito et al. 2013). The commonly named "true truffles" belong to this genus. The latter exclusively includes hypogeous fungi, which establish symbiotic relationships (ectomycorrhizae) with different host trees within the families *Betulaceae*, *Fagaceae*, *Pinaceae*, and even *Tiliaceae*. In the genus *Tuber*, according to the *Index Fungorum*, more than 225 taxa are recognized (Jeandroz et al. 2008). In Europe, about 30 species have been found and described. Due to their famous organoleptic properties, some of them, such as *Tuber magnatum* (the Alba truffle) and *Tuber melanosporum* (the Périgord truffle), have high economic values. *Tuber* species have been generally differentiated and named based on ascomata and their phenotypic characteristics, using morphological features of asci and spores. But, with the increasing use of molecular tools in biology, species identification is usually supported by phylogenetic approaches. Nevertheless, even though new classifications based on molecular markers of the family *Tuberaceae* and the genus *Tuber* have recently been proposed (Bonito et al. 2010a; Jeandroz et al. 2008), ambiguities in species delimitation and phylogenetic placement remain for some species. *Tuber aestivum* Vittad. (the Summer truffle), for instance, described for the first time in 1831 by Carlo Vittadini (Vittadini 1831), and commonly present in Europe, has a maturation period during the summer and a light brown gleba (spore-bearing) with a black peridium. However, in 1887, Adolphe Chatin described a new truffle species, *Tuber uncinatum* Chatin. (the Burgundy truffle), very similar to *T. aestivum* (Chatin 1887). According to his description, the new species mainly differed from *T. aestivum* by the presence of hooks in the spore reticulum. This criterion was later shown to be an artifact, but for more than 2 centuries, scientists have been divided concerning the taxonomic classification of *T. aestivum* and *T. uncinatum* as other morphological and ecological criteria suggested considerable differences between these two taxa (Chevalier and Frochot 1997; Riousset

Electronic supplementary material The online version of this article (doi:10.1007/s13127-013-0146-2) contains supplementary material, which is available to authorized users.

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et al. 2001). Other morphology-based studies concluded that *T. uncinatum* and *T. aestivum* constitute a single species (Gandeboeuf et al. 1994; Mouches et al. 1981; Urbanelli et al. 1998). Using molecular marker genes, three major studies have focused on possible genetic differentiation between the two putative taxa. In 2002, Mello et al. using phylogenetic analyses mainly based on the Internal Transcribed Spacers (ITS), assigned the two forms to two different taxa (Mello et al. 2002). Paolocci et al. (2004), using RFLP analyses, ITS, beta-tubulin and elongation factor alpha sequences from a subset of the samples, concluded that they belong to a single species. The samples analyzed by Mello et al. (2002) and Paolocci et al. (2004) originated mostly or even exclusively from Italy; therefore, it seems unlikely that the whole genetic diversity of the two forms across their geographic range from Sweden to Spain through central Europe was addressed in these studies. Weden et al. (2005) analyzed possible correlations between the spore reticulum (which is the morphological character commonly used to distinguish *T. aestivum* and *T. uncinatum)* and ITS sequences in a broader geographical sample of fruit bodies. These authors came to the conclusion that the spore reticulum is not a useful diagnostic character and that *T. aestivum* and *T. uncinatum* were intermingled in the phylogenies, thus indicating conspecificity.

The contrasting conclusions obtained in the three cited studies may have been due to the differences in the geographic origin of the samples, the number of phylogenetic markers used or to the phylogenetic methods used. It is evident that a limited geographic sampling area can bias results toward genetic homogeneity. The small number of marker genes used may not have provided sufficient resolution for all taxa analyzed or may have resulted in weakly supported phylogenies. Indeed, gene history cannot always be equated to species history, for instance, due to horizontal gene transfer, deep coalescence or gene duplication (Maddison and Knowles 2006).

Due to the difficulties in using morphological and biological species concepts in fungi, which however are widely useful for many plants and animals, phylogenetic species recognition approaches have been proposed for fungi, requiring DNA sequences of multiple marker genes (Taylor et al. 2000). The advantage of these approaches is that they define precise tests for the probability that two taxa are conspecific.

It has to be stated that in spite of the long debate over the conspecificity of *T. aestivum* and *T. uncinatum*, this question to date has not been stringently addressed using multiple marker genes and a sufficiently broad geographic sampling. Therefore, our goal was to investigate the genetic diversity among *Tuber* samples assigned either to the *T. aestivum* morphotype or to the *T. uncinatum* morphotype by robust and powerful methods using multiple marker genes. *Tuber* ascomata were sampled from numerous European locations, and we performed a range of phylogenetic analyses using nine different genetic markers from five genes commonly used for phylogenetic analysis. We used the robust maximum likelihood and Bayesian inference methods for phylogenetic analyses and performed coalescent analyses, which are powerful tools to infer species history. Finally, several tests were used to study the potential genetic structure and geographic correlation within the sampling.

Materials and methods

Sampling, amplification and sequencing

Fungal samples were prepared from 26 *exsiccata* coming from natural habitats and previously assigned to either *T. aestivum* or *T. uncinatum* according to morphological criteria. Morphological aspects of spores were characterized, including spore dimensions and the reticulum and alveole shape (Chevalier et al. 1979). A lower reticulum height and less regular alveoles were considered to be characteristic for *T. aestivum*. Two outgroup taxa, *T. macrosporum* and *T. magnatum,* were added. Details about the samples analyzed are provided in Table 1. The *exsiccata* were deposited in the herbarium of the University of Lille (LIP). Genomic DNAs of the ascomata were isolated from 20 mg of dried gleba of each sample using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extracts were eluted in 190 μl of Buffer AE supplied in the kit. DNA concentration was estimated using a NanoDrop (Thermo Scientific) spectrophotometer. Nine different loci were amplified by polymerase chain reaction (PCR) from ascoma extracts: internal transcribed spacers 1 and 2, a fragment each of the nuclear ribosomal large subunit (nu LSU), the nuclear ribosomal small subunit (nu SSU), the mitochondrial ribosomal large subunit (mt LSU) and the mitochondrial ribosomal small subunit (mt SSU), the beta-tubulin (*β-tubulin*) gene, elongation factor 1-α (*EF1α*) and the *RPB2* gene, which encodes the second largest subunit of RNA polymerase II (RPB2_7_11). All PCR amplifications were performed using 50 ng of template DNA in a final volume of 20 μl containing 10X PCR Buffer (Invitrogen), 1 U of *Taq* DNA polymerase (Invitrogen), 0.125 mM of each dNTP, 0.5 μM of each forward and reverse primer, and sterile water to adjust the volume. For each locus, primer sequences, $MgCl_2$, DMSO and BSA concentrations (if added) and cycling conditions are listed in Table 2. One-directional Sanger sequencing was performed by GATC Biotech (Konstanz, Germany). Sequences were edited and manually trimmed using BioEdit 7.0.5.3 (Hall 1999). Sequences were queried against the NCBI public database GenBank using the BLASTN algorithm (Altschul et al. 1990) to verify that sequences were from *Tuber* or closely related to it for markers lacking *Tuber* reference sequences.

Table 1 Identity of samples, type indication and geographic location

ID	Type	Country	Location[§]	Collector	Harvesting date	Donor
CH2	*T. uncinatum*	France-Charente	Jarnac	G. Chevalier	2010	G. Chevalier
CH4	*T. uncinatum*	France-Charente	Jarnac	G. Chevalier	2010	G. Chevalier
D4	*T. uncinatum*	France-Côte d'Or	Daix	V. Molinier & H. Frochot	November 2010	V. Molinier & H. Frochot
D5	*T. uncinatum*	France-Côte d'Or	Daix	V. Molinier & H. Frochot	November 2010	V. Molinier & H. Frochot
E38	*T. uncinatum*	Hungary	Debrecen	Unknown	Unknown	G. Chevalier
E43	*T. uncinatum*	Luxembourg	Rumelange	Unknown	17 November 2002	G. Chevalier
E58	*T. uncinatum*	Switzerland	Lausanne	M. Groux	20 November 2000	G. Chevalier
E60	*T. uncinatum*	Turkey	Ankara	Unknown	1 July 1997	G. Chevalier
E70	*T. uncinatum*	UK	Bedford	Unknown	October 2004	G. Chevalier
E98	*T. uncinatum*	Romania	Bucharest	Unknown	November 2006	G. Chevalier
E101	*T. uncinatum*	Slovakia	Bratislava	Unknown	November 2003	G. Chevalier
E104	*T. uncinatum*	Sweden	Roma	C. Weden	November 2006	G. Chevalier
E122	*T. aestivum*	Spain	Granada	R. Ribes	January 2008	G. Chevalier
F1	*T. aestivum*	France-Corse	Santa-Lucia-di-Moriani	Unknown	June 2002	G. Chevalier
F4	*T. aestivum*	France-Alpes Maritimes	Menton	G. Chevalier	March 2003	G. Chevalier
F16	*T. aestivum*	France-Dordogne	Saint-Pantaly-d'Excideuil	M. Aynaud	Unknown	G. Chevalier
F23B	*T. aestivum*	France-Drôme	Montjoyer	P. Tabouret	Unknown	G. Chevalier
F25	*T. aestivum*	France-Hérault	Colombiers	M. Brayes	June 1992	G. Chevalier
F31	*T. aestivum*	France-Lot	Gignac	J. Delsol	June 1996	G. Chevalier
F65	*T. uncinatum*	France-Savoie	Gaillard	Unknown	3 November 2000	G. Chevalier
F74	*T. aestivum*	France-Vaucluse	Richerenches	Unknown	1 July 1987	G. Chevalier
F85	*T. uncinatum*	France-Yonne	Dyé	M. Jalade	23 August 2001	G. Chevalier
F95	*T. aestivum*	France-Gironde	Bordeaux	M. Castroviejo	April 2004	G. Chevalier
F103	*T. aestivum*	France-Bouches du Rhône	Saint-Rémy-de-Provence	L. Riousset	Unknown	G. Chevalier
F166	*T. uncinatum*	France-Aube	Vailly	M. Poinsot	October 2006	G. Chevalier
F171	*T. aestivum*	France-Aude	Quillan	Unknown	March 2001	G. Chevalier
MAC*	*T macrosporum*	Italy	NA	Gian Carlo Ponzi	2010	G.C Ponzi
MAG*	*T magnatum*	Italy	NA	Unknown	Unknown	C. Murat

*Outgroup samples

[§] Due to the confidentiality between authors and truffle providers, only names of the closest town or village are indicated

NA = Not available

Sequence alignments and phylogenetic analyses

Sequences were aligned using Clustal W program (Thompson et al. 1994) and manually corrected. DNA sequences generated in this study have been deposited in EMBL as accessions HE601903-HE601930 and HE602558-HE602780. First, alignments from each gene were used to perform phylogenetic analyses using maximum likelihood (ML) and Bayesian inference (BI) in parallel. Second, all sequences from the different genes for each sample were concatenated together in a supermatrix to perform multigenic analyses using Mesquite v.2.75 software (Maddison and Maddison 2011). ML and BI were also used for this global analysis. Models of sequence evolution for the nine genes separately and also for the supermatrix data set were evaluated using the program JModel Test (Posada 2008). Negative log likelihoods of different models of nucleotide substitution were compared using the Akaike information criterion (AIC) model selection criterion (Posada and Buckley 2004). Maximum likelihood and Bayesian analyses were inferred using PhyML 3.0 online (Guindon et al. 2010) and MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), respectively. Branch support was assessed by using the nonparametric bootstrapping under maximum likelihood, and posterior

Table 2 Target loci, primer sequences and special PCR conditions used during this study

Locus	Primers and sequences (5'-3')[a]	References	BSA, DMSO and $MgCl_2$ concentrations	PCR conditions
β-Tubulin	Bt2a: GGTAACCAAATCGGTGCTG CTTTC	(Glass and Donaldson 1995)	$MgCl_2$ 1.5 mM	95 °C: 3 min
	Bt2b: ACCCTCAGTGTAGTGACCC TTGGC	(Glass and Donaldson 1995)		35×(94 °C: 30 s, 59 °C: 45 s, 72 °C: 1 min)
	Btspect: GTCGGGGAACTCTTCACG GATCTTRGAG	(Paolocci et al. 2004)		72 °C: 10 min
EF1α	EF1aTub_for2	(Bonito et al. 2013)	BSA: 0.9 µg / µl	94 °C: 1 min 30 s
	EF1aTub_rev1	(Bonito et al. 2013)		24×(94 °C: 30 s, 55 °C: 30 s (-0.4 °C each cycle), 72 °C: 1 min 30 s (+0.02 min each cycle)
				12×(94 °C: 30 s,
				45 °C: 30 s, 72 °C: 2 min)
				72 °C: 10 min
ITS1	ITS1: TCCGTAGGTGAACCTGCGG	(White et al. 1990)	$MgCl_2$ 1.5 mM	94 °C: 3 min
	ITS2: GCTGCGTTCTTCATCGATGC	(White et al. 1990)	DMSO: 10 %	35×(94 °C: 1 min, 56 °C: 1 min, 72 °C: 1 min)
				72 °C: 10 min
ITS2	ITS3: GCATCGATGAAGAACGCAGC	(White et al. 1990)	$MgCl_2$ 1.5 mM	94 °C: 3 min
	ITS4: TCCTCCGCTTATTGATATGC	(White et al. 1990)	DMSO: 10 %	35×(94 °C: 1 min, 56 °C: 1 min, 72 °C: 1 min)
				72 °C: 10 min
mt LSU	ML3: GCTGGTTTTCTACGAAACAT ATTTAA	(White et al. 1990)	Nothing added	94 °C: 3 min
	ML4: GAGGATAATTTGCCGAGTTCC	(White et al. 1990)		35×(94 °C: 45 s, 50 °C: 45 s, 72 °C: 1 min)
				72 °C: 10 min
mt SSU	MT1: TACGTGCCAAGCAGTCGC GGTAATACG	(Guillemaud et al. 1996)	Nothing added	94 °C: 3 min
	MT2: TACTCTTGAGGTGGAATG CTTACAC	(Guillemaud et al. 1996)		40×(94 °C: 45 s, 55 °C: 45 s, 72 °C: 1 min)
				72 °C: 10 min
nu LSU	LR1: GCATATCAATAAGCGGAGGA	(van Tuinen et al. 1998)	Nothing added	94 °C: 3 min
	NDL22: TGGTCCGTGTTTCAAGACG	(van Tuinen et al. 1998)		35×(94 °C: 30 s, 58 °C: 30 s, 72 °C: 1 min)
				72 °C: 10 min
nu SSU	SSU1: CAGAGGTGAAATTCTTGGAT	(Molitor et al. 2010)	Nothing added	94 °C: 3 min,
	SSU2: TGTGTACAAAGGGCAGGG	(Molitor et al. 2010)		35×(94 °C: 45 s, 50 °C: 45 s, 72 °C: 2 min)
				72 °C: 10 min.
RPB2 7_11	RPB2V_7_11_F1: GGCAAATATTCTT TACTACC	In this study	Nothing added	94 °C: 3 min
	RPB2V_7_11_R1: CTCCTTCAAACC CTCGTAATG	In this study		15×(94 °C: 45 s, 55 °C: 45 s, 72 °C: 1 min)
				20×(94 °C: 45 s, 50 °C: 45 s, 72 °C: 1 min)
				72 °C: 10 min

[a] IUPCA degenerate nucleotides: M, AC; R, AG; W, AT; Y, CT

probabilities were assessed under Bayesian inference. Detailed information on the implementation of all phylogenetic methods is listed in Supplementary Table 1.

Incompatibility of the phylogeny obtained from the data with the hypothesis of monophyly of *T. aestivum* and *T. uncinatum* was assessed using the Wilcoxon signed rank test

(Templeton 1983) as implemented in PAUP*4.0 (Swofford 2002).

Coalescent analyses

The software BEAST was used to perform coalescent analysis gene by gene but also with the supermatrix data (Drummond and Rambaut 2007; Drummond et al. 2012). For all cases, a relaxed clock with an uncorrelated lognormal distribution (Drummond et al. 2006) and a coalescent constant size process as tree prior were used. We performed two independent runs for 150 millions of generations, sampling every 1,000 generations, 10 % of which were discarded as burn-in. To check for convergence (ESS>200), Tracer software (Rambaut and Drummond 2007) was used. The results were obtained in the TreeAnnotator software (Rambaut and Drummond 2006) and visualized in Figtree (Rambaut 2009).

Diversity indices: polymorphism vs. divergence

The 26 *T.aestivum-uncinatum* samples were analyzed according to the two putative taxa *T.aestivum* and *T. uncinatum*. For each independent analysis, the polymorphism within each group and the degree of divergence between them and toward each outgroup taxon were calculated.

For each target locus and for the super matrix, diversity indices [polymorphic sites (v), singleton variable sites (s), haplotype diversity (H), nucleotide diversity (π) and Fst] were calculated to study the polymorphism within each of the two target groups (*T. aestivum* and *T. uncinatum* groups) (polymorphism) and the genetic divergence between them and the outgroup taxa (divergence). All the diversity indices were calculated using DnaSP v5 software (Librado and Rozas 2009).

Structure analyses

Structure software was used to determinate the potential numbers of clusters existing in our data without an *a priori* point of view. Structure estimates the log probability of the data for each value of K (number of clusters). A series, if independent runs were performed, used K=1-25 populations, a burn-in of 40,000 Markov chain Monte-Carlo (MCMC) iterations and a data collection period of 1,500,000 MCMC iterations. We used the admixture model in which the fraction of ancestry from each cluster is estimated for each individual. Each simulation of K was replicated 20 times. K was chosen on the basis of the second order rate of change of the log likelihood function (ΔK) with respect to K. ΔK was plotted against K (Evanno et al. 2005).

Geographical correlation test

A Mantel test was performed here using XLSTAT (*Addinsoft*, New York City, *NY*, USA) to study the correlation between the genetic distance and geographical distance within the *T.aestivum/uncinatum* group including the 26 individuals. The genetic distance matrix was obtained using Mega v.5 (Tamura et al. 2011) by a pairwise comparison for each couple of sequences. The geographical distance matrix was obtained using the number of kilometers between two samples (i.e., their respective location) via their respective global positioning system (GPS) data. Ten thousand random permutations were performed during this test.

Partition homogeneity test

To test for significant non-congruence among the different marker genes, a partition homogeneity test was performed using PAUP*4.0 (Swofford 2002). The test was performed between the following partitions: nuclear ribosomal gene regions, beta-tubulin, EF1-α, rpb2 and mitochondrial ribosomal genes.

Results

Amplification, phylogenetic and coalescent analysis

For *Tuber aestivum-uncinatum* samples, fragments of 519, 648, 791, 287, 252, 356, 536, 670 and 663 bp were amplified from ITS1 and ITS2, the nuLSU gene, nuSSU gene, mtLSU gene, mtSSU gene, β-tubulin gene, EF 1-α gene and RPB2 7-11 genes, respectively.

PCR amplifications were successful for all isolates and genes except for the amplification of the β-tubulin gene from *T. macrosporum*. This was coded in the supermatrix as "missing data."

For the ITS1 and ITS2, it was necessary to add DMSO (10 %) to obtain sequences of good quality, indicating a high GC content, which is confirmed by sequence analyses (Table 3a). These results are congruent with those of Paolocci et al. (2004). In the combined (concatenated) data set matrix of the 26 taxa consisting of 4,722 characters, 103 sites were polymorphic (2.18 %), and 64 were singleton variable sites.

In some cases, polymorphism for a given gene was very low, resulting in a lack of resolution in the phylogenetic trees (see Suppl. Fig. 1). Clusters of sequences found using one gene were rarely recovered with another gene and generally relatively poorly supported. The partition homogeneity test showed significant discordance (P=0.01) between the phylogenies based on nuclear ribosomal genes and EF 1-α, but not for any of the other possible combinations. It has been disputed whether genes giving discordant phylogenetic

Table 3 Genetic diversity indices within the *Tuber aestivum / uncinatum* group (3.a) ; divergence between *Tuber aestivum/ uncinatum* groups and *Tuber macrosporum* (MAC) and *Tuber magnatum* (MAG) (3.b) and Fst values calculated between pre-assigned *Tuber aestivum* and pre-assigned *uncinatum* groups (3c) for each target locus separately and the supermatrix (concatenated genes)

	3a						3b		3c
Target gene	G+C content (%)	Polymorphic sites (v)	Singleton variable sites (s)	Haplotypes (nH)	Haplotype diversity (H)	Nucleotide diversity (π) (per site)	Nucleotide divergence to MAC (%)	Nucleotide divergence to MAG (%)	Nucleotide divergence between MAG and MAC (%)	Fst values between aestivum group and uncinatum group
nuLSU	54.3	11 (2.11 %)	10	8	0.674	0.00228 (±0.00055)	0.07916	0.12573	0.11569	0
nuSSU	50.5	7 (1.08 %)	4	6	0.652	0.00184 (±0.00045)	0.00572	0.00572	0.0062	0.06015
mtLSU	32.6	3 (0.38 %)	2	4	0.345	0.00047 (±0.00017)	0.01324	0.03057	0.02372	0.03571
mtSSU	34.8	1 (0.35 %)	1	2	0.077	0.00027 (±0.00024)	0.01066	0.00716	0.00703	0
ITS1	67.5	17 (6.75 %)	7	17	0.951	0.02068 (±0.00169)	0.49122	0.6014	0.68067	0.11299
ITS2	61.8	17 (4.79 %)	8	9	0.748	0.00844 (±0.00184)	0.39121	0.36989	0.35543	0.06139
β-Tubulin	48.1	4 (0.75 %)	2	5	0.622	0.00156 (±0.00029)	-	0.09708	-	0.05473
EF 1-α	46.5	34 (3.58 %)	23	20	0.978	0.00756 (±0.00146)	0.12262	0.07762	0.12124	0.01214
RPB2 7-11	47.3	9 (1.36 %)	7	4	0.545	0.00219 (±0.00067)	0.1064	0.04662	0.09131	0.06825
Super matrix	47.4	103 (2.18 %)	64	26	1	0.00383 (±0.00032)	0.09683	0.09289	0.10578	0.05902

signals can be analyzed together (Cunningham 1997); in any case, we chose to present the results of the concatenated data set (Fig. 1) side by side with the single gene phylogenies, some of which have a low resolution. In the phylogenetic analyses for genes concatenated in a super matrix, maximum likelihood and Bayesian trees showed the same topology (Fig. 1). A single tree is presented with bootstrap values and posterior probabilities greater than 70 % and 0.95, respectively. Neither trees from the concatenated data set nor the single-gene phylogenies showed a separation of *T. aestivum* and *T. uncinatum* or only larger clusters exclusively composed of one or the other type. In fact, both types appear to be randomly distributed over the trees. The Wilcoxon signed-rank test showed that parsimony trees obtained under the constrained of monophyletic *T. uncinatum* and *T. aestivum,* respectively, were significantly worse ($P<0.026$) than those obtained without the constraint.

For the gene-by-gene analysis, different topologies were obtained (Supplementary Fig. 1), but in the majority branches within the *aestivum-uncinatum* cluster were not well supported in ML and in BI.

Regarding phylogenetic analyses for genes concatenated in a super matrix, maximum likelihood and Bayesian trees were identical with the same topology (Fig. 1). A unique tree is presented with the bootstrap values and posterior probabilities greater than 70 % and 0.95, respectively. EF 1-α sequences were included.

Coalescent analyses were performed for 150 million generations (two independent runs). The coalescent analyses conducted with the concatenated super matrix data resulted in the same topology as the phylogenetic analyses (Fig. 2). Only two samples (F25 and E43) were related to group 1 in the coalescent analyses and with group 2 in the phylogenetic analyses. Analyses of single genes resulted in different topologies of low support (Supplementary Fig. 2).

Diversity indices calculation

Regarding the genetic diversity of the 26 samples within *T. aestivum* group, and when taking into account each target locus, the diversity indices such as polymorphic sites, haplotype diversity and nucleotide diversity were low (Table 3a). Indeed, on average, we found an average of 2.35 % variable sites (from 0.35 % for mtSSU to 6.75 % for ITS1). The values ranged from 2 to 20 for the number of haplotypes with on average 8.33 haplotypes per locus. The average of the nucleotide diversity was 0.005, ranging from 0.00027 to 0.020. We observed heterogeneity in the genetic diversity between target loci. Mitochondrial DNA seemed to be more stable in this taxon than nuclear DNA. Indeed, few haplotypes were observed for the mt SSU and mt LSU, with the weakest nucleotide diversity of the nine markers analyzed. This is congruent with previous results dealing with *T. aestivum* (Guillemaud

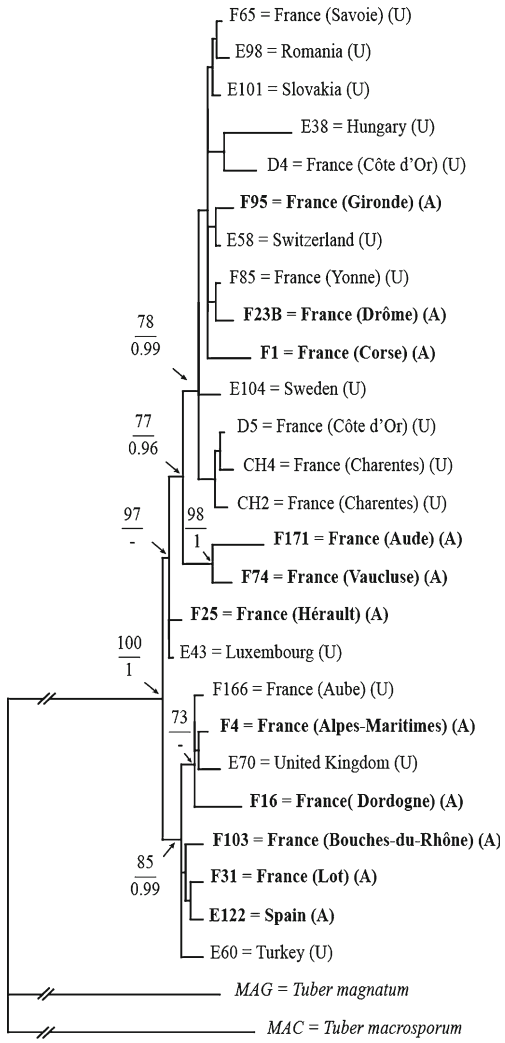

Fig. 1 Phylogenetic relationships among the 26 *Tuber aestivum-uncinatum* isolates inferred using maximum likelihood (ML) and Bayesian inference (BI) from the concatenated nine-gene data set (4,722 bp total). The same topology was obtained for both phylogenetic analyses after 1,000 bootstrap replicates for ML and 2,000,000 generations for BI using the GTR+G model for both analyses. The tree is rooted with *T. macrosporum* and *T. magnatum* (in italics). Only bootstrap values higher than 70 % (*number above*) and posterior probabilities higher than 0.95 (*number below*) are indicated. The two pre-assigned types *T. aestivum* and *T. uncinatum* are indicated by *A* (*boldface*) and *U*, respectively. The geographic origin is indicated after for each sample ID

et al. 1996). ITS1 and ITS2 showed the highest nucleotide diversity values in our analysis, with ITS1 being more variable than ITS2. Regarding the protein-coding regions, on one hand, the elongation factor 1α gene showed a considerable degree of polymorphism with 20 different haplotypes for 26 total individuals with a nucleotide diversity reaching 0.00756. On the other hand, the β-tubulin gene and RPB2 7-11 gene had very low nucleotide diversity (0.00156 and 0.00219, respectively) with only five and four haplotypes, respectively. This relative heterogeneity between different protein gene coding regions confirms the usefulness of multigenic analysis to avoid interpretation errors. The super matrix data analysis confirmed the low diversity within this fungal group with only 103 out of 4,722 variable sites (2.18 %) and nucleotide diversity per site reaching 0.00383. However, no two samples showed identical sequences; thus, each haplotype was unique (e.g., haplotype diversity = 1.0). When comparing the *T. aestivum-uncinatum* group with *T. macrosporum* and *T. magnatum* (Table 3b), the supermatrix showed a divergence of 9.68 % and 9.28 %, and 10.57 % between *T. macrosporum* and *T. magnatum,* respectively. These three different species are well differentiated using the molecular tools.

We used the fixation index, F_{ST}, which is an estimation of population differentiation by measuring the diversity of randomly chosen alleles within the same sub-population relative to that found in the entire population. It is often expressed as the proportion of genetic diversity due to allele frequency differences among populations. This comparison of genetic variability within and between populations is frequently used in the field of population genetics, with values range from 0 to 1. A zero value implies complete panmixis, that is, that the two populations are interbreeding freely, and a value of one would imply the two populations are completely separate.

When the different values were calculated between the "*aestivum pre-assigned group*" and "*uncinatum pre-assigned group,*" the F_{ST} values were very weak (from 0 to a maximum of 0.06) (Table 3c). According to Wright's diversity scale, those values indicate no differentiation between these two potential populations.

Genetic structure analysis and geographical correlation test

After performing structure analysis according to the method by Evanno et al. (2005), two clusters were defined without *a priori*. Nevertheless, ΔK values were very weak with low differences between them. These model-based clustering analyses showed a typical pattern of unstructured populations. This point was confirmed by the fact that for K=2, the proportion of the sample assigned to each population was roughly symmetric (1/K, i.e., 0.5 in each population) (Fig. 3). For others K values (3-17), similar patterns were observed, meaning that the sample assigned to each population was nearly symmetric (1/K) (data not shown). All these points confirmed that it is not possible to differentiate the *T. aestivum uncinatum* samples into two subgroups.

For the geographical correlation test, a correlation index R equal to 0.026 with a *p*-value>0.05 (Supplementary Fig. 3) was obtained. We cannot reject the null hypothesis indicating an absence of correlation between the two matrices, meaning that there is no significant correlation between the geographical and genetic matrices.

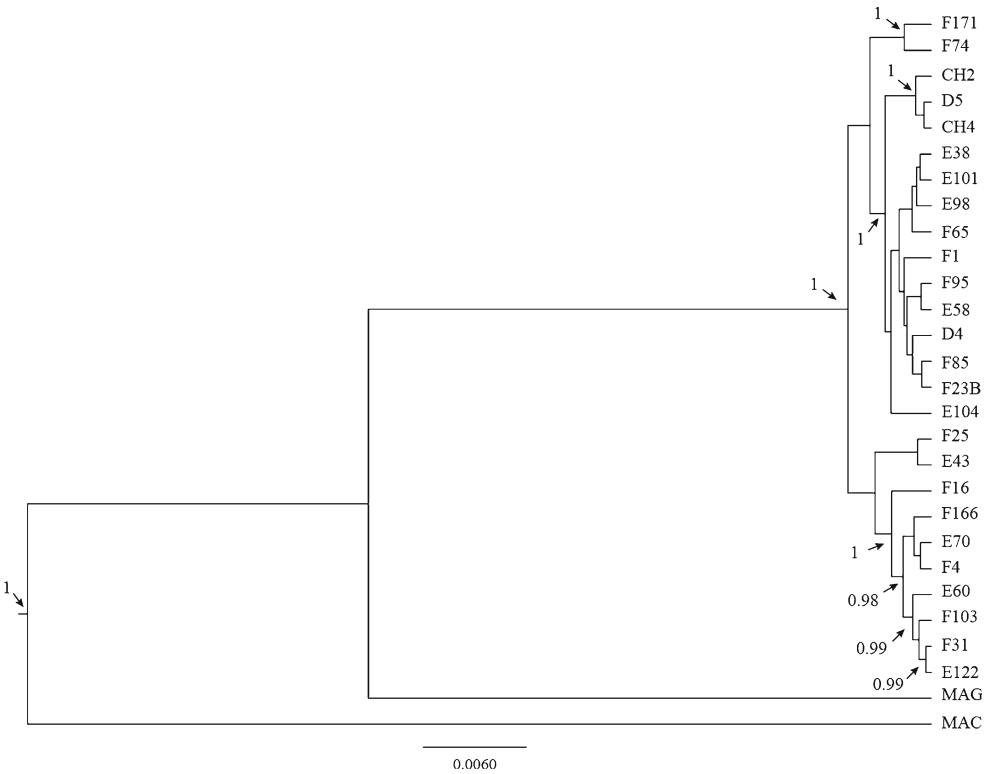

Fig. 2 Coalescent tree reconstruction for all concatenated genes. Only posterior probabilities higher than 0.95 are indicated. The tree is rooted with MAC *(T. macrosporum)* and MAG *(T. magnatum)*

Discussion

The question addressed in this study, the possible conspecificity of *T. aestivum* and *T. uncinatum*, has been disputed for a long time. Although several studies have used molecular markers to answer this question, with sometimes contradictory conclusions, this is the first study to test this problem using a rigorous multilocus phylogenetic approach on a broad geographic sampling of morphotypes assigned to the two putative species.

Morphological characters for separation have proven to be unreliable as they show considerable variation in the *T. uncinatum/aestivum* group across sampling times and sampling sites. For diagnosing reproductive isolation as a criterion for two separate species, a single marker gene is considered insufficient, necessitating a multigene approach (Rokas et al. 2003). We used nine molecular marker genes that have been successfully used to distinguish other truffle species (Jeandroz et al. 2008; Paolocci et al. 2004; Wang et al. 2006; Bonito et al. 2010a; Roux et al. 1999; Bonito et al. 2010b) and other fungi (Gardes and Bruns 1993; Hansen et al. 2005; Larsson and Jeppson 2008; Matheny 2005; Matheny et al. 2002; Spatafora et al. 2006; Stockinger et al. 2009).

Phylogenetic analyses yielded no support for a separation of *T. uncinatum* and *T. aestivum* either in single gene phylogenies or based on the concatenated data set. Indeed, even if some polymorphism was found, its level was far below that found when comparing *T. aestivum/uncinatum* with *T. macrosporum* or *T. magnatum*. A partition homogeneity test for non-congruence of the phylogenies of the different gene loci, indicative of a recombining population and proposed as one indicator of conspecificity (Geiser et al. 1998), showed significant discordance between the two genes.

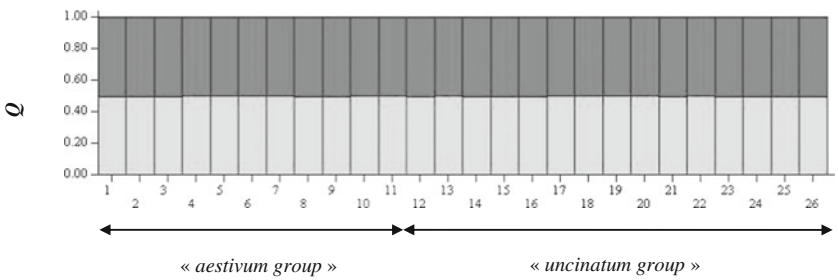

Fig. 3 Structure analysis. Summary plot of estimates of Q (membership) in the two defined clusters for the European data set with the 9 markers in the 26 individuals with K=2. Each individual is represented by a *single line* broken into K *gray-colored segments*, with lengths proportional to each of the K inferred clusters

Based on these analyses, we conclude, in accordance with some previous studies, that *T. uncinatum* and *T. aestivum* are conspecific. The observed genetic differences do not explain and do not justify the existence of two different species, in agreement with morphological (Weden et al. 2005) analyses and aroma studies (Splivallo et al. 2012). The desire to separate these two taxa is more the expression of a cultural or regional attachment rather than based on scientific evidence.

Following the priority rules of botanical nomenclature, the name *T. aestivum* has priority because *T. aestivum* was described by Vittadini in 1831, long before *T. uncinatum* (Chatin 1887).

In the future it will be interesting to address the possible differentiation of *T. aestivum* to different environments across its wide range, using more sensitive molecular markers, such as microsatellites.

In France, local legislation regulates the harvesting periods. For example, legislation generally requires that *T. aestivum* must be harvested between June and September, whereas *T. uncinatum* can be collected and sold between September and January. Evidently, this will have to be adjusted to assure the harvest of high-quality truffles (i.e., optimal maturity) across different regions, taking into account specific local growing conditions.

Acknowledgments This study was supported by the Burgundy Regional Council. We are grateful to Henri Frochot for providing *Tuber aestivum* samples. We thank Gian Carlo Ponzi and Claude Murat for providing *Tuber macrosporum* and *Tuber magnatum* samples, respectively. We are grateful to Gregory Bonito for providing primer sequences for the elongation factor 1α gene. We also thank Régis Courtecuisse for accepting our exsiccata samples in the LIP herbarium.

The authors declare that the experiments comply with the current laws of France.

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