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Genetic structure of *Tuber mesentericum* Vitt. based on polymorphisms at the ribosomal DNA ITS

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Abstract *Tuber mesentericum* fruit bodies are in increasing demand on the food market and are an important economic resource for southern Italy, their major production area. Because molecular studies on this truffle species are very scarce, we analyzed ITS1 and ITS2 nucleotide variability of 126 ascocarps of *T. mesentericum* collected in different European areas, mainly southern Italy. The results of haplotype distribution, analysis of molecular variance, and spatial analysis of molecular variance analyses show strong genetic structuring of the samples collected in the different geographic areas, confirmed by parsimony and distance analyses. In particular, the Italian samples seem to be a well-distinguished group.

Keywords *Tuber mesentericum* · Truffle · Genetic structuring · ITS

Introduction

Tuber mesentericum Vitt. is an ectomycorrhizal fungus that generates edible fruit bodies that are a product in increasing demand on the food market. Its distribution is spread throughout Europe, with the center of production localized in Italy, in particular in the area surrounding Bagnoli Irpino (Province of Avellino, inland of Naples), where *T. mesentericum* is very abundant. It is known as the "Bagnoli black truffle" and represents an important economic resource for this rural area (Palenzona et al. 1976).

Many factors influence truffle morphology, including age, host tree species, and environmental conditions; thus, the typing of truffle ascocarps should always be supported by molecular methods (Murat et al. 2005).

In the past, *T. mesentericum* was sometimes considered a subspecies of *T. aestivum* Vitt. (Trappe 1979), but some morphological studies have defined it as a true species (Rauscher et al. 1995), and recent molecular phylogeny analyses have shown that *T. mesentericum* forms a well-distinguished species, sister group to *T. aestivum* (Wedén et al. 2005). Immature fruit bodies of *T. mesentericum* and *T. aestivum* can easily be confused, but at full spore maturity, *T. mesentericum* emits a strong and very distinct naphthalene-like scent, earning it the former name *T. bituminatum* (Berkeley and Broome 1851). In fact, it can also be distinguished from the other truffle species on the basis of its volatile compounds (Pacioni et al. 1991; Gioacchini et al. 2005).

Increasing interest has been focused recently on the genetic characterization of some truffle species. For example, internal transcribed spacer (ITS) sequence analysis demonstrated that it is not possible to separate *T. aestivum* from *T. uncinatum* and that the Gotland (Sweden) population of *T. aestivum* is genetically distinct as compared to other European specimens (Wedén et al. 2004, 2005), and revealed a geographical association of haplotypes in *T. melanosporum* (Murat et al. 2004); moreover, microsatellites variability analysis showed the presence of a genetic and phylogeographic structure in *T. magnatum* (Rubini et al. 2005). The almost complete lack of molecular studies on *T. mesentericum* has lead us to investigate the genetic variability of ITS1 and ITS2 nucleotide sequences

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in this species, focusing our attention on samples collected in some European sites and in the major production area, the Campania Region of Italy, to understand if among them there is a genetic and geographic differentiation.

Materials and methods

Sample collection

A total of 126 fruit bodies were examined (Table 1, Fig. 1). Of these, 81 (from the Campania Region, Italy) were collected by many different truffle hunters working mainly in Bagnoli Irpino and in the surrounding areas (Campania Region, Italy) that performed the first morphological determination. Unfortunately, they just provided generic geographic information about the collection sites, and we cannot exclude that some samples could have been derived from the same genet. Successive morphological attribution of these samples was performed in the laboratory on the basis of shape and dimension of asci, number of spores per ascus, shape, and dimension of spores (Violante et al. 1997). The high number of ascocarps from the Campania Region (and, in particular, from Bagnoli Irpino) is due to the interest of the regional and local institutions to promote molecular studies on this truffle species that represents an important economic resource for this rural area.

Dried pieces of fruit bodies from different European countries were kindly supplied and determined by Dr. Chevalier (France, Luxemburg, Spain, Italy–Spoleto), Dr. Souzart (France), Dr. Frochot (France), Dr. Zoltan (Hungary), Dr. Bernolt (Switzerland, Germany), Dr. Clare (France), Dr. de Miguel (Spain), and Dr. Wedén (Sweden).

DNA extraction, amplification, and sequencing

The samples examined in the present study and their collection areas are listed in Table 1 and Fig. 1 and were taken from either fresh ascocarps (all the Italian samples) or small pieces of dried tissue. DNA extraction was conducted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Polymerase chain reaction amplification was performed with primers TartF3 (5′-gtaggtgaacctgcggaagg-3′) and TartR1 (5′-gatgattcact gagttctgca-3′) for ITS1 and primers TartF2 (5′-tgcagaact cagtgaatcatc-3′) and TartR2 (5′-ccctacctgatccgaggtca-3′) for ITS2, under the following conditions: 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C (for ITS1) or 52°C (for ITS2) for 1 min, 72°C for 1 min, and 72°C for 7 min. The products were run on 2% agarose gel, and the amplification fragments were collected and eluted from the gel and directly sequenced in both directions with the Big Dye Terminator Cycle Sequencing Kit v 1.1 (Applied Biosystems) using the same primers cited above. The sequence reactions were run on an ABI 310 sequencer (Applied Biosystems). Amplification and sequencing reactions were carried out twice to avoid any amplification and sequencing artifacts.

Data analysis

Nucleotide sequences were visually checked and the basic local alignment search tool (BLAST) analysis was conducted to determine the sequence homology. Sequences were examined as a contig including ITS1 and ITS2 obtained, eliminating from the whole sequenced fragments the nucleotide sequences of rDNA 18S, 5.8S, and 28S at the 5′ and 3′ termini. The alignments were produced using ClustalW and then manually edited. Samples from Spain and Germany were not included in some of the successive analyses because of the small sample size (two and three ascocarps, respectively) that could have strongly affected the estimation of genetic diversity and haplotype frequencies.

Haplotype frequencies calculation, shared haplotypes among groups, and analysis of molecular variance (AMOVA) were carried out with ARLEQUIN v.2.00 (Schneider et al. 2000) using the F_{ST} statistics. The Mantel test of geographic differentiation was performed to verify the correlation between the geographical distance matrix [Ln(distances in kilometers)] and genetic distance matrix (F_{ST} pairwise comparison), using the same software. A simulated annealing procedure implemented in the spatial analysis of molecular variance (SAMOVA) software (Dupanloup et al. 2002) was used to define groups of samples that are geographically homogeneous and differentiated from each other. The simulations were conducted with K (number of groups) ranging from two to six and repeating each simulation annealing process 100 times.

A maximum parsimony analysis was conducted adding to the alignment the ITS sequences of the samples from Spain and Germany and nine ITS sequences of *T. aestivum* from the GenBank, used as an outgroup (Table 1). Using PAUP v. 4.08 (Swofford 2002), the heuristic search was conducted with four random addition sequence replicates, tree bissection–reconnection branch swapping, and the MULTREES option off. Gaps were treated as missing characters. Bootstrap analysis was performed with 1,000 replicates. A neighbor-joining analysis was carried out with the Jukes–Cantor distance option.

Results

The morphological analysis conducted on the samples has shown that all the ascocarps have phenotypic characters corresponding to *T. mesentericum*.

Table 1 List of the samples examined

Sample code	Species	Collection area	Collection date	Accession number	
011	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261177	AB261178
012	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261179	AB261180
013	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261181	AB261182
014	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261183	AB261184
015	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261185	AB261186
016	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261187	AB261188
017	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2002	AB261189	AB261190
018	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261191	AB261192
019	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261193	AB261194
020	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261195	AB261196
021	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261197	AB261198
022	*T. mesentericum*	Montella (Southern Italy)	2003	AB261199	AB261200
023	*T. mesentericum*	Montella (Southern Italy)	2003	AB261201	AB261202
024	*T. mesentericum*	Montella (Southern Italy)	2003	AB261203	AB261204
025	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261205	AB261206
026	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261207	AB261208
027	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261209	AB261210
028	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261211	AB261212
029	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261213	AB261214
030	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261215	AB261216
031	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261217	AB261218
032	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261219	AB261220
033	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261221	AB261222
034	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261223	AB261224
035	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261225	AB261226
036	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261227	AB261228
037	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261229	AB261230
038	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261231	AB261232
039	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261233	AB261234
040	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261235	AB261236
041	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261237	AB261238
042	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261239	AB261240
043	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261241	AB261242
044	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261243	AB261244
045	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261245	AB261246
046	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261247	AB261248
047	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261249	AB261250
048	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261251	AB261252
049	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261253	AB261254
050	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261255	AB261256
051	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261257	AB261258
052	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261259	AB261260
053	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261261	AB261262
054	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261263	AB261264
055	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261265	AB261266
056	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261267	AB261268
057	*T. mesentericum*	Colliano (Southern Italy)	2003	AB261269	AB261270
058	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261271	AB261272
059	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261273	AB261274
060	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261275	AB261276
061	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261277	AB261278
062	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261279	AB261280
063	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2003	AB261281	AB261282
064	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261283	AB261284
065	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261285	AB261286
066	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261287	AB261288

Table 1 (continued)

Sample code	Species	Collection area	Collection date	Accession number	
067	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261289	AB261290
068	*T. mesentericum*	Le Montat (France)	Unknown	AB261359	AB261360
069	*T. mesentericum*	Tanlay (France)	1988	AB261341	AB261342
070	*T. mesentericum*	Jussy (France)	1984	AB261343	AB261344
071	*T. mesentericum*	Massangis (France)	1996	AB261345	AB261346
072	*T. mesentericum*	Troussey (France)	1994	AB261347	AB261348
073	*T. mesentericum*	Meuse (France)	1991	AB261349	AB261350
074	*T. mesentericum*	Meuse (France)	1996	AB261351	AB261352
075	*T. mesentericum*	Lot (France)	2001	AB261361	AB261362
076	*T. mesentericum*	Richelieur (France)	2001	AB261363	AB261364
077	*T. mesentericum*	Peyrins (France)	2002	AB261365	AB261366
078	*T. mesentericum*	Dordogne (France)	1993	AB261367	AB261368
079	*T. mesentericum*	Reybiro à Cahors (Luxemburg)	1984	AB261381	AB261382
080	*T. mesentericum*	Reybiro à Cahors (Luxemburg)	1984	AB261383	AB261384
081	*T. mesentericum*	Reybiro à Cahors (Luxemburg)	1984	AB261385	AB261386
082	*T. mesentericum*	Reybiro à Cahors (Luxemburg)	1984	AB261387	AB261388
083	*T. mesentericum*	Reybiro à Cahors (Luxemburg)	1984	AB261389	AB261390
084	*T. mesentericum*	Daix (France)	2005	AB261353	AB261354
085	*T. mesentericum*	Fresnois (France)	2005	AB261355	AB261356
086	*T. mesentericum*	Tarsul (France)	2005	AB261357	AB261358
087	*T. mesentericum*	Felsòtàrkàny (Hungary)	2000	AB261391	AB261392
088	*T. mesentericum*	Felsòtàrkàny (Hungary)	2000	AB261393	AB261394
089	*T. mesentericum*	Zöld-hàz (Hungary)	1994	AB261395	AB261396
090	*T. mesentericum*	Zöld-hàz (Hungary)	1994	AB261397	AB261398
091	*T. mesentericum*	Eszergom (Hungary)	2004	AB261399	AB261400
092	*T. mesentericum*	Mecsek (Hungary)	1997	AB261401	AB261402
093	*T. mesentericum*	Mecsek (Hungary)	1997	AB261403	AB261404
094	*T. mesentericum*	Navarra (Spain)	2002	AB261405	AB261406
095	*T. mesentericum*	Tèruel (Spain)	1975	AB261407	AB261408
096	*T. mesentericum*	Cahors (France)	1977	AB261369	AB261370
097	*T. mesentericum*	Ariège (France)	2002	AB261371	AB261372
098	*T. mesentericum*	Ariège (France)	2002	AB261373	AB261374
099	*T. mesentericum*	Ariège (France)	2002	AB261375	AB261376
100	*T. mesentericum*	Ariège (France)	2002	AB261377	AB261378
101	*T. mesentericum*	Ariège (France)	2002	AB261379	AB261380
102	*T. mesentericum*	Gotland (Sweden)	2000	AJ888043*	
103	*T. mesentericum*	Gotland (Sweden)	2000	AJ888044*	
104	*T. mesentericum*	Gotland (Sweden)	1999	AJ888045*	
105	*T. mesentericum*	Gotland (Sweden)	2003	AJ888046*	
106	*T. mesentericum*	Gotland (Sweden)	2000	AJ888047*	
107	*T. mesentericum*	Gotland (Sweden)	1999	AJ888048*	
108	*T. mesentericum*	Basel (Switzerland)	Unknown	AB261409	AB261410
109	*T. mesentericum*	Birsfelden (Switzerland)	1955	AB261411	AB261412
110	*T. mesentericum*	Birsfelden (Switzerland)	1955	AB261413	AB261414
111	*T. mesentericum*	Birsfelden (Switzerland)	1955	AB261415	AB261416
112	*T. mesentericum*	Birsfelden (Switzerland)	1970	AB261417	AB261418
113	*T. mesentericum*	Basel (Switzerland)	1924	AB261419	AB261420
114	*T. mesentericum*	Bann (Germany)	1955	AB261421	AB261422
115	*T. mesentericum*	Bann (Germany)	1955	AB261423	AB261424
116	*T. mesentericum*	Oberangers (Germany)	1955	AB261425	AB261426
118	*T. aestivum*	Marche (Central Italy)	Unknown	AJ888090*	
119	*T. aestivum*	Unknown	Unknown	AJ888093*	
120	*T. aestivum*	Tèruel (Spain)	1975	AB261427	AB261428
121	*T. aestivum*	Montauban sur Ouvèze (France)	2003	AB261429	AB261430
122	*T. aestivum*	Abruzzo (Central Italy)	1996	AY226042*	
123	*T. aestivum*	Unknown	Unknown	AY226040*	

Table 1 (continued)

Sample code	Species	Collection area	Collection date	Accession number	
124	*T. aestivum*	Lombardia (Northern Italy)	1999	AY226041*	
125	*T. aestivum*	France	1999	AY226039*	
126	*T. aestivum*	Abruzzo (Central Italy)	1997	AY226038*	
127	*T. aestivum*	Abruzzo (Central Italy)	1997	AY226037*	
128	*T. aestivum*	Abruzzo (Central Italy)	1999	AY226036*	
129	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261291	AB261292
130	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261293	AB261294
131	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2004	AB261295	AB261296
132	*T. mesentericum*	Colliano (Southern Italy)	2004	AB261297	AB261298
133	*T. mesentericum*	Colliano (Southern Italy	2004	AB261299	AB261300
134	*T. mesentericum*	Colliano (Southern Italy)	2004	AB261301	AB261302
135	*T. mesentericum*	Colliano (Southern Italy)	2004	AB261303	AB261304
136	*T. mesentericum*	Colliano (Southern Italy)	2005	AB261305	AB261306
137	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261307	AB261308
138	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261309	AB261310
139	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261311	AB261312
140	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261313	AB261314
141	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261315	AB261316
142	*T. mesentericum*	Montella (Southern Italy)	2005	AB261317	AB261318
143	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261319	AB261320
144	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261321	AB261322
145	*T. mesentericum*	Colliano (Southern Italy)	2005	AB261323	AB261324
146	*T. mesentericum*	Colliano (Southern Italy)	2005	AB261325	AB261326
147	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261327	AB261328
148	*T. mesentericum*	Colliano (Southern Italy)	2005	AB261329	AB261330
149	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261331	AB261332
150	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261333	AB261334
151	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261335	AB261336
152	*T. mesentericum*	Bagnoli Irpino (Southern Italy)	2005	AB261337	AB261338
153	*T. mesentericum*	Spoleto (Central Italy)	2005	AB261339	AB261340

The accession numbers marked with an asterisk are referred to sequences previously present in GenBank. The left and right columns of the accession numbers are referred to ITS1 and ITS2 nucleotide sequences, respectively.

ITS amplification gave fragments approximately 235 (ITS1) and 210 (ITS2) base pairs long. No heterozygote was found among any of the sequences examined. BLAST analysis showed that all but two sequences were *T. mesentericum*: one sample from Spain (code number 120) and one from France (code number 121) were found to be *T. aestivum*.

Sequence nucleotide diversity within samples ranges from 0.007 (Sweden) to 0.091 (Luxemburg), and the differences observed are both nucleotide substitutions and indels (Table 2).

Out of 126 sequences of *T. mesentericum* examined, 39 different haplotypes were found. The Italian and the Swedish samples do not share haplotypes with any other geographical groups (Table 3). Although the other samples share one or more haplotypes with different frequencies, each geographical group shows at least one private haplotype, absent in all the other samples (Table 3).

The Mantel test shows no significant correlation between geographic and genetic distances.

The AMOVA analysis shows evidence of genetic structuring, with high and significant values of total differentiation (F_{ST}=0.714, the correlation among random ITS haplotypes within populations relative to the correlation of random pairs drawn from the whole sample). The highest percentage of variation is among populations (71.36%; Table 4).

SAMOVA analysis (Dupanloup et al. 2002) identifies, for a prespecified number of groups of populations, the geographical groups that are most differentiated from one another. The grouping of populations is based on conducting a hierarchical analysis of F_{ST} analogues and maximizing the proportion of total genetic variance between groups (F_{CT} analogue). The SAMOVA analysis confirms the genetic structuring of the geographic groups, with the highest F_{CT} value (0.777) corresponding to the simulation of six groups.

Parsimony analysis conducted on ITS1 and ITS2 sequences gave one most parsimonious tree (tree length 244 steps, consistency index 0.831, retention index 0.981);

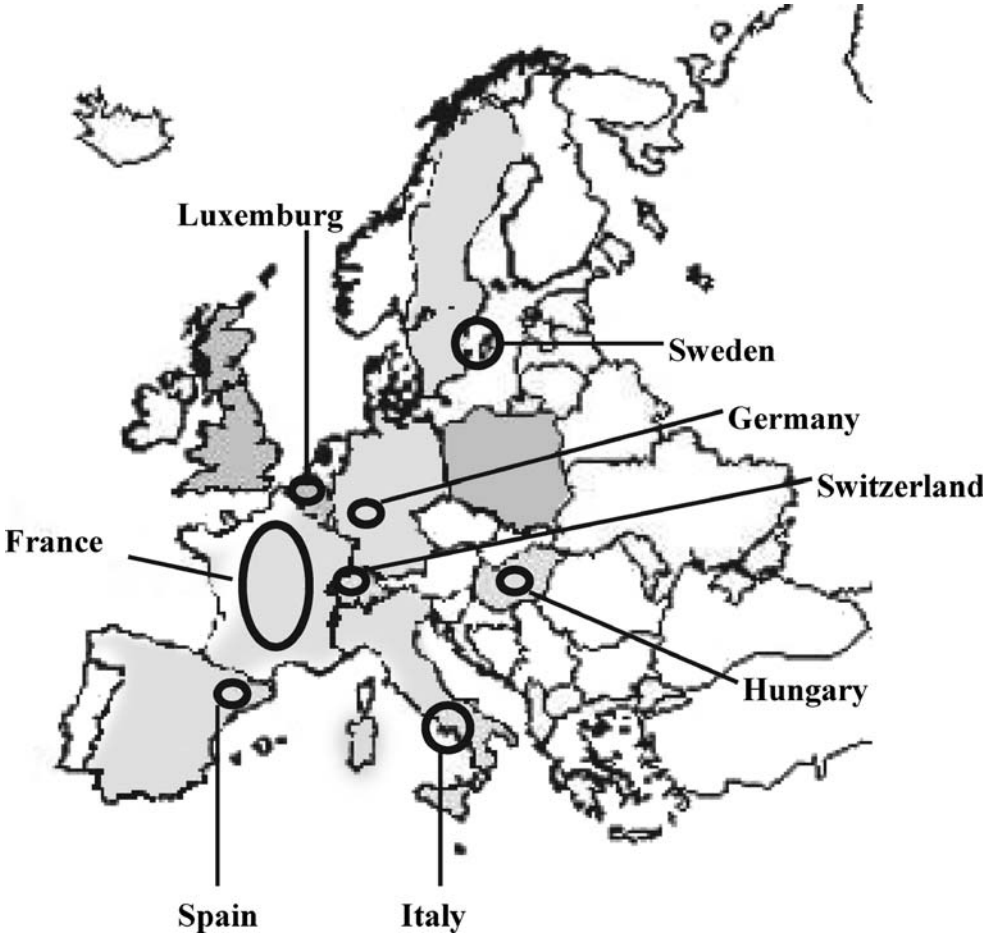


Fig. 1 Distribution range (in *gray*) of *T. mesentericum* in Europe. The *circles* represent the areas of collection of the samples examined

the bootstrap tree is shown in Fig. 2a. The neighbor-joining tree and the bootstrap neighbor-joining tree are shown in Fig. 2b and c, respectively. Both parsimony and distance trees show the same topology and group together all the 16 haplotypes from the 82 Italian samples. The samples from the other regions are not grouped strictly by their geographic origin.

Discussion

The geographical distribution and environmental requirements of truffles vary according to species: some are widely distributed, whereas others have a more restricted distribution; some have pronounced morphological and molecular variability, whereas others have little intraspecific polymorphisms in either morphological or genetic traits (Rubini et al. 2005).

Geographical distribution of *T. mesentericum* is quite wide throughout Europe, but not ubiquitous (Fig. 1). No significant morphological difference was found among *T. mesentericum* ascocarps based on their geographic origin, and the finding of two samples of *T. aestivum* misclassified as *T. mesentericum* strongly confirms the importance of coupling morphological and molecular analyses to characterize a truffle ascocarp.

The lack of correspondence between genetic and geographic distance could be due to the absence of current gene flow among populations (in particular Italy and Sweden) followed by genetic drift that leads to random genetic divergence of populations. This observation is confirmed by the absence of shared haplotypes of these two populations, although the parsimony tree analysis shows that some haplotypes from France, Germany, Hungary, Luxemburg, Spain, and Switzerland group with the Italian haplotypes, indicating past gene flow among these populations.

Table 2 Nucleotide diversity (±standard deviation) values and number of indels within groups of *T. mesentericum*

Group	Number of samples	Nucleotide diversity	Indels
Italy	82	0.029±0.015	21
France	20	0.060±0.030	14
Luxemburg	5	0.091±0.056	52
Hungary	7	0.069±0.039	14
Sweden	6	0.007±0.004	3
Switzerland	6	0.057±0.034	11

Table 3 Haplotype frequencies within groups of *T. mesentericum*

Haplotype name	IT	FR	LUX	HUN	SWE	SWI
I	0.207	0	0	0	0	0
II	0.463	0	0	0	0	0
III	0.012	0	0	0	0	0
IV	0.036	0	0	0	0	0
V	0.024	0	0	0	0	0
VI	0.012	0	0	0	0	0
VII	0.024	0	0	0	0	0
VIII	0.012	0	0	0	0	0
IX	0.110	0	0	0	0	0
X	0.012	0	0	0	0	0
XI	0.012	0	0	0	0	0
XII	0.012	0	0	0	0	0
XIII	0.024	0	0	0	0	0
XIV	0.012	0	0	0	0	0
XV	0.012	0	0	0	0	0
XVI	0.012	0	0	0	0	0
XVII	0	0.100	0	0	0	0
XVIII	0	0.100	0.600	0.143	0	0.167
XIX	0	0.400	0	0	0	0.500
XX	0	0.050	0	0	0	0
XXI	0	0.050	0	0	0	0
XXII	0	0.050	0	0.143	0	0
XXIII	0	0.250	0	0	0	0
XXIV	0	0	0.200	0	0	0
XXV	0	0	0.200	0	0	0
XXVI	0	0	0	0.286	0	0
XXVII	0	0	0	0.143	0	0
XXVIII	0	0	0	0.286	0	0
XXIX	0	0	0	0	0.167	0
XXX	0	0	0	0	0.167	0
XXXI	0	0	0	0	0.167	0
XXXII	0	0	0	0	0.333	0
XXXIII	0	0	0	0	0.167	0
XXXIV	0	0	0	0	0	0.333
Total number	16	7	3	5	5	3

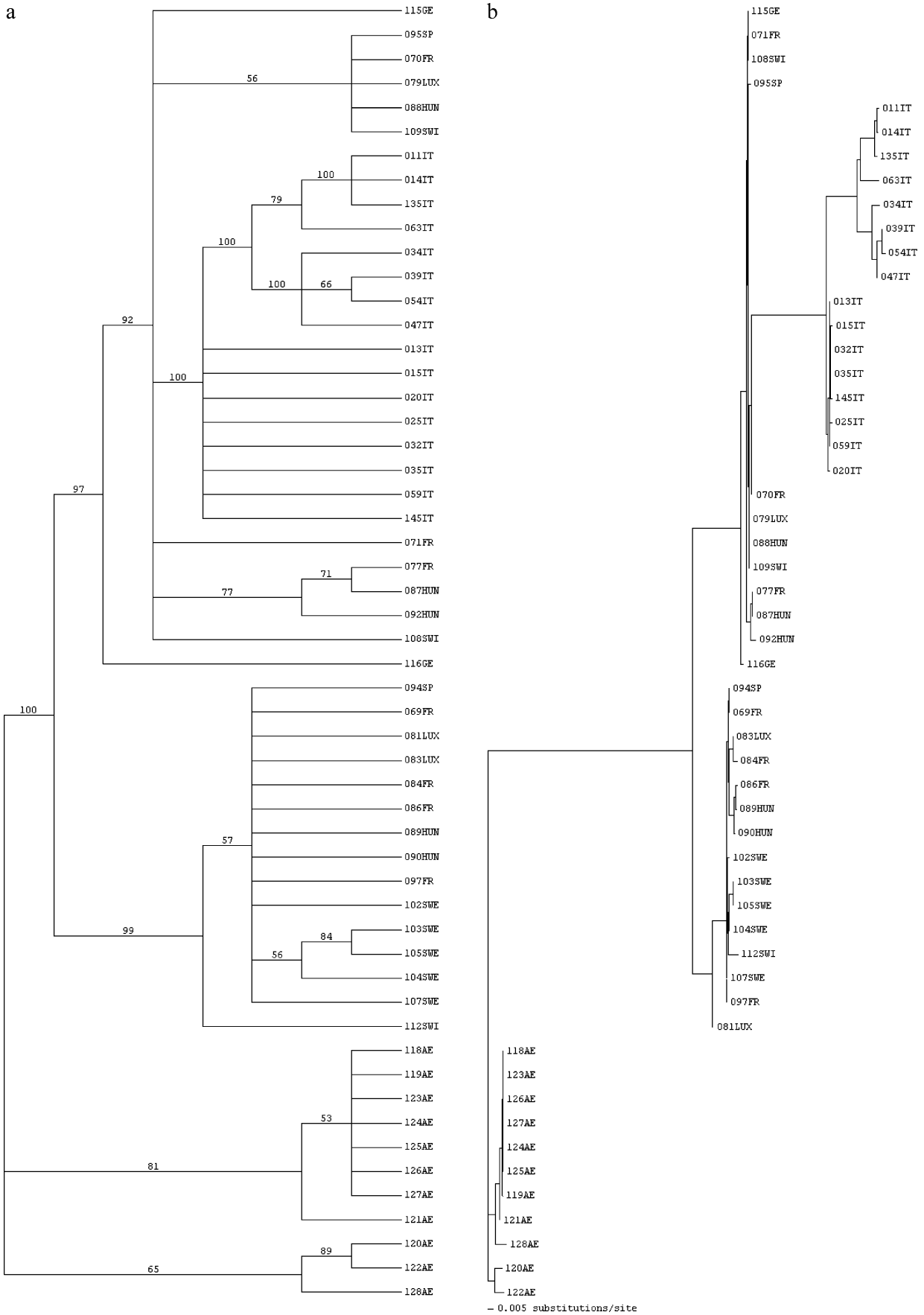
IT Italy; *FR* France; *LUX* Luxemburg; *HUN* Hungary; *SWE* Sweden; *SWI* Switzerland.

The haplotype distribution shows that the southernmost (Italy) and the northernmost (Sweden) samples do not share haplotypes with any other geographic groups, and that all the other samples are also recognizable for the presence of specific haplotypes. The presence of one haplotype with high frequency in most groups further suggests the presence of genetic drift followed by genetic differentiation. The high intraspecific genetic polymorphism observed in the 126 samples of *T. mesentericum* is in agreement with the recent results reporting high intraspecific heterozigosity (He=0.22) using ITS length analysis, micro- and mini-satellites variability, and random amplified polymorphic DNA markers on 11 ascomata of *T. mesentericum* from four collection sites located in Southern Italy with respect to the He values of other truffle species (*T. borchii*, *T. magnatum*, and *T. brumale*; Pomarico et al. 2007).

Table 4 Results of the AMOVA analysis conducted on the six geographical groups of *T. mesentericum*

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	Fixation index	*P*
Among populations	5	1563.565	22.239	71.36	F_{ST} 0.714	<0.001
Within populations	120	1071.126	8.926	28.64		
Total	125	2634.690	34.888			

df degrees of freedom, *P* probability


a
115GE
095SP
070FR
079LUX
088HUN
109SWI
011IT
014IT
135IT
063IT
034IT
039IT
054IT
047IT
013IT
015IT
020IT
025IT
032IT
035IT
059IT
145IT
071FR
077FR
087HUN
092HUN
108SWI
116GE
094SP
069FR
081LUX
083LUX
084FR
086FR
089HUN
090HUN
097FR
102SWE
103SWE
105SWE
104SWE
107SWE
112SWI
118AE
119AE
123AE
124AE
125AE
126AE
127AE
121AE
120AE
122AE
128AE
b
115GE
071FR
108SWI
095SP
011IT
014IT
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063IT
034IT
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047IT
013IT
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077FR
087HUN
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069FR
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084FR
086FR
089HUN
090HUN
102SWE
103SWE
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104SWE
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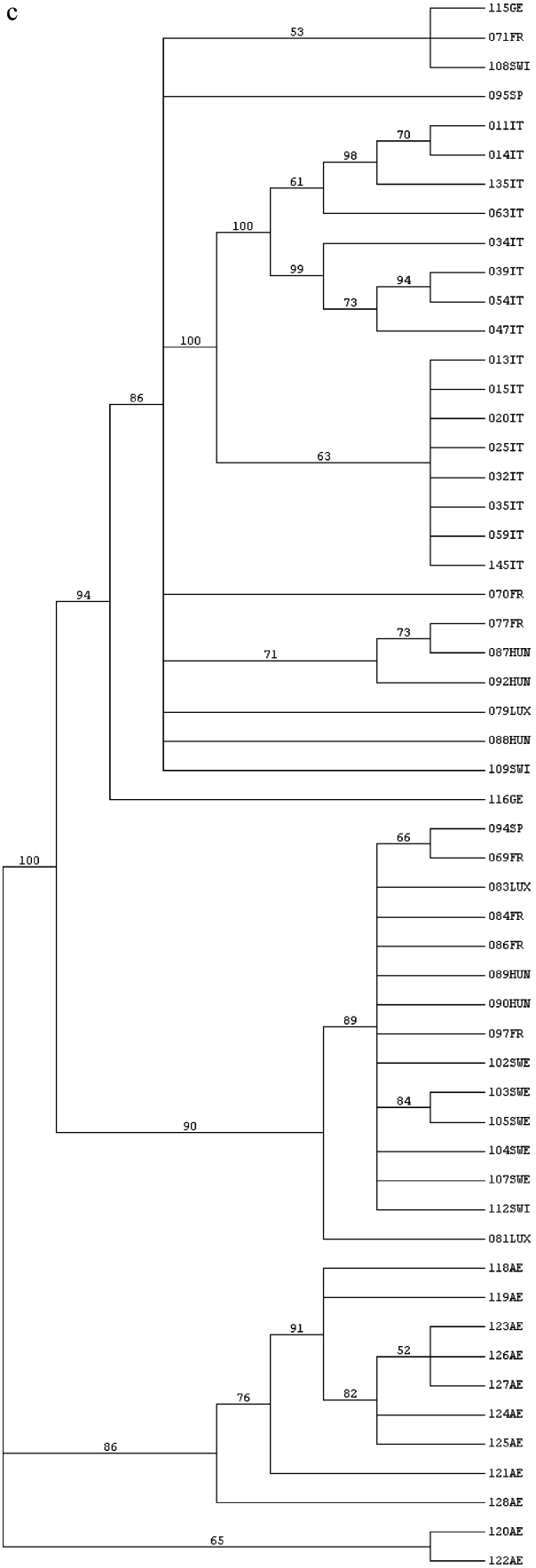

◀ **Fig. 2** **a** Maximum parsimony bootstrap tree, **b** neighbor-joining tree, and **c** neighbor-joining bootstrap tree of the *T. mesentericum* samples. Identical samples are represented once. *Numbers* refer to the bootstrap values. *T. aestivum* samples are used as the outgroup. *FR*, France; *GE*, Germany; *HUN*, Hungary; *IT*, Italy; *LUX*, Luxemburg; *SP*, Spain; *SWE*, Sweden; *SWI*, Switzerland; *AE*, *T. aestivum*

The F_{ST} statistics, AMOVA, and SAMOVA analyses indicate strong genetic differentiation among populations, and the Italian samples have most haplotypes but not the highest nucleotide diversity. The AMOVA analysis shows that 71.36% of the total variation is attributable to differences among populations and 28.64% to differences within populations. These and the SAMOVA results further suggest that there is limited or absent current genetic exchange among populations. Although the parsimony and distance analyses show no clustering of the groups reflecting their geographic origin, the Italian samples appear to be a well-defined and statistically supported group, confirming its differentiation from all the other samples.

The present analysis represents the first molecular characterization of *T. mesentericum* samplings from different European areas and is in agreement to the growing evidence that populations of different *Tuber* species are genetically structured across Europe (Murat et al. 2004; Rubini et al. 2005). Further studies are needed, including larger samplings and different loci analysis, to understand the phylogeography and biology of this truffle species.

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