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Phylogeny and phylogeography of the *Tuber brumale* aggr.

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Abstract

The *Tuber brumale* (winter truffle) is a black truffle reported from most European countries, belonging to the *Melanosporum* group. Its significance in the economy is ambivalent as the winter truffle has been shown to be a frequent contaminant species in the orchards of the Perigord truffle and occasionally in those of the summer truffle, yet owing to its delicate fragrance, its trade is worthy of note. The phylogeny and phylogeography of economically important truffles are relatively well-explored; however, no thorough research has been published on these aspects of the winter truffle. Therefore, here, we report the first phylogeographic analyses based on samples representing the entire distribution of the species. ITS sequences were used in this survey for haplotype and coalescent analyses, while phylogenetic analyses were based on the ITS, LSU and PKC loci. According to all loci, the samples clustered into two big clades imply the existence of two phylogenetic species. Based on our results, one of these appears to be endemic to the Carpathian Basin. In the other more widespread species, two main phylogeographic groups can be distinguished that show east-west separation with a zone of overlap in the Carpathian Basin, suggesting that they survived the latest glacial period in separate refugia.

Introduction

The genus *Tuber*, containing hypogeous species indigenous in the Northern hemisphere, comprises the largest group in the ascomycete family Tuberaceae in the order Pezizales (Bonito et al. 2010, 2013). Numerous gastronomically and economically remarkable fungi belong here including the winter truffle (*Tuber brumale* Vittad.) which is one of the most significant members of the genus. Its reputation is demonstrated by the fact that in French marketplaces, the name ‘truffle’ is reserved exclusively for *Tuber melanosporum* Vittad. (Perigord truffle) and *Tuber brumale* (Douet et al. 2004). The organoleptic features of the winter truffle are also similar to that of *T. melanosporum* resulting in a prominent gastronomic status (Kiss et al. 2011). Phylogenetically, the winter truffle is closely related to the Perigord truffle (Bonito et al. 2013), the genome of which has been fully sequenced (Martin et al. 2010). *T. brumale* forms ectomycorrhizae and, like most species in the genus, is not strictly host-specific (Chevalier and Frochot 1997; Hall et al. 2007). It is one of those representatives of *Tuber* that can become non-native colonizers in regions distant from their original habitats (Bonito et al. 2010) and frequently appear in cultivated truffle orchards (Chevalier and Frochot 1997; Benucci et al. 2011; Belfiori et al. 2012; Olivier and Savignac 2012; Guerin-Laguette et al. 2013). Several articles have issued the effects of *T. brumale* on other truffles; negative, suppressing effects are underlined mainly by handbooks (Chevalier and Frochot 1997; Sourzat 2011; Chevalier and Sourzat 2012; Olivier et al. 2012). Mamoun and Olivier (1993) have revealed that negative impacts are greatly dependent on environmental factors such as irrigation strategies. The competitive properties of the economically important truffle species against other ectomycorrhizal fungi vary, as can be concluded from the compositional and quantitative differences of mycorrhizal communities in truffeorchards (Belfiori et al. 2012). Eleven major clades are currently recognised within the genus *Tuber* (Bonito et al. 2013), including the *Melanosporum* major clade which further branches into two lineages (Jeandroz et al. 2008). The first one contains the *T. melanosporum*, *Tuber regiomontanum* Guevara, Bonito, and Rodríguez (Guevara et al. 2008; Bonito et al. 2013) and *Tuber indicum* Cooke and Masee species complex whilst the second lineage comprises *Tuber pseudoexcavatum* Wang et al. and *T. brumale*. The same origin of the latter two species is confirmed by, inter alia, a 300-bp long insertion in the internal transcribed spacer 1 (ITS1) region (Wang et al. 2006). According to Wang et al. (2006), the most recent common ancestor (MRCA) of the *Melanosporum* clade probably existed between China and Europe. First, the *T. pseudoexcavatum*/*T. brumale* lineage diverged and the ancestor of *T. brumale* commenced its migration towards Europe while that of *T. pseudoexcavatum* moved towards China. The ancestors of *T. melanosporum* and *T. indicum* diverged later, but followed similar migration routes to Europe and China, respectively, thus also forming a vicariant species pair. On the other hand, according to Bonito et al. (2013), the radiation of the *Melanosporum* clade began in North America 79 million years ago (Ma) connected to host plants of the family Pinaceae, followed by migration towards Asia and to Europe. Furthermore, the low nucleotide variability found solely in

European species suggests a strong bottleneck effect in their history (Murat et al. 2004; Wang et al. 2006). *T. brumale* is widespread in almost all of Europe except in boreal and arctic regions (Hollós 1911; [Ławrynowicz 1992](#); Montecchi and Sarasini 2000). In 1995, it was also found in New Zealand on account of human-mediated propagation (Ho et al. 2008). It is thought to be rare or absent in some countries (England, Germany, Poland and Scandinavia; Benjamin Stielow, Christina Wedén, Maria Ławrynowicz, Marie-Anne French pers. comm.) and common in others (France, Hungary, Italy, Romania, Serbia and Slovenia; Grebenc 2008; Marjanović 2008; [Bratek et al. 2013](#); and Alessandra Zambonelli and Gérard Chevalier pers. comm.). It should be noted that winter truffles are dog preferred hypogeous fungi (DPH; [Bratek et al. 2013](#)), and their collection for trade may severely influence estimations of their abundance. [Beheregaray \(2008\)](#) showed that 3,049 articles dealing with phylogeography were published between 1987 and 2006 with as few as 13 concerned with fungi (e.g., Hibbett 2001; [Geml et al. 2006](#)), which highlights the need of fungal phylogeographical research. [Lumbsch et al. \(2008\)](#) also mention the scarce use of phylogeographical methods in mycology, although several surveys were released on the subject afterwards (e.g., Linzer et al. 2008; Moncalvo and Buchanan 2008; [Bergemann et al. 2009](#); [Ghikas et al. 2010](#); Grubisha et al. 2012). Still, fungi are vastly understudied with respect to phylogeography and taxonomic diversity. On the basis of all these, it is not surprising that the winter truffle is one of the most intensely studied Tuber species. The assessment of its distribution and genetic variability could reveal important findings for the ecological, conservation and biological and economical research of this mushroom. The aim of this work is to characterize the phylogeographic structure of the *T. brumale* species complex covering its entire known distribution. In our molecular study, 194 specimens from 19 countries were analyzed to test for correlations between spatial and genetic patterns, including the estimation of gene flow among geographic areas and the detection of evolutionary lineages undergoing speciation.

Materials and methods

Sampling

Our sample includes 194 localised findings of *T. brumale* and *T. brumale* var. *moschatum* (Bull.) Hall et al. (Online Resources 1, 2). Most of these originate from the known native distributional area of this species (Europe), with the exception of three findings (two from Iran and one from New Zealand). Our goal was to perform an even and representative sampling, with a large enough sample size, covering the biogeographical area of the winter truffle as entirely as possible. Fresh or dried ascomata from different countries were determined and kindly provided by collectors from the Carpathian-Pannonian region and also by Alessandra Zambonelli, Amer Montecchi, Chriss

Chrysopoulos, Diamandis Stephanos, Gérard Chevalier, Joseph Maria Vidaland Stanislav Glejdura. These, 136 specimens in all, were deposited in Zoltán Bratek's herbarium (ZB).

Isolation of DNA, PCR and sequencing

DNA was isolated from dried or fresh sporocarps with DNeasy Plant Mini Kit (Qiagen) or DNA Mini Kit (Plant)(Geneaid) according to the instructions of the manufacturers with minor modifications. Three nuclear loci were analysed for phylogeny; these are the ribosomal internal transcribed spacer (ITS), the large subunit (LSU) regions of the ribosomal DNA repeat and the variable segment of the protein kinase C (PKC) locus. The LSU is closely linked to the ITS, while the PKC is not linked to the previous two. Primers used for the amplification of the ITS region were normally ITS1F, ITS4 (White et al. 1990; Gardes and Bruns 1993), and in case of problematic samples, ITS5, ITS6 and ITS7 (Bertini et al. 1999). For the LSU region LROR, LR3 (Vilgalys and Hester 1990), for the PKC region PKC1F, PKC1R (Ambra and Macino 2000) primers were used. PCRs were performed with a final volume of 50 µl: the components are as follows: DreamTaq Green Buffer (Fermentas) (20 mM MgCl₂, 5.0 µl), dNTP mix (Fermentas) (2 mM, 5.0 µl), primers for ITS (0.01 mM, 1.0 µl) or LSU (0.01 mM, 1.0 µl) or PKC (0.05 mM, 1.0 µl), Milli-Q water (12.75 µl), DreamTaq polymerase (Fermentas) (5 unit/µl, 0.25 µl) and template DNA dissolved in Milli-Q water (25.0 µl). Thermocycling was carried out under the following conditions: 94 °C for 10 min, 33 cycles of 94 °C for 30 s, 51 °C (ITS) or 54 °C (LSU) or 58 °C (PKC) for 30 (alternatively 60) s and 72 °C (elongation) for 45-120 s (depending on the types of primers). ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems) was applied for sequencing. Capillary gel electrophoresis was accomplished by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the instructions of the manufacturer (Biomet Ltd.). Restriction fragment length polymorphism (RFLP) was used for the quick, simple and cheap detection of lineages identified earlier. HinfI and MboI restriction endonucleases were applied on the basis of preliminary *in silico* digestions (SnapGene® software, GSL Biotech; available at snapgene.com) of sequenced ITS loci. ITS products of 2 µl were digested overnight at 37 °C with 1.25 units of one of the endonucleases per reaction. Restriction fragments were separated in 3 % agarose gels. On the whole, 108 samples were sequenced for at least one locus, 28 samples were identified with RFLP and 58 samples were collected through NCBI (<http://www.ncbi.nlm.nih.gov/>). Thus, our phylogenetic analyses included 136 ITS region, 31 LSU region, and 24 PKC sequences.

Phylogenetic and haplotype analyses

Electropherograms were checked with the program FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA; <http://www.geospiza.com>). Alignment of sequences was carried out with the software MAFFT (Katoh

and Toh 2008). Alignments were manually corrected with MEGA 5 (Tamura et al. 2011) when needed. File formats were transformed with the online application ALTER (Glez-Pena et al. 2010). Phylogenetic analyses were executed with PAUP (Swofford 2003) and MrBayes 3.1.2. (Huelsenbeck and Ronquist 2001) program packages. Filograms generated with maximum parsimony (MP) were calculated in two ways, that is, with 'missing' and 'new state' gap handling. Tree-bisection-reconnection (TBR) branch-swapping option was chosen for heuristic searches with 100 bootstrap replicates for each locus. The suitable substitution matrices for Bayesian analyses were selected with MrMODELTEST v2.3 (Nylander 2004). With regard to Akaike Information Criterion (AIC), the best fit likelihood models were found to be the HKY+G, GTR+G and GTR for ITS, LSU and PKC, respectively. Phylogenetic reconstructions performed by MrBayes ran in four chains with 12,000,000 generations for ITS and 10,000,000 generations for LSU and PKC. Every 100th generations were sampled. Average standard deviations of split frequencies were 0.007224 for ITS, 0.003314 for LSU and 0.001210 for PKC. Phylogenetic trees were visualized by FigTree v1.3.1 (Rambaut 2009). To test for congruence of gene genealogies, partition homogeneity test (PHT) was used within the program PAUP (Farris et al. 1995; Swofford 2003). Paired nucleotide divergence values were also calculated with PAUP selecting the P-distance option. Haplotype diversity was determined with the DNAsp software (Librado and Rozas 2009). Haplotypes were recognized by the program Collapse (Posada 2011). The haplotype networks were made using the TCS program (Clement et al. 2000) and visualized with Cytoscape (Shannon et al. 2003). The distribution of the main lineages, based on the 194 samples, was visualized by Quantum GIS mapping software (<http://qgis.org>). To better understand the causes of haplotype patterns, DNAsp was used to calculate Tajima's D (Tajima 1989), Fu's F_s (Fu 1997), Fu and Li's D^* and Fu and Li's F^* (Fu and Li 1993) neutrality tests (based on 1,000 coalescent simulations).

Molecular dating

Divergence times of *Melanosporum* clade members were estimated with BEAST version 1.8 (Drummond et al. 2012). We employed a secondary calibration approach based on Bonito et al. (2013) to compensate the absence of fossil records in the *Melanosporum* group. For the molecular clock analysis, the ITS1 region was neglected because ITS1 is too divergent; thus, the 5.8S, ITS2 and LSU dataset was analyzed. The clock model and substitution models were unlinked. jModeltest (Posada 2008) was used to select the substitution models for each locus (5.8S: HKY; ITS2: N93+G; LSU: GTR+I). We created two monophyletic taxon sets, one containing the species of the *Melanosporum* clade and the other containing only *T. brumale* and *T. pseudoexcavatum* sequences. Analyses were run under an uncorrelated lognormal relaxed molecular clock, setting the tree prior to

the birth-death speciation process. We used normal prior distributions for the node with value of Melanosporum clade (mean=79.7 Ma, SD=5 Ma). Priors were set to the default except the clock rates (substitution per site per million years) of unlinked regions which were set as follows: 5.8S=3.02–4, ITS2=2.07–3 and LSU=6.5–4 (Bonito et al. 2013). The MCMC analysis was run for 50,000,000 generations, and every 5,000th tree was sampled. To check if the effective sample sizes reached the appropriate value, we used TRACER version 1.5 (Rambaut and Drummond 2009). We calculated the maximum clade credibility tree using TreeAnnotator version 1.8 (available as part of the BEAST package) after the initial 10 % of the trees were discarded.

Coalescent analyses of *T. brumale* populations

Identical ITS sequences were collapsed into haplotypes using SNAP Map (Aylor et al. 2006) after excluding insertion or deletions (indels) and infinite-sites violations. The analyses presented here assume an infinite sites model under which a polymorphic site is caused by exactly one mutation and there can be no more than two segregating bases. Base substitutions were categorized as phylogenetically uninformative or informative and as transitions or transversions. Site compatibility matrices were generated from each haplotype dataset using SNAP Clade and Matrix (Markwordt et al. 2003; Bowden et al. 2008) to examine compatibility/incompatibility among all variable sites, with any resultant incompatible sites removed from the dataset. Genetic differentiation among geographical populations was analysed using SNAP Map, Seqtomatrix and Permttest (Hudson et al. 1992) implemented in SNAP Workbench (Price and Carbone 2005). Permttest is a nonparametric permutation method based on Monte Carlo simulations that estimates Hudson's test statistics (KST, KS and KT) under the null hypothesis of no genetic differentiation. KST is equal to $1 - KS/KT$, where KS is a weighted mean of K_1 and K_2 (mean number of differences between sequences in haplogroups I and II, respectively), and KT represents the mean number of differences between two sequences regardless of the subpopulation to which they belong. The null hypothesis of no genetic differentiation was rejected at $p < 0.05$. For this test, *T. brumale* specimens were assigned to geographical groups (Western vs. Eastern Europe) that corresponded to haplogroups I and II, respectively. We used MDIV (Nielsen and Wakeley 2001), implemented in SNAP Workbench (Price and Carbone 2005), employing both likelihood and Bayesian methods using Markov chain Monte Carlo (MCMC) coalescent simulations to estimate the migration (M), population mean mutation rate (Theta) and divergence time (T). Here, M equals $2' N_e$ the net effective population size (N_e) multiplied by m (migration rate), while Theta is $4' N_e$ multiplied by m (mutation rate) (Watterson 1975). Ages were measured in coalescent units of $2N$, where N is the population size. This approach assumes that all populations descended from one panmictic population that may or may not have been followed by

migration. For each dataset, the data were simulated assuming an infinite sites model with uniform prior. We used 2,000,000 steps in the chain for estimating the posterior probability distribution and an initial 500,000 steps to ensure that enough genealogies were simulated before approximating the posterior distribution. Subsequently, we reconstructed the genealogy with the highest root probability, the ages of mutations and the time to the most recent common ancestor (TMRCA) of the sampling using coalescent simulations in Genetree v. 9.0 ([Griffiths and Tavaré 1994](#)).

Results

Phylogenetic analyses

A total of 108 samples were successfully sequenced, another 58 sequences of *T. brumale* and 69 sequences of closely related species were downloaded from databases of NCBI (Online Resource 1). We gathered 140 ITS, 92 LSU and 28 PKC sequences for subsequent phylogenetic analyses. Thus, the sample size was sufficient to study the phylogeny of *T. brumale* and accomplish our above-mentioned objectives. The constructed parsimony trees, based on ITS, LSU and PKC regions, are shown in Table 1. Phylogenetic trees of the same locus revealed identical topologies regardless of method (Bayesian or Parsimony) and types of gap handling with only one exception: the tree based on ITS with gaps scored as a new state. This deviation was a result of an approximately 300 bp long insertion located in the ITS of *T. brumale* mentioned in the "Introduction". On the LSU tree (Fig. 1), the samples represent a separate monophyletic cluster containing two clades (clades A and B) of high support within the *Melanosporium* group. Two further groups can be distinguished (I and II) within clade A; however, only group II can be considered as monophyletic. The ITS and PKC trees (Fig. 2, Online Resource 4) confirm the highly supported clades (A and B). However, unlike the LSU and ITS trees, clade A does not separate into two groups on the PKC tree. Therefore, calculating a concatenated tree was inadvisable, which was also affirmed by the significant result of the PHT test ($p=0.0004$). Nucleotide distance between groups I and II of clade A, as calculated by the P-distance method, suggests an intraspecific variability (2.02 %) below the generally applied limit of 3 % (Nilsson et al. 2008; [Bonito et al. 2010](#)), while the distance between clades A and B (9.32 %) is large enough to be regarded as interspecific (Table 2). Clades A and B as well as haplotype groups I and II in clade A could be clearly identified based on RFLP fragment patterns acquired with the use of MboI and HinfI restriction enzymes; both of these enzymes have three to five restriction sites in the ITS segment. As DNA fragments shorter than 100 bp cannot be sufficiently separated using 3 % agarose gel, only fragments of 100 to 550 bp were informative. With regard to the enzyme MboI, these are the 121, 303 and 368 bp fragments for clade B; fragments of 227, 231 and 306 bp for haplogroup I in clade A; and fragments of 142, 225, 231 and 307 bp for haplogroup II in clade A. With regard to HinfI, 223 and

528 bp for clade B; 140, 226 and 402 bp for haplogroup I in clade A; and 227 and 540 bp for haplogroup II in clade A. Clades A and B could be distinguished using MboI as the 368-bp long fragment was only present in clade B. Detection of the two haplogroups in clade A was made possible by fragments sized 140 and 402 bp digested by HinfI, both unique for haplogroup I.

Phylogeography and haplotype analysis

One hundred thirty-six sequences were involved in ITS haplotype analyses: 119 sequences for clade A and 17 for clade B, both examined separately (Fig. 3). Altogether, 17 haplotypes were found in clade A and two haplotypes in clade B. As was seen on the ITS and LSU trees, two distant haplogroups can be observed on the haplotype network of clade A, which are 16 mutation steps far from each other. The most abundant haplotype in haplogroup I is C1 with a frequency of 84.1 % from which the rest of the haplotypes branch in a star-like shape with distances of 1-2 mutations. Haplotype diversity (Hd) of haplogroup I is 0.29. In contrast, Hd of haplogroup II is 0.72, and the most common haplotype belonging here (K1) has a frequency of 43.2 %; thus, haplotypes are more evenly distributed. Samples of clade B cluster in two haplotypes of similar size, resulting in a Hd value of 0.53. Phylogeographic analyses were performed based on 194 samples including sequences needed for phylogeny as well as partial sequences and samples identified with RFLP. Clade A was found to be distributed across Europe, whilst clade B turned out to be restricted to the Carpathian-Pannonian region (Fig. 3, Online Resource 2). This means that the distribution of the two clades is sympatric. Representatives of haplogroup I (clade A) were detected from Western Europe to the eastern and southern parts of Hungary. On the other hand, specimens of haplogroup II were shown from locations that lie to the east of the Alps. Therefore, the distribution of the two haplogroups within clade A is to be considered parapatric with a contact zone in the Carpathian Basin. Several interesting haplotypes were identified within clade A such as a sample from New Zealand (EU753268) shown from roots. This belongs to the most frequent haplotype (C1) and was presumably transported overseas from France within inoculated seedlings. Likewise, all specimens from the Carpathian Basin belong to haplotype C1. Haplotype C2, found only in Italy, is a curiosity having an ITS1 region with a 33-bp long deletion. The two sequences identified from Iranian root samples are unique that only constitute haplotype L. Tajima's D , Fu's F_s , Fu and Li's D^* and Fu and Li's F^* were non-significant for haplogroup II, clade A and clade B (with 95 % confidence intervals). For haplogroup I, all of the tests were significant ($D = -0.07$, $p = 0.003$; $F^* = -0.17$, $p = 0.03$; $F_s = -0.17$, $p = 0.02$) except the Fu and Li's D^* .

Molecular dating

The estimates of divergence times and the chronogram are shown in Online Resource 5. The result of the posterior MRCAs was 78.0 Ma, and the posterior clock rates of unlinked regions were $5.8S=1.97-4$, $ITS2=4.56-3$ and $LSU=8.51-4$. These values are similar to the results of Bonito et al. (2013). The split of the *T. brumale* clade A from clade B likely occurred 17.9 Ma (95 % high posterior density, 10.9–25.7 Ma).

Coalescent analyses of *T. brumale* populations

Estimates of Hudson's test statistics (KST, KS and KT) using nonparametric permutation method indicated strong genetic differentiation among haplogroups I and II. The genetic differences within and between populations were $KS=5.9036$ and $KT=7.1813$, respectively, resulting in $KST=0.1780$, $p<0.0001$. In our combined approach, MDIV was used to estimate gene flow and to determine if the diversity patterns in the Western and Eastern populations of clade A were the result of retention of ancestral polymorphism or recent gene flow. After recoding indels and removing infinite-sites violations from the original ITS datasets, there were 13 ITS haplotypes in populations of *T. brumale*, respectively (Table 3). MDIV showed evidence for no gene flow ($M=0$) and statistically significant, non-zero population divergence time ($0<T$) (Online Resource 6). Coalescent genealogies generated by Genetree simulations confirmed the historical population division between haplogroups I and II. Even though the initial settings were based on the conservative null hypothesis of a panmictic population with a moderately high level of migration ($M=0.1$) between geographic populations, the separation of the two haplogroups was evident on the genealogy with inferred mutational history and variation between and within geographical regions (Fig. 4).

Discussion

Phylogenetic and phylogeographic analyses of *T. brumale* were carried out in this study using 194 samples identified as *T. brumale* or *T. brumale* var. *moschatum* attempting to cover the entire biogeographic area of this species. Two of the three utilized phylogenetic markers (ITS and LSU) are closely linked, yet because of their functional differences, their mutation rates differ greatly. PKC is not linked with the aforementioned sites and its evolution is slow (Wang et al. 2006). The separation of clades A and B was highly supported by all examined loci, and they are recognised as distinct phylogenetic species based on gene genealogical concordance sensu Taylor et al. (2000). The distinction of haplogroups I and II within clade A is supported by both ITS and LSU while it is unsupported by PKC. This incongruence is further demonstrated by the significant outcome of the PHT test. The congruent distinction of clades A and B and the incongruent distinction of haplogroups I and II stand on opposite sides of the 'species boundary', i.e. the former two are different species

while the latter two are the same. The lack of segregation of haplogroups I and II observed on the PKC tree could be a consequence of either current recombination or recent separation with the ancestral polymorphic alleles still present. Coalescence analyses favour recent separation, according to which gene flow between the two haplogroups ceased some time ago. Recent separation is also supported by the notable distance in ITS (2.02 %) and the divergence time not being zero. The picture is much clearer concerning the segregation of clades A and B. The genetical distance of ITS between them (9.3 %) exceeds 3 % indicating that they are to be considered separate phylogenetic species ([Nilsson et al. 2008](#); [Bonito et al. 2010](#)), which is corroborated by the gene genealogical concordance of the three loci. The sympatric distribution of the two clades and their reciprocal monophyly indicates a lack of genetic exchange between them ([Bickford et al. 2007](#)). Furthermore, the results of molecular dating indicate that the split of clades A and B was 17.9 Ma (Online Resource 5) which is similar to the divergence time of *T. melanosporum* and *T. indicum* species complex ([Bonito et al. 2013](#)). According to these results, clades A and B can be considered as two different phylogenetic species. Some previous studies showed morphological differences between *T. brumale* and *T. brumale* var. *moschatum* (for examples, see [Riousset et al. 2001](#)), but neither our molecular results nor [Gandeboeuf et al. \(1994, 1997\)](#) support this differences. Nota bene, both *T. brumale* and *T. brumale* var. *moschatum* were described in Western Europe, a geographic region where *T. brumale* s.l. is genetically homogeneous (haplogroup I) according to our results. Thus, it is unlikely that these putative morphological differences are an outcome of speciation. Other studies (e.g. [Montecchi and Sarasini 2000](#); [Ceruti et al. 2003](#)) failed to show any morphological differences between ascomas of clades A and B, though they gathered specimens from locations where the geographic areas of these two clades overlap. Therefore, they might be assumed as members of a cryptic species complex, i.e. distinct phylogenetic lineages previously lumped under the same name due to the lack of observed morphological differences ([Bickford et al. 2007](#)). Several other *Tuber* taxa, including *Tuber borchii* Vittad. ([Bonuso et al. 2009](#)) and *T. indicum* ([Chen et al. 2011](#)), have also turned out to contain cryptic species. *T. indicum* is a close relative of *T. brumale* aggr. In the case of *T. brumale* aggr., it is possible that examining character combinations will solve the morphological differentiation of the two clades. A more thorough morphological investigation shall be the subject of future research. The formal taxonomic treatment of these taxa is also a promising direction, but until there is no novel morphological species description, *T. brumale* should be treated as a species aggregate. For this reason and because of the phylogenetic species differentiation of clades A and B, it is more appropriate to use *T. brumale* aggr. instead of *T. brumale* Vittad. Previous studies underline the low genetic variability of *T. brumale* s.l. Using isoenzyme methods and random amplified polymorphic DNA (RAPD), [Gandeboeuf et al. \(1994, 1997\)](#) suggested that the intraspecific variability of *T. brumale* s.l. was lower than that of other members of *Tuber*. [Giomaro et al. \(2002\)](#) found no variability in the

ITS region of the species in question, which was confirmed later by Pomarico et al. (2006). Wang et al. (2006) showed that among the main representatives of the *Melanosporum* group, nucleotide divergence was lower in European species (*T. melanosporum*, 0.0–0.3 %, *T. brumale* s.l., 0.0–0.1 %) than in Asian species (*T. indicum*, 3.2 %; *T. pseudoexcavatum*, 1.0 %). These authors attributed this to a bottleneck effect occurring in the course of migration toward Europe. A further bottleneck effect is assumed to have taken place during the previous glacial period in the case of European *T. melanosporum* populations (Bertault et al. 2001; Murat et al. 2004). Bonito et al. (2010) showed less than 0.5 % intraspecific variation in *T. brumale*. Here, we present a higher ITS variability in *T. brumale* s.l. than was previously believed. Our data shows that intraspecific ITS variabilities of clades A and B are 0.91 and 0.07 %, respectively, meaning that clade B does harbour low variability while that of clade A is relatively high. Intraspecific ITS variabilities of haplogroups I and II are 0.032 and 0.201 %, pointing out that intergroup differences contribute the most to the genetic variation found in clade A. In the *Tuber* genus, three other species show ITS variability similar to that of clade B (less than 0.2 %; *Tuber puberulum*, *Tuber castellanoi* and *Tuber oregonense*) and one species similar to that of clade A (<1 %; *Tuber mesentericum*) (Bonito et al. 2010). Low nucleotide and haplotype variability and the starshaped structure of the haplotype network of haplogroup I suggest recent expansion (Slatkin and Hudson 1991). The significant negative results of Tajima's D , Fu's F_s and Fu and Li's F^* tests support the recent expansion of this haplogroup. Based on the present distribution of haplogroup I, it is likely that its populations survived the last glacial in Western European refugia. Looking at the phylogeographical investigations of potential host plant of *T. brumale* aggr., similarities appear in the haplotype patterns of the winter truffle and its hosts. For instance, dominance of one haplotype was observed in the case of white oak species (*Quercus* spp.) and the common hazel (*Corylus avellana*) in France. These host trees survived the last glacial in Iberian refugia (Palmé and Vendramin 2002; Petit et al. 2002a, b). In contrast to this, beech (*Fagus* spp.) and hornbeam (*Carpinus* spp.) species are thought to have recolonised Europe mainly from the Italian Peninsula and the Balkan region, although these species had refugia in the Iberian Peninsula as well (Taberlet et al. 1998; Grivet and Petit 2003; Magri 2008). The recolonization of haplogroup I from two refugia (Iberian and Italian Peninsulas) is in conflict with the star-shaped structure of its haplotype network and the low genetic diversity of this group because should the two lineages of different refugia intermingle, the resulting diversity would be higher (Petit et al. 2003). Haplogroup II is more diverse, its nucleotide and haplotype variability is higher and the structure of its haplotype network is more complex. This greater diversity could be a result of surviving the last glacial in Eastern European refugia; the Balkan Peninsula harboured several refugia and recolonization routes as it has been identified in the case of other species (Petit et al. 2003; Magri 2008; Hatziskakis et al.

2009). Despite the large number of samples, the other phylogenetic species (clade B) was only found within the Carpathian Basin. A notable number (20) of *T. brumale* aggr. samples collected to the east and south-east of the Carpathian Basin were at our disposal, but they all belonged to clade A. This suggests that clade B is indeed distributed solely in the Carpathian Basin and represents a fungus endemic to this region. The low nucleotide and haplotype diversity of clade B can indicate a strong bottleneck effect; however, the non-significant results of Tajima's D , Fu's F_s , Fu and Li's D^* and Fu and Li's F^* tests do not support this assumption. Nevertheless, clade B could have survived the last glacial in a Carpathian refugium, a scenario also proposed concerning some tree species (Bordács et al. 2002; [Petit et al. 2003](#); Magri 2008). It can be concluded, therefore, that the Carpathian Basin and the Balkan region are diversity hotspots not only for plant and animal species but also for certain macrofungi as well, e.g. the genus *Tuber* ([Sica et al. 2007](#); [Marjanović et al. 2010](#)). These regions may be home to more undescribed endemic *Tuber* species. Digestion of the ITS region of *T. brumale* s.l. with *Hinf*I was utilized earlier by Giomaro et al. (2002) in order to quickly identify samples of this species. However, this enzyme is only capable of separating haplogroups I and II in clade A, while *Mbo*I, used in our research, made it possible to distinguish clade A from clade B. The RFLP method allows for the time- and cost-efficient screening of *T. brumale* aggr. samples, which could contribute to surveys on populations of *T. brumale* aggr. in un- or undersampled regions such as the Middle East, Ukraine, Russia and Spain. In the present study, we identified an endemic phylogenetic sister species of *T. brumale* aggr. (clade B) and detected two remarkably different haplotype groups within clade A of *T. brumale* aggr. This finding provides new proof for the existence of hardly recognizable (cf. cryptic/pseudocryptic) macrofungi species for which there are several other examples (e.g. [Geml et al. 2006](#); [Bonuso et al. 2009](#); Li et al. 2010; Van de Putte et al. 2010; [Chen et al. 2011](#); Stielow et al. 2011; Sheedy et al. 2013; Urban et al. 2013). Furthermore, the assessment and protection of biodiversity require knowledge about species that are complicated to distinguish merely on morphological grounds. In addition, the discovery of endemic species, as the new *Tuber* sp. was shown here to be endemic to the Carpathian Basin, plays an important role in settling the proper conservation status of a specific area. Also, our work exemplifies the importance of sufficiently large sample size and broad geographical spread in sampling to achieve reliable approximations of the genetic diversity of a certain species. Our results may contribute to further physiological and ecological investigations of *T. brumale* aggr., leading to a deeper understanding of the biology and cultivation of truffles.

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Fig. 1 Bayesian phylogenetic tree of *Melanosporium* major clade based on LSU. Bayesian posterior probabilities (PP) and maximum parsimony (MP) bootstrap values divided by / are displayed above branches, only values where PP >0.95 and MP bootstrap support >70 % are shown. Numbers of OTUs on collapsed branches parenthetically follow species names. See accession numbers in Online Resource 3. Clades A and B of *Tuber brumale* as well as specimens belonging to haplogroups I or II are marked. Specimens of *Tuber brumale* aggr. are shown with their identifiers listed in Online Resource 1

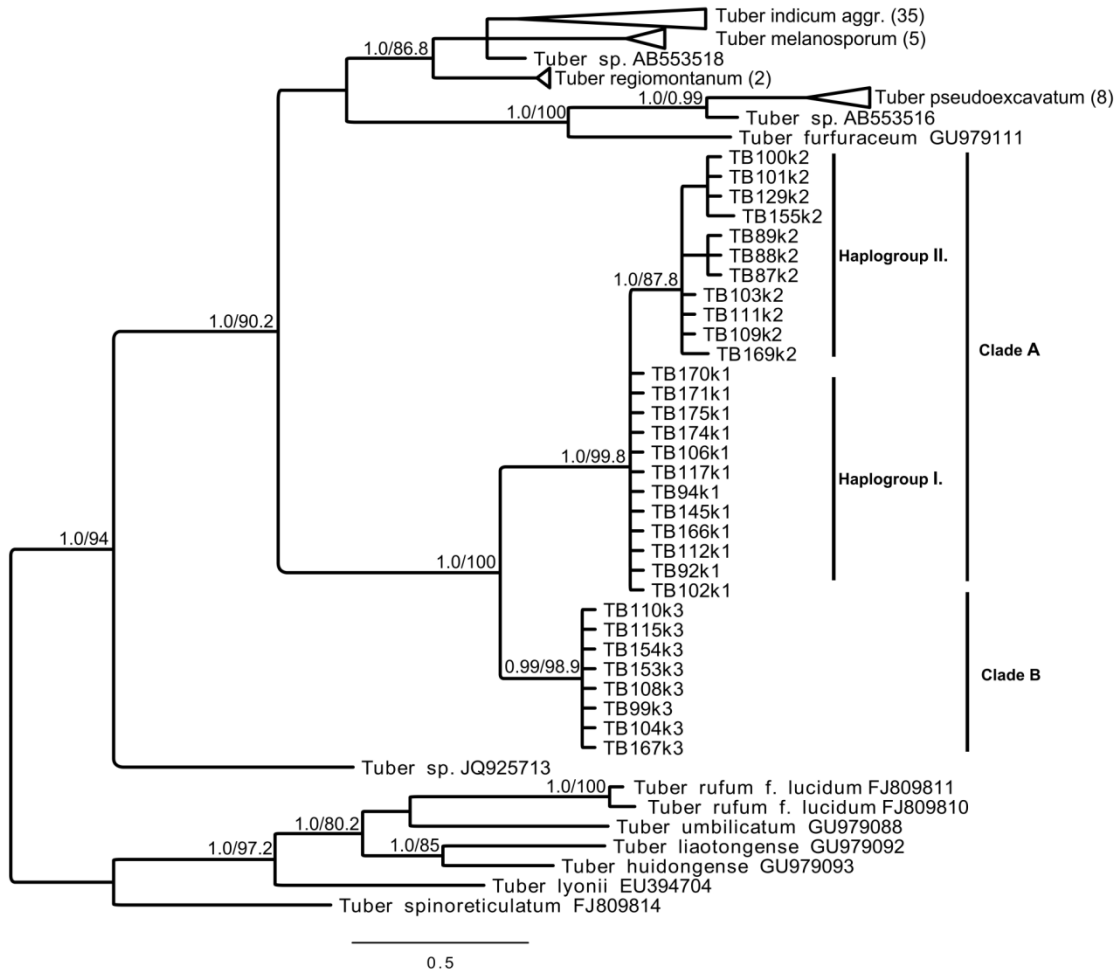


Fig. 2 Bayesian phylogenetic tree of *T. brumale* aggr. based on PKC. Bayesian posterior probabilities (PP) and maximum parsimony (MP) bootstrap values divided by / are shown above branches. Only values where PP >0.95 and MP bootstrap support >70 % are shown. Specimens of *Tuber brumale* aggr. are shown with their identifiers listed in Online Resource 1

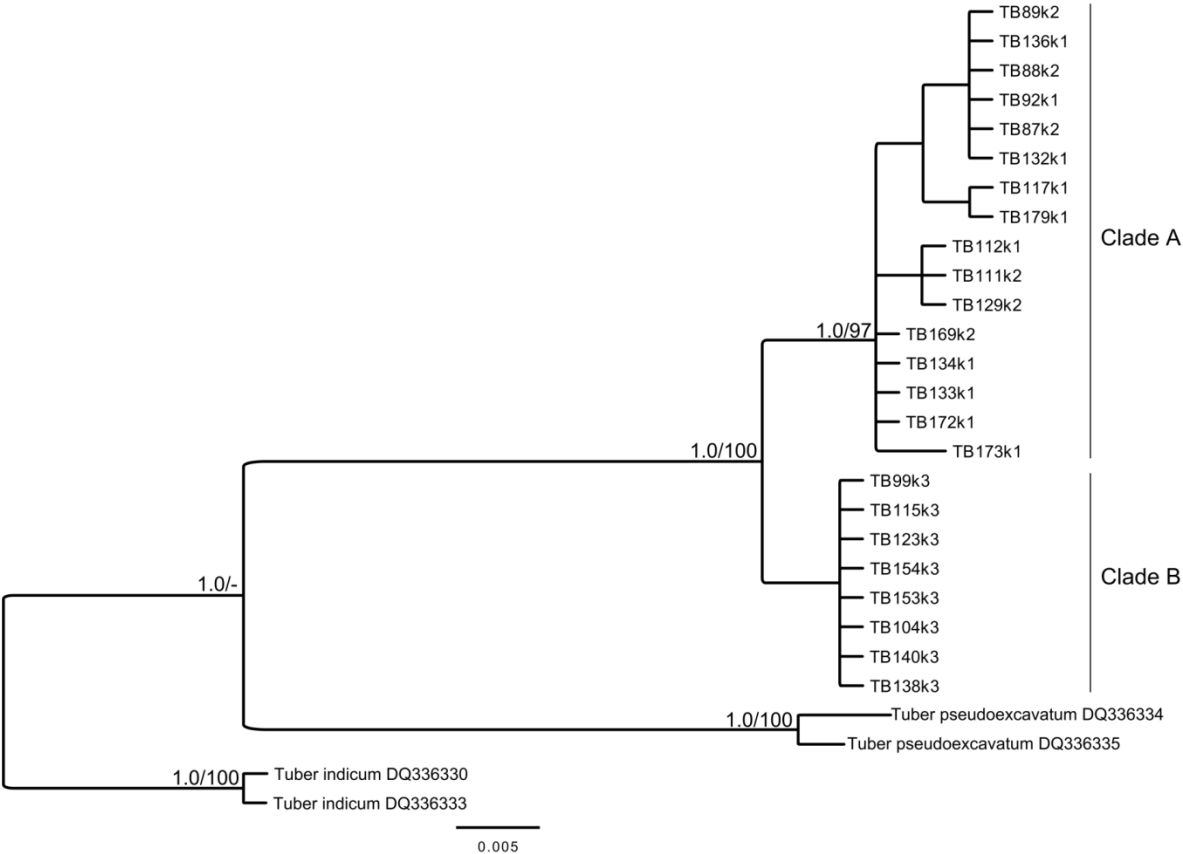


Fig. 3 Haplotype network and distribution of *Tuber brumale* aggr. in Europe based on the 17 haplotypes separated by the collapse program. Haplotypes designated with letters only were found to be identical regardless of coalescent analyses method, except that haplotypes A and B were merged by the collapse program, while haplotypes signified with both letters and numbers were recognised only by the collapse program. The sizes of circles are proportional to the number of samples. Specimens not depicted on the map are TB20k1 (New Zealand), TB169k2 (Turkey), TB46k2 (Iran) and TB47k2 (Iran). Maps were generated applying the QuantumGIS mapping software (<http://qgis.org>) using the world map of APRS™ (<http://aprsworld.net/gisdata/world/>)

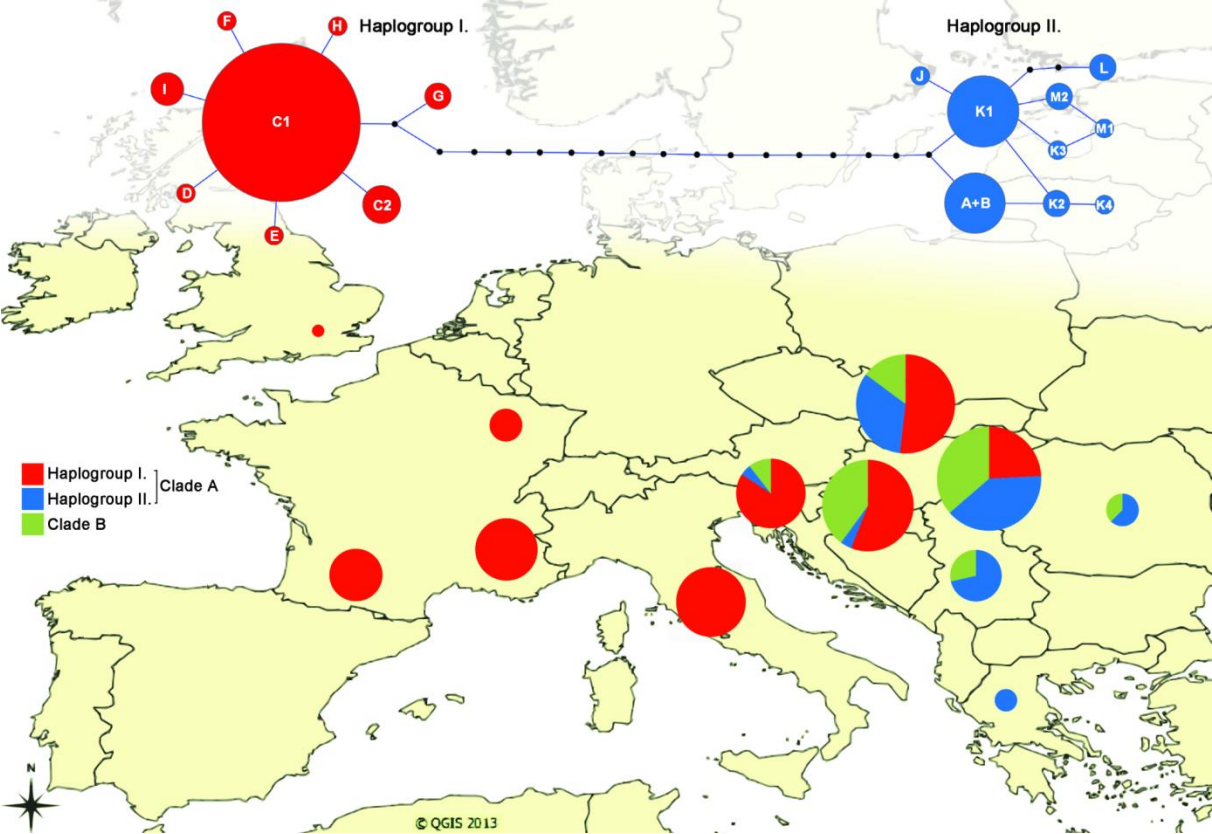


Fig. 4 Coalescent-based genealogy of *T. brumale* with the highest root probability (likelihood scores: $L=1.9518 \times 10^{-17}$, $SD=1.1488 \times 10^{-17}$) showing the distribution of mutations for the ITS region. The inferred genealogy is based on 2,000,000 simulations of the coalescent with a Watterson's (1975) estimate of $\theta=1.9$. The time scale is in coalescent units of $2N$, where N is the population size. Mutations and bifurcations are time ordered from the top (past) to the bottom (present). Mutation designations correspond to the site numbers in Table 3. The numbers below the tree designate the distinct haplotypes and their observed frequencies in total and in the different geographical regions, where 0 = Eastern, 1 = Western Europe

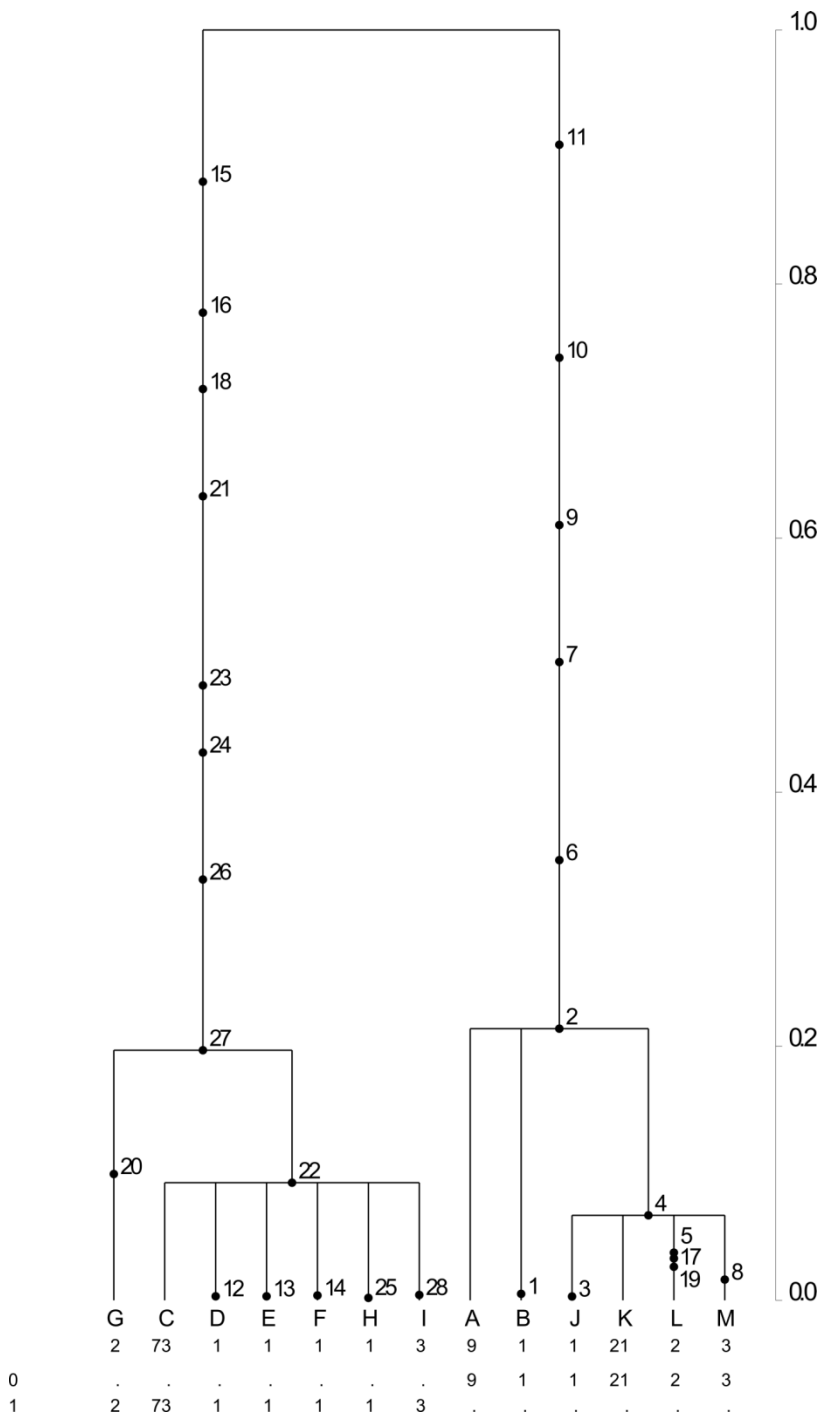


Table 1 Data for phylogenetic trees calculated with the maximum parsimony method Region

| Region | gap threatment | Length | CI | RI | RC | outgroup | ingroup | <i>T. brumale</i> | PIS | total charaters |
|--------|----------------|--------|-------|-------|-------|----------|---------|-------------------|-----|-----------------|
| ITS | missing | 215 | 0.926 | 0.992 | 0.918 | 4 | 136 | 136 | 176 | 762 |
| | 5th char. | 722 | 0.910 | 0.981 | 0.892 | 4 | 136 | 136 | 507 | 762 |
| LSU | missing | 301 | 0.631 | 0.942 | 0.595 | 7 | 85 | 31 | 121 | 523 |
| | 5th char. | 310 | 0.639 | 0.942 | 0.601 | 7 | 85 | 31 | 126 | 523 |
| PKC | missing | 77 | 0.987 | 0.994 | 0.981 | 4 | 24 | 24 | 65 | 735 |
| | 5th char. | 86 | 0.988 | 0.994 | 0.983 | 4 | 24 | 24 | 73 | 735 |

Further features displayed are the length of trees, the number of total characters, the number of parsimony informative sites (PIS), the number of outgroups and ingroups and the number of samples belonging to *Tuber brumale* aggr. among ingroups

CI consistency index, RI retention index, RC rescaled consistency index

Table 2 P distance matrix based on ITS sequences Clade/haplogroup

| clade/haplogroup | I. | II. | B | A |
|------------------|--------|--------|--------|--------|
| Haplogroup I. | 0.032% | 2.024% | 9.258% | |
| | 0.073% | 0.094% | 0.085% | |
| Haplogroup II. | | 0.201% | 9.450% | |
| | | 0.166% | 0.254% | |
| Clade B | | | 0.070% | 9.317% |
| | | | 0.067% | 0.181% |
| Clade A | | | | 0.909% |
| | | | | 0.979% |

The upper values in cells are averages and the lower values are standard deviations

Table 3 Polymorphic sites in the ITS haplotypes of *Tuber brumale* collapsed after recoding indels and removing infinite-sites violations from the original ITS dataset for the subsequent coalescent analyses. Haplotype designations, position, site number, and designation of the given mutation are as shown in Fig. 4. Position refers to that in the original alignment, site type refers either transition (t), transversion (v), deletion (-) change with regard to the consensus sequence

| <i>T. brumale</i> | |
|---------------------------|--|
| Position | 1111222233344556666777 1236602241469056004837789013 7361901219053057126583513640 |
| Site number | 111111111122222222 123456789012345678901234567 |
| Site Type | -vttvtvtvtvt-ttv--tttt-vvtv |
| Character Type | i-i-ii-iii-- ii- -iii -ii- |
| Consensus | 1AGAGACGTTAAC1ATT21ACTA2TATT |
| Haplotypes (Frequency) | |
| A (9) | .T...TT.ACG...GC.1..T.G1.CC. |
| B (1) | 2T...TT.ACG...GC.1..T.G1.CC. |
| C (73) |C..... |
| D (4) |C.....C..... |
| E (1) |T.....C..... |
| F (1) |2.....C..... |
| G (2) |G..... |
| H (1) |C..A... |
| I (3) |C..... |
| J (1) | .TAG.TT.ACG...GC.1..T.G1.CC. |
| K (21) | .T.G.TT.ACG...GC.1..T.G1.CC. |
| L (2) | .T.GATT.ACG...GCG12.T.G1.CC. |
| M (3) | .T.G.TTAACG...GC.1..T.G1.CC. |