The Journal of Animal & Plant Sciences, 27(2): 2017, Page: 528-541
ISSN: 1018-7081

MORPHOLOGICAL, PHYSICO-CHEMICAL AND MOLECULAR INVESTIGATIONS ON *TUBER BELLONAE* FROM BASILICATA - ITALY

S. M. Mang[1], T. Zotta[2], I. Camele[1], R. Racioppi[3], M. D'Auria[3] and G. L. Rana[1]

[1]Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, V. le Ateneo Lucano, 10, 85100 Potenza, Italy.
[2]Istituto di Scienze dell'Alimentazione - CNR, via Roma, 64, 83110 Avellino, Italy. [3]Dipartimento di Scienze, Università degli Studi della Basilicata, V. le Ateneo Lucano, 10, 85100 Potenza, Italy.
[*]Corresponding Author E-mail: ippolito.camele@unibas.it

ABSTRACT

Morphological, physico-chemical and molecular investigations were accomplished on ascomata of *Tuber bellonae* from Basilicata (southern Italy) identified on the basis of their macro- and microscopic features. *Tuber aestivum* and *T. mesentericum* were used as comparative truffle species. An accurate comparison of ascospore dimensions, in 4-spored asci, a morphological character previously used to distinguish the above described truffles, was also carried out. The volatile organic compounds (VOCs) from *T. bellonae* were investigated by solid phase micro extraction technique coupled to gas chromatography and mass spectrometry (SPME-GC-MS). The following loci were used for molecular analysis: a) the beta-tubulin gene, b) the $(GTG)_5$ microsatellite, c) the M13 minisatellite. The ascospore volume of *T. bellonae* in 4-spored asci resulted significantly bigger than those of *T. aestivum* and *T. mesentericum*. Twenty-three VOCs were detected. Out of these, only 1,4-dimethoxy-2-methylbenzene, never found in *T. aestivum* and *T. mesentericum*, could be considered the main VOC marker discriminating *T. bellonae*. High polymorphism level was revealed in *T. bellonae* by both microsatellite and minisatellite markers. Finally, preliminary results on partial β-tubulin gene sequence of *T. bellonae* specimens suggest that *T. bellonae* it is closely related to *T. aestivum* and *T. mesentericum,* probably holding an intermediate position between them.

Key words: β-tubulin gene, molecular phylogeny, morphology, VOCs, *Tuber bellonae.*

INTRODUCTION

Basilicata, a region of southern Italy, is very rich in fungal biodiversity. In fact, about 83-85 taxa of hypogeous and semi-hypogeous fungi, including 29 *Tuber* entities were discovered in its territory in the last 14 years (Rana *et al*., 2008, 2012, 2015 and unpublished data). Among the *Tuber* species, *T. bellonae* Quél. (1887) can be considered, at present, a very less distributed and rare taxon in either Basilicata (Rana *et al*., 2008) or in few other truffle producing countries. It was initially found and described by Quélet in Provence (France) and successively evidenced in Italy, South-East of France and Spain (Pacioni and Fantini, 1997; Montecchi and Sarasini, 2000; Riousset *et al*., 2001) and, some years ago, in three humid and cold areas of central-southern Poland (Ławrynowicz *et al*., 2008).

Pacioni and Fantini (1997), who first studied and described the taxon in Italy, seemed to have used only very few ascomata originated in Sardinia and Spain and selected, among macro- and microscopic features useful for differentiating the species from *T. mesentericum* Vittad. and *T. aestivum* Vittad., the following ones: a) the average ascospore volume in tetrasporic asci: 15,000 μm^3 instead of 10,000 μm^3 and 5,000 μm^3, the last two measured in the single last two *Tuber* species, respectively; b) the ascospores shape and ornamentation: spherical (22.5–55 μm), rarely sub-spherical, enclosed by a reticulate-alveolate exosporium with net meshes delimited by 4–6 walls 5–7.5 μm high; c) a peridium constituted by very small and flat warts, almost never transversely striated; d) the odour, at first phenolic (3-methyl-anisole), then grateful due to the presence of etil-metyl-chetone, without any phenolic component; e) the colour of the gleba, light-brown with numerous, irregularly flexuous and subtle sterile veins; f) the ovoid (77–95 × 7–115 μm) asci, supported by an hooked pedicle and containing 1–6 spores.

The following additional and sometimes contrasting writings are, nevertheless, available on *T. bellonae*: 1) the odour of *T. bellonae*, according to Riousset *et al*. (2001) and Ławrynowicz *et al*. (2008), should be clearly phenolic and never grateful; 2) despite of the initial phenolic odour, Pacioni and Fantini (1997) though *T. bellonae* was more close to *T. aestivum* than to *T. mesentericum*, and also wrote that "some clones of *T. aestivum*, produce ascomata with a basal cavity and, in others, the relative amount of phenolic components is rather high"; 3) about ascospore shape, Riousset *et al*. (2001) reported that, in *T. bellonae* it is "spherique". On the other hand, samples of *T. bellonae* from Poland (Ławrynowicz *et al*., 2008) do not seem characterized by

ascospores that match with features underlined by the above mentioned authors because at least 40–50 % of them have a sub-spherical or ovoid form.

What above specified, along with the presence/absence of a cavity at ascoma's base and the existence, according to Pacioni and Fantini (1997), of hypothetical clones or variants of *T. mesentericum* with a striated peridium wart surface renders impossible to differentiate the above described taxa only using morphological characters. Discriminating factors seem to be, at contrary, the ascospore volume in tetrasporic asci and, overall, the enzyme electrophoretic pattern (Pacioni and Fantini, 1997; Pacioni and Pomponi, 1991).

In Basilicata, several hundred ascomata of *T. aestivum* [including *T. aestivum* (Chatin) Montecchi *et* Borelli 1990 fo. *uncinatum*] and *T. mesentericum* were macroscopically and microscopically examined since 1997-98 (Rana, unpublished data). Wart surface was always clearly and horizontally striated in the first species and, although less clearly also in the second one. Odour was always grateful in *T. aestivum* and in its genetically identical form *uncinatum* (Wedèn *et al*., 2005) whereas markedly phenolic and persistent in *T. mesentericum*. Ascospores were mostly ellipsoid or ovoid in mature asci of both species. The exosporium reticulum was rather regular in *T. aestivum* with pentagonal (but also rhomboid and esagonal) meshes larger than those of *T. mesentericum* which, according to Montecchi and Sarasini (2000) and Riousset *et al*. (2001), were *vice versa* irregular in shape and often interrupted. In addition, other ascomata, protected by a peridium with small and flat warts with surface weekly striated in horizontal sense, having a pleasant fungal odour, an exosporim reticulum like that of *T. mesentericum* and a percentage of spherical ascospores varying from 20 to 50–55 % were sometimes encountered. It has been hypothesized that these ascomata, could belong to *T. bellonae* which so far seems a not well defined black truffle taxon. For what above underlined and for verifying this hypothesis, it seemed opportune to accomplish some comparative micro- and macroscopic observations, VOCs studies and molecular analyses on typical ascomata of the three above mentioned *Tuber* species.

Molecular studies seem particularly helpful to assess the genetic diversity within and among taxa and furnish valuable information to better classify truffles (Halász *et al*., 2005; Frank *et al*., 2006; Bonito, 2010). Markers employed to assess the genetic variation in truffles and to serve as tools for a correct species identification are: RAPDs (see, among others, Rossi *et al*., 2000; Bertault *et al*. 2001; Wedèn *et al*., 2004); RFLPs (Rossi *et al*., 2000; Bertault *et al*., 2001; Paolocci *et al*., 2004), AFLPs (Murat *et al*., 2008; Riccioni *et al*., 2008), microsatellites and minisatellites (see, among others, Rubini *et al*., 2004; Pomarico *et al*., 2007) and rDNA of the Internal Transcribed Spacer (ITS) [see, among others, Mello *et al*., 2002, 2011; Bonito *et al*., 2010; Chen *et al*., 2011; Zambonelli *et al*., 2012].

The genetic diversity of *Tuber* species was extensively investigated during the last three decades and generally resulted closely linked to species. The high intra-polymorphism, detected in either *T. aestivum* or *T. mesentericum* compared to other truffles, let Pacioni and Pomponi (1991) to hypothesize that they are two species complexes. Despite all published works, knowledge available for *T. aestivum* and *T. mesentericum* (see, among others, Pomarico *et al*., 2007; Sica *et al*., 2007; Ławrynowicz, 2009; Hobart, 2011; Stobbe *et al*., 2012) is still incomplete because it is unknown how many effective types/subspecies could be present within each of these species complexes. In fact, another species, i.e. *T. sinoaestivum* sp. nov. (Zhang and Liu 2012, in Zhang *et al*., 2013), was recently found within *T. aestivum* in southwestern China (Zhang *et al*., 2012). Furthermore, presence of species complexes was also reported for *T. excavatum* Vittad. 1831, *T. gennadii* (Chatin) Patouillard 1903 (= *Terfezia gennadii* Chatin) and *T. candidum* Harkn. 1899 (Bonito, 2009). Apart from the previously revealed molecular markers, β-tubulin gene has been also extensively employed in species recognition and phylogeny of filamentous ascomycetes (Glass and Donaldson, 1995) and truffles (see among others, Paolocci *et al*., 2004; Jeandroz *et al*., 2008; Gryndler *et al*., 2011).

Due to its rarity in nature and its insignificant commercial value when compared to other truffles, *T. bellonae* has been less studied and thus, its classification is consequently still unclear and controversial. Furthermore, *T. bellonae* was compared to other *Tuber* species in only a very limited number of scientific papers (Gryndler *et al*., 2011; Božac *et al*., 2012; Zambonelli *et al*., 2012).

In this context, the present study was undertaken with the following goals: 1) to cast light on the microscopic features and VOCs of *T. bellonae* present in Basilicata; 2) to estimate the genetic variation of *T. bellonae* and its related species *T. aestivum* and *T. mesentericum* using as molecular markers the beta-tubulin gene, the $(GTG)_5$ microsatellite and the M13 minisatellite; 3) to assess phylogenetic relationships among the above mentioned truffles species and try to determine the taxonomic position of *T. bellonae*.

MATERIALS AND METHODS

Biological material: For morphological and molecular assays, ten mature ascomata for each of the three *Tuber* species i.e. *T. bellonae*, *T. aestivum* and *T. mesentericum*, collected in Potenza and Matera provinces of Basilicata in various years, were used (Table 1 and Figure 1). The host plants associated with the above mentioned truffle

species were mainly *Quercus* spp. and/or *Fagus sylvatica* L. In few cases oak and *Pinus* spp. were found together in natural beds of *T. bellonae* and *T. aestivum*. For the appartenance to *T. bellonae*, only specimens containing more than 40 % spherical spores (observed at 100×) were selected.

Morphological analysis: Truffle species identification was achieved on the basis of spore morphological traits according to Montecchi and Sarasini (2000), Riousset *et al*. (2001), Pacioni and Fantini (1997). To avoid eventual changes in ascospore size, distilled water was always used as mounting medium for microscopic slide preparation. For acquisition of bright field images, an Eclipse 80i microscope (Nikon, Japan), equipped with a 5 mega-pixels colour cooled digital camera (DS-5Mc, Nikon) and a DS-L1 camera controller (Nikon) interfaced with a PC, were used. External surface of truffle peridium was observed at 25× with a Zeiss stereomicroscope. Peridium structure was observed at 1000× using an Axioscop Carl Zeiss optical microscope equiped with a DS-U1 camera (Nikon). Ascospores were observed with a 10×/0.25 Plan Nikon objective to catch a greater number of spores in each focal field. Ascus and ascospore images were recorded employing a NIS-Element BR 3.0 software package (Nikon). From each ascoma sample, two microscope slides were prepared and micrographs of ascus and spores contained in 5 randomly selected fields were acquired. The following parameters were measured for each truffle species: the length (minimum, maximum and average values) of the major and minor ascospore axes (without ornamentations); the percentage and dimensions of asci with different spore number; the percentage of spherical, sub-spherical and ellipsoid ascospores in 1-, 2-, 3- and 4-spored asci; in the case of *T. aestivum* 5-spored asci were also considered; the ascospore volume (average value measured among spherical, sub-spherical and ellipsoid ascospores) in each ascus type (Table 2). The formula 4/3 π r3 (where r is the sphere radius) was used to calculate the volume of spherical ascospores; for sub-spherical and ellipsoid ones the formula 4/3 π a b2 (where a and b are the major and minor ellipsoid semi-axes, respectively) was preferred.

A total number of 915 ascospores (412 of *T. bellonae*; 207 of *T. aestivum*, and 296 of *T. mesentericum*) was analysed and, on the basis of Q, i.e. length/width quotient (Pacioni and Fantini, 1997), classified as spherical (Q from 1.00 to 1.02), sub-spherical (Q from 1.03 to 1.29) and ellipsoid (Q > 1.29).

A more stringent valuation, with Q values ranging from 1.00 to 1.002 for spherical spores and from 1.003 to 1.29 for sub-spherical ones was also accomplished. However, for comparative purposes, the Q values established by Pacioni and Fantini (1997) were used.

Analysis of Volatile Organic Compounds: Analysis of VOCs in *T. bellonae* (Table 1) was accomplished by SPME-GC-MS as described in details by Mauriello *et al*. (2001) and D'Auria *et al*. (2006). VOCs were commonly determined using thin slices (0.5 g) of each collected sample after a maximum 48 h storage at 4 °C in hermetically closed vials. VOCs detected were finally compared to those found in ascomata of *T. aestivum* and *T. mesentericum* in the past (Mauriello *et al*., 2001).

DNA extraction, PCR and sequencing: Ascomata, previously brushed under tap water to detach soil debris, were superficially sterilized with alcohol (90 %), deprived of peridium, cut in thin slices and frozen at – 20 ºC until molecular analyses were performed. Genomic DNA (gDNA) was isolated from 100 mg of gleba using DNeasy Plant Mini DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. The extracted gDNA concentration was determined using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, Delaware, U.S.A.). Subsequently, the extracted DNA was used as a template for β-tubulin gene amplification using the Bt2a+Bt2b primer pair and following the protocol of Glass and Donaldson (1995). Finally, the amplicons were purified, concentrated and directly sequenced as reported for the ITS region by Mang and Figliuolo (2010) with a minor change, e. g. β-tubulin gene primers Bt2a+Bt2b were used for either amplification or sequencing reactions. Partial β-tubulin gene sequences of *T. bellonae* were queried against those available in National Center for Biotechnology Information (NCBI) GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the BLASTN algorithm (Altschul *et al*., 1997).

M13 minisatellite amplification and analysis: Minisatellite variation analysis was carried out using the universal primer M13 (GAGGGTGGCGGTTCT) and amplifying the repetitive gDNA sequence (Latouche *et al*., 1997). PCR reactions were performed as suggested by Williams *et al*. (1990) with the following minor modifications: the final volume of PCR reaction was 25 µl, and contained 1X PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 U Taq DNA polymerase (Invitrogen, New York, U.S.A.), 0.2 µM M13 universal primer and 20 ng of gDNA template. Control experiments were included without template DNA.

Amplifications were performed with an *iCycler* (BioRad, Hercules, California, U.S.A.) and consisted of the following steps: 2 min at 95 °C, 1 min at 94 °C for 35 cycles, 1 min at 45 °C, 1 min at 72 °C and 5 min at 72 °C. Finally, amplified DNA fragments were separated in a 2 % agarose gel electrophoretic run in 1 X TBE buffer at 100 V, stained with ethidium bromide (EB), visualized under an EuroClone UV transilluminator (EuroClone Ltd., Milan, Italy) and finally photographed using an

OCD Video Camera Module. Every assay was performed at least twice. All clear bands at given molecular weight levels were considered while faint ones were not scored. Fragment sizes were estimated using a 1-kb DNA Ladder (Invitrogen). After a preliminary visual analysis of the electrophoretic profile of each taxon, the relative mobility of the amplified fragments was recorded using a binary code. Finally, Nei's genetic distance indices (Nei and Li, 1979) were calculated to evaluate the intra- and inter-taxa polymorphism (Peakall and Smouse, 2006, 2012).

$(GTG)_5$ microsatellite amplification and analysis*:* The $(GTG)_5$ primer reported by Buscot *et al*. (1996) was used to amplify the gDNA of all *Tuber* taxa analyzed. PCR reactions were performed as described by Guenaoui *et al*. (2013). Control experiments were carried out without template DNA. An *iCycler* (BioRad) was employed for the microsatellites amplification using a "touchdown" program as described by Don *et al*. (1991). Amplification products were analyzed by electrophoresis in 2 % agarose gel run in 1X TBE buffer at 100 V, subsequently stained with EB and photographed. Each assay was performed at least twice. Finally, PCR products analysis was carried out in the same mode reported for the M13 minisatellite.

Statistical analysis of molecular markers data: The analysis of binary haploid genetic data obtained with M13 and $(GTG)_5$ microsatellite markers was carried out with help of GenALEx 6.501 program (Peakall and Smouse, 2006, 2012). The Nei's genetic distance was calculated using a dataset containing combined data of both the above mentioned markers and considering three populations corresponding to the three analyzed tuber taxa. Subsequently, the distance matrix with all pair-wise comparisons was used as an input for a further principal components analysis (PCA) via covariance matrix with standardization to achieve a graphical output of the first two coordinates exhibiting the genetic relationships among the investigated taxa (Peakall and Smouse, 2006).

Phylogenetic analyses: Forty-one β-tubulin gene sequences were used in the phylogenetic analysis. Eight sequences were generated from this research and other 33 sequences were retrieved from EMBL-EBI European Nucleotide Archive database as shown in Table 1. In order to determine the phylogenetic position of *T. bellonae*, the eight partial β-tubulin gene sequences obtained in this study were compared with the 33 partial β-tubulin gene sequences downloaded from EMBL-EBI database. In addition, the partial β-tubulin gene sequence of *T. maculatum* Vittad. (FN555425), from NCBI database, was selected and used as the outgroup (Table 1). Phylogenetic analyses were carried out using MEGA version 6.0 software (Tamura *et al*., 2013), treating gaps as missing data, excluding ambiguous positions (complete deletion option) and using the CLUSTAL alignment method. Following sequence alignment, the evolutionary distances interpreted as units of the number of base substitutions per site were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). Codon positions included were 1st+2nd+3rd+Noncoding. The evolutionary history was inferred using the following methods: UPGMA (Sneath and Sokal, 973), Neighbor-Joining (NJ) (Saitou and Nei, 1987), Minimum Evolution (Rzhetsky and Nei, 1992) and Maximum Parsimony (MP) (Eck and Dayhoff, 1966). A bootstrap consensus un-rooted tree (cut-off value 70 %) was inferred from 1000 replicates to represent the evolutionary history of the investigated *Tuber* taxa (Felsenstein, 1985).

RESULTS

Morphology: Macroscopic and microscopic features of ascomata of the three *Tuber* species investigated, except for spherical spores percentage found in *T. bellonae* (Table 2), were like those described by Montecchi and Sarasini (2000) and Riousset *et al*. (2001). Transversal striae of peridium pyramidal wart faces were very evident, less clear and almost absent in *T. aestivum*, *T. mesentericum* and *T. bellonae*, respectively (Figure 2A–C). Peridium structure was pseudo-parenchymatic in the *Tuber* species studied, with polygonal, spherical and oval elements dimensions of which decreased from *T. aestivum* through *T. mesentericum* to *T. bellonae*. Peridium of the last species exhibited some kidney-shaped cells and all the species showed dark brown outer peridium layer cells (data not shown). Data regarding the other microscopic features of the truffles analysed in this study are reported in Table 2. Average ascus dimensions (width and length) (peduncle excluded) differed among the considered species: in particular, asci of *T. aestivum* had an ovate shape (axes: 66 and 98 µm) and the smallest size, while those of *T. bellonae*, oblong or ellipsoid (73 and 104 µm), and of *T. mesentericum*, elliptical (67 and 107 µm), were significantly larger. The smallest asci width generally characterized 1-spored asci of *T. aestivum*, while the major length was found in 4-spored asci of *T. mesentericum* (Table 2). Ascospore surface was, in *T. aestivum*, reticulate-alveolate with almost regular polygonal meshes, often containing one central crest, whereas appeared irregularly reticulate-alveolate in the other two species/taxa (Figure 3 A–C).

Compared to *T. mesentericum* (4 % spherical, 10 % sub-spherical and 87 % ellipsoid spores) and *T. aestivum* (9 % spherical, 32 % sub-spherical and 59 % ellipsoid), in which ellipsoid ascospores were predominant, *T. bellonae* was characterized by spherical (53 %) and sub-spherical (43 %) ascospores, even if a small percentage (4 %) of elliptical spores was found. Using a Q ranging from 1.00 to 1.002 for selecting spherical ascospores, their percentage in *T. bellonae*

decreased to 41 % and that of sub-spherical ones consequently increased up to 55 %. The percentage of spherical, sub-spherical and ellipsoid spores varied among the ascus type (1-, 2-, 3-, 4- and 5-spored asci). *T. aestivum* had, on average, ascospore dimensions slightly smaller than those of *T. mesentericum*, while *T. bellonae* had the ascospore length (average value of major axis) ranging from that of *T. mesentericum* to that of *T. aestivum* (Table 2). The average value of minor axis of *T. bellonae* spores, on the contrary, was significantly larger because of higher number of spherical and sub-spherical spores. In all three species, 2- and 3-spored asci were predominant. Among the analysed samples, a significant percentage (15.7 %) of 5-spored asci were only found in *T. aestivum* which, actually, exhibited also 1 % of 6-spored asci (data not shown). The average ascospore volumes in 4-spored asci of *T. bellonae*, *T. mesentericum* and *T. aestivum* were 49,379 μm^3; 19,584 μm^3 and 18,309 μm^3, respectively. The average ascospore diameter (42.1 µm), calculated in this study for *T. bellonae*, based on total spore number, is close to that obtained by Ławrynowicz et al. (2008) i. e. 38.7 µm (min 22.5 max 55 µm), even if these authors did not specify if they considered only the spherical spores.

VOCs: Fresh ascomata of *T. bellonae* used in this study had a fungal pleasant odour. When they were analysed by SPME-GC-MS showed 23 twenty three volatile compounds reported in Table 3. The main VOCs always found in *T. bellonae* were 1-methoxy-3-methylbenzene (peak area from 6.74 to 66.32 %) and 1,4-dimethoxy-2-methylbenzene (peak area from 3.70 to 19.43 %). Even if with a smaller peak area (ranging from 0.17 to 2.11 %), 3,5-dimethoxytoluene was found in 91 % of tested ascomata. Dimethylsulphide, a VOC found in the past in 100 % and 80 % of *T. mesentericum* and *T. aestivum* ascomata (Mauriello et al. 2001), respectively, was only detected in sample number 10. In one instance (sample number 2), SPME-GC-MS analysis, accomplished after 1 month storage at 4 °C, revealed the presence of hexanal as a probable oxidation product; 3-methyl-butanal and 3-methyl-butanol, which were not present in the fresh samples were also detected, whereas all other VOCs present in fresh samples exhibited a lower peak area.

Partial β-tubulin gene amplification and sequencing: Partial β -tubulin gene was efficiently amplified from gDNA of all three considered *Tuber* species always giving a PCR product of about 500 bp (Figure 4). Only amplification products of *T. bellonae* were directly sequenced and finally, eight β-tubulin gene sequences, were deposited into the EMBL-EBI database under the following accession numbers: HF947920, HF947921, HF947922, HF947923, HF947924, HF947925, HF947926, and HF947927 (Table 1).

(GTG)5 microsatellite length polymorphism: PCR amplification with the $(GTG)_5$ primer produced fragments ranging from 250 to 1400 bp. An average number of 6 amplified fragments was detected using this marker. But, no species-specific profile was revealed in the three *Tuber* taxa analyzed. Microsatellite $(GTG)_5$ analysis, due to the high number and different size of bands observed, indicated that there is a quite high genetic diversity among the specimens of either *T. bellonae* or the other two *Tuber* species. Finally, based on the $(GTG)_5$ profile, at moment, it can be assumed that an elevated polymorphism degree could be present within the rare species *T. bellonae* although more ascomata (if possible considering the already mentioned rarity of this taxon in nature) should be analyzed to support this assumption.

M13 minisatellite variation: The pattern of M13 minisatellite marker consisted of fragments ranging in size from about 400 to 1200 bp. An average number of 4 electrophoretic bands was obtained with this marker. For all the Tuber taxa analysed, M13 was also polymorphic at either inter- or intraspecific level. Results of this preliminary molecular investigation are consistent with either those reported by Pomarico et al. (2007) who found a high expected heterozygosity for M13 locus in *T. aestivum* and *T. mesentericum* in respect of other three Tuber species (*T. borchii* Vittad., *T. magnatum* and *T. brumale* Vittad.) or those of Pacioni and Pomponi (1991) who detected a high genetic heterogeneity in both *T. aestivum* and *T. mesentericum* based on multiloci isozyme patterns. Even if M13 marker seemed useful to distinguish different *Tuber* species and different profiles were detected for the three taxa here investigated, it should not be considered species-specific as it also showed a high intra-specific polymorphism.

PCA: Genetic analysis using M13 and $(GTG)_5$ markers data via PCA did not show any clear separation among the three *Tuber* taxa studied. Furtermore, as it is shown in Figure 5, only a scanty separation of *T. mesentericum* specimens from those of *T. bellonae* and *T. aestivum* was evidenced.

Phylogeny: A possible similar evolutionary history of *T. bellonae* was traced with all methods followed (UPGMA, NJ, ME and MP). Therefore, only that of NJ is here presented. The consensus tree showed an unique group (Clade I) of *T. aestivum -T. mesentericum* complex which also includes *T. bellonae* with moderate statistical support (bootstrap values about 50 %). This clade resulted consistently separated from *T. maculatum*, which, as expected, performed as a distant species (Fig. 6). Furthermore, within the big clade I, the eight specimens belonging to *T. bellonae* occupied two positions, e.g. four were placed near *T. aestivum* (subgroup A) and the other four were positioned close to *T. mesentericum* samples

(subgroup B). The only strong bootstrap support (97 %) detected was for 4 *T. bellonae* sequences placed within *T. aestivum* specimens (subgroup A) while a lower bootstrap support (< 50 %) was observed for the other 4 ascomata of *T. bellonae* (subgroup B) closer to *T. mesentericum*. In this context, the precise phylogenetic relationship among *T. bellonae*, *T. aestivum* and *T. mesentericum* still remain unclear. This is perhaps, due to the fact that the nucleotide variation present on only β-tubulin gene is surely insufficient to allow separation of such closely related taxa.

Table 1. *Tuber* species employed in this study and their partial β-tubulin gene sequences GenBank accession number

Species	Geographical origin (Place / Province)	Collecting date (mdy)	GenBank accession no.
T. bellonae	Muro Lucano / Potenza	12/14/2005	-
T. bellonae	Campomaggiore / Potenza	01/12/2006	**HF947920**
T. bellonae	Brienza / Potenza	01/13/2006	**HF947921**
T. bellonae	Pignola / Potenza	11/22/2005	**HF947922**
T. bellonae	Pignola / Potenza	03/06/2006	**HF947923**
T. bellonae	Campomaggiore / Potenza	02/09/2006	**HF947924**
T. bellonae	Tricarico / Matera	02/28/2006	**HF947925**
T. bellonae	Muro Lucano / Potenza	01/18/2006	-
T. bellonae	Abriola / Potenza	11/11/2011	**HF947926**
T. bellonae	Pierfaone / Potenza	11/17/2011	**HF947927**
T. aestivum	Pignola / Potenza	06/06/2006	-
T. aestivum	Gorgoglione / Potenza	05/24/2006	-
T. aestivum	Campomaggiore / Potenza	07/01/2006	-
T. aestivum	Muro Lucano/ Potenza	05/31/2006	-
T. aestivum	Pignola / Potenza	03/06/2006	-
T. aestivum	Forenza / Potenza	07/03/2005	-
T. aestivum	Tricarico / Matera	06/07/2005	-
T. aestivum	Tricarico / Matera	04/24/2005	-
T. aestivum	Ruoti / Potenza	05/31/2006	-
T. aestivum	Abriola / Potenza	11/30/2009	-
T. aestivum	-	-	AY226030*
T. aestivum	-	-	DQ336290*
T. aestivum	-	-	AF516813*
T. aestivum	-	-	AF516810*
T. aestivum	-	-	AY226029*
T. aestivum	-	-	AF516811*
T. aestivum	-	-	AF516812*
T. aestivum	-	-	AY170361*
T. aestivum	-	-	AF516801*
T. aestivum	-	-	AY226035*
T. aestivum	-	-	AY226033*
T. aestivum	-	-	AF516802*
T. aestivum	-	-	AF516807*
T. aestivum	-	-	AF516809*
T. aestivum	-	-	AY226031*
T. aestivum	-	-	AF516806*
T. aestivum	-	-	AY226034*
T. aestivum	-	-	AF516805*
T. aestivum	-	-	AY226032*
T. aestivum	-	-	AF516803*
T. aestivum	-	-	AF516804*
T. aestivum	-	-	DQ336291*
T. aestivum	-	-	GU979150*
T. aestivum	-	-	GU979152*
T. mesentericum	Gorgoglione / Potenza	12/26/2009	-
T. mesentericum	Brienza / Potenza	02/16/2006	-
T. mesentericum	Corleto Perticara / Potenza	12/13/2005	-
T. mesentericum	Abriola / Potenza	11/11/2011	-
T. mesentericum	Abriola / Potenza	12/06/2012	-

T. mesentericum	Pignola / Potenza	12/18/2012	-
T. mesentericum	Abriola / Potenza	11/30/2009	-
T. mesentericum	Villa d'Agri / Potenza	09/06/2006	-
T. mesentericum	Muro Lucano / Potenza	01/12/2006	-
T. mesentericum	Pignola / Potenza	03/06/2006	-
T. mesentericum	-	-	AY170362*
T. mesentericum	-	-	AF516816*
T. mesentericum	-	-	AF516817*
T. mesentericum	-	-	AF516818*
T. mesentericum	-	-	AY170364*
T. mesentericum	-	-	AY170363*
T. mesentericum	-	-	AF516814*
T. mesentericum	-	-	AF516815*
T. maculatum	-	-	FN555425*

The accession numbers with asterisk specify sequences downloaded from EMBL-EBI European Nucleotide Archive and NCBI nucleotide database. The minus symbol indicates that no data were available. Newly generated sequences accession numbers are evidenced in bold.

Table 2. Ascospore axes and volume in 1-4 spored asci and ascus type dimension of *Tuber bellonae*, *Tuber mesentericum* and *Tuber aestivum* from Basilicata

T. bellonae	**Length of ascospore axes (µm)**				
	Major Ø (min-max-average) 22.2 – 61.2 – 41.6		**Minor Ø (min-max-average) 21.7 – 58.6 – 40.1**		
	Ascus type dimension and %				
	1-spored (length=79 µm; width=73 µm) 17.9 %	**2-spored** (length=104µm; width=75 µm) 26.0 %	**3-spored** (length=100 µm; width=85µm) 39.0 %	**4-spored** (length=103 µm; width=99 µm) 17.1 %	
	Ascospore morphology and % in 1-4 spored asci				
	27 % spherical 50 % sub-spherical 23 % ellipsoid	44 % spherical 51 % sub-spherical 5 % ellipsoid	60 % spherical 38 % sub-spherical 2 % ellipsoid	52 % spherical 44 % sub-spherical 4 % ellipsoid	
	Average ascospore volume in 1-4 spored asci				
	50,276 µm^3	49,822 µm^3	49,490 µm^3	49,379 µm^3	
T. mesentericum	**Length of ascospore axes (µm)**				
	Major Ø (min-max-average) 29.0 – 60.9 – 44.9		Minor Ø (min-max-average) 23.7 – 43.6 – 33.6		
	Ascus type dimension and %				
	1-spored (length=75µm; width=67 µm) 17.0 %	**2-spored** (length=90 µm; width=76 µm) 26.6 %	**3-spored** (length=99 µm; width=79 µm) 36.2 %	**4-spored** (length=107 µm; width=87 µm) 20.2 %	
	Ascospore morphology and % in 1-4 spored asci				
	0 % spherical 0 % sub-spherical 100 % ellipsoid	2.0 % spherical 3.4 % sub-spherical 94.6 % ellipsoid	1.0 % spherical 17.4 % sub-spherical 81.6 % ellipsoid	5.0 % spherical 16.4 % sub-spherical 78.0 % ellipsoid	
	Average ascospore volume in 1-4 spored asci				
	20,676 µm^3	20,297 µm^3	19,796 µm^3	19,584 µm^3	
T. aestivum	**Length of ascospore axes (µm)**				
	Major Ø (min-max-average) 21.1 – 59.8 – 40.4			Minor Ø (min-max-average) 17.0 – 47.2 – 32.2	
	Ascospore morphology and % in 1-5 spored asci				
	1-spored (length=70 µm; width=66 µm) 14.3 %	**2-spored** (length=80 µm; width=61 µm) 27.1 %	**3-spored** (length=90µm; width=68 µm) 25.7 %	**4-spored** (length=99µm; width=70µm) 16.1 %	**5-spored** (length=98 µm; width=70 µm) 15.7 %
	Average ascospore volume in 1-5 spored asci				
	0 % spherical 60 % sub- spherical 40 % ellipsoid	18 % spherical 21 % sub-spherical 61 % ellipsoid	13 % spherical 27 % sub-spherical 60 % ellipsoid	7% spherical 32 % sub-spherical 61 % ellipsoid	4 % spherical 42 % sub- spherical 54 % ellipsoid
	Average ascospore volume in 1-4 (5) spored asci				
	19,732 µm^3	18,636 µm^3	18,598 µm^3	18,309µm^3	17,056 µm^3

Table 3. Volatile organic aroma compounds determined in *Tuber bellonae* by SPME-GC-MS

Compound	Retention time (min)	Sample number / Area (%) of the single compound									
		1	2	3	4	5	6	7	8	9	10
2-methyl-1,3-butadiene	1.66	4.21	1.63	4.21				7.52		6.03	
Dimethylsulfide	1.68										14.95
3-methyl-butanal	2.51	0.46		0.46	4.48		2.45	1.96	2.46	0.58	
3-methyl-1-butanol	3.55	0.12		0.12	0.51		0.18	0.18	0.38	0.12	
Hexanal	4.70				0.20				0.20		
2-heptanone	6.51						0.84			0.85	
1-octen-3-ol	8.39				11.52				11.29		
3-octanone	8.61			0.03				0.06	0.82		
1-methoxy-3-methylbenzene	9.25	66.32	69.50	66.32	16.45	65.57	7.47	64.81	17.14	6.74	45.29
1-undecene	10.62			0.19				1.29			
3-methylphenol	10.75										0.14
3-ethyl-5-methylphenol	11.12		0.88			0.90	0.32		0.08	0.22	2.17
2,3-dimethoxytoluene	12.37	0.51	0.21	0.51		0.22	0.05	0.49		0.15	
3,4-dimethoxytoluene	13.47	1.35	1.94	1.35		1.84	0.24	1.34		0.28	0.21
1,4-dimethoxy-2-methylbenzene	13.65	13.57	19.43	13.57	3.55	18.36	9.43	13.47	3.70	9.62	4.75
3,5-dimethoxytoluene	13.99	2.11		2.11	0.34	0.75	0.31	2.09	0.17	0.35	0.48
2,5-dimetoxyethylbenzene	14.98	14.98									0.07
4-butylindan-5-ol	16.32	16.32		0.04							
1,2,3-trimethoxy-5-methylbenzene	16.72	0.40	0.64	0.40		0.63	3.60	0.43		0.50	
1,3,5-trimethyl-2-cyclopentylbenzene	18.56			0.03			0.10	0.04			
2,6-diisopropylnaphtalene	20.44	0.02	0.06	0.02	0.07	0.16	0.27			0.12	0.02
1,7-diisopropylnaphtalene	20.52	0.04	0.06	0.04				0.07		0.13	0.02
1,3-diisopropylnaphtalene	21.07	0.03	0.05	0.03	0.06	0.14	0.12	0.03		0.06	0.04

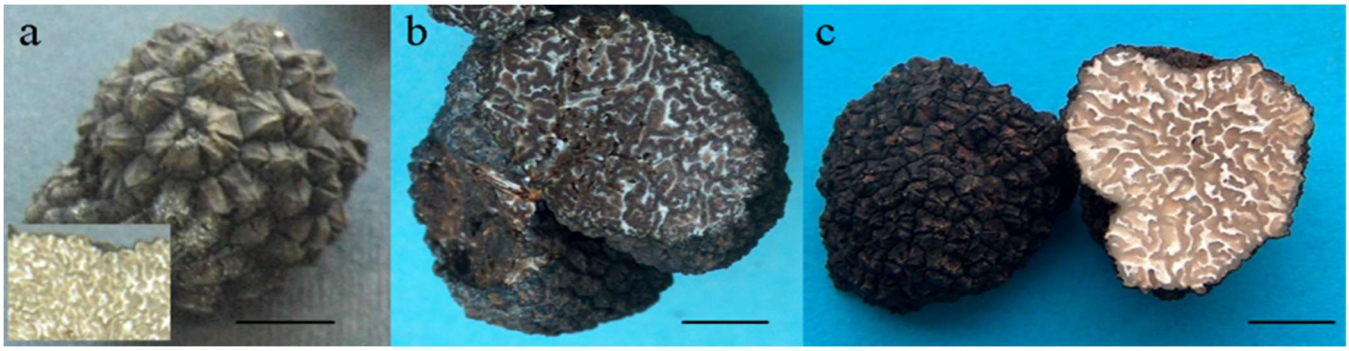

Figure 1. External and internal aspects of ascomata of *T. aestivum* (A), *T. mesentericum* (B) and *T. bellonae* (C). Scale bars: A-C = 1 cm.

Figure 2. Transversal striae of peridium pyramidal wart faces of *T. aestivum* (A), *T. mesentericum* (B) and *T. bellonae* (C). Scale bars: A = 3 mm; B = 1 mm; C = 0.8 mm.

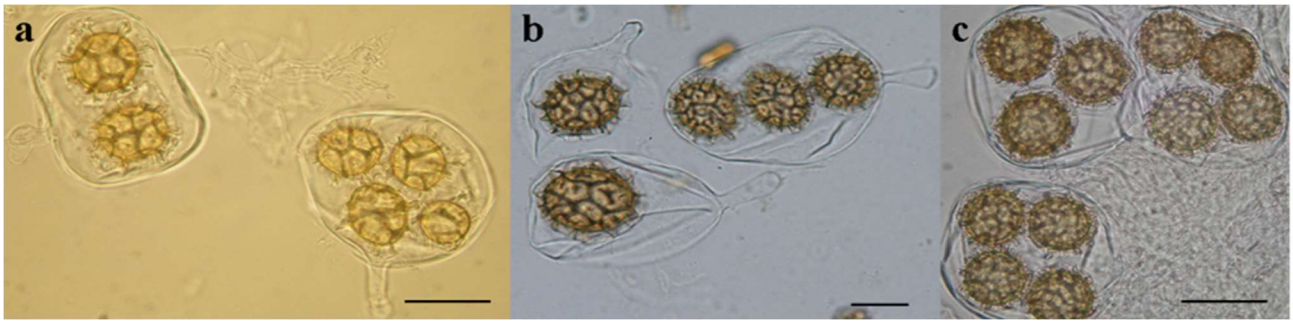

Figure 3. Asci and spores of *T. aestivum* (A), *T. mesentericum* (B) and *T. bellonae* (C). Scale bars: A, B = 25 µm; C = 50 µm.

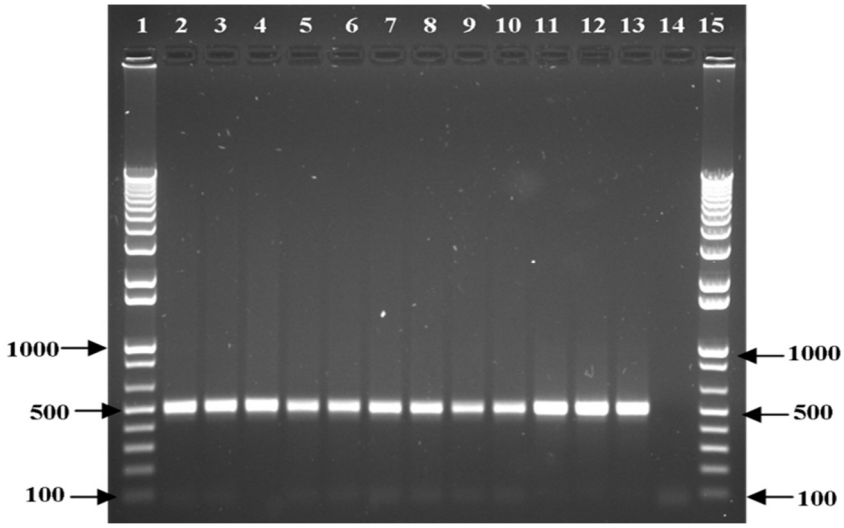

Figure 4. Electropherogram obtained after PCR amplification of 12 genomic DNA samples isolated from *T. bellonae*, *T. aestivum* and *T. mesentericum* using the β-tubulin primers Bt2a+Bt2b. Lanes: 1 and 15 = Marker 1Kb Plus DNA ladder (Invitrogen); 2–11 = *T. bellonae*; 12 = *T. aestivum*; 13 = *T. mesentericum* and 14 = negative control (no DNA template).

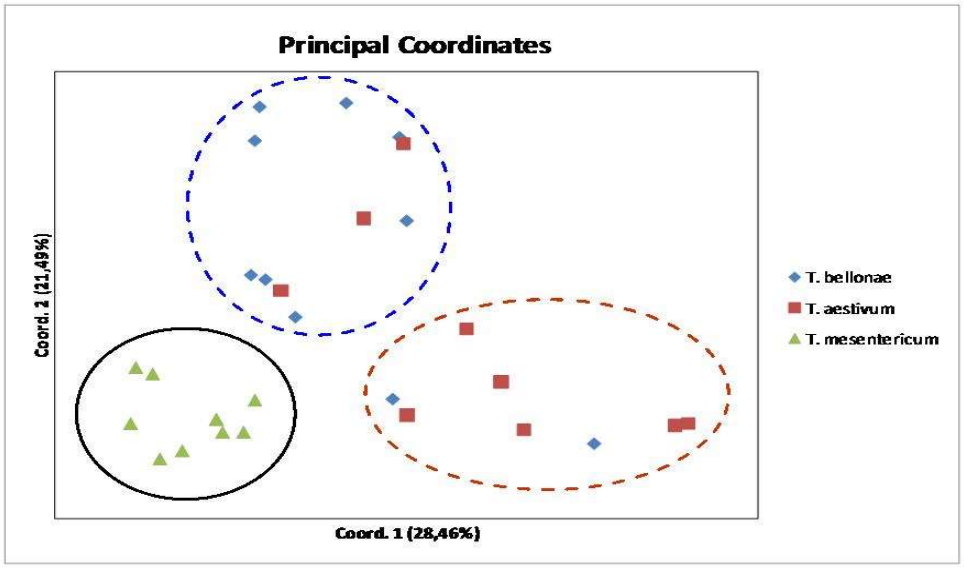

Figure 5. Genetic relationships among 30 specimens of *T. bellonae*, *T. aestivum* and *T. mesentericum* from Basilicata detected using M13 minisatellite and $(GTG)_5$ microsatellite loci. The first two coordinates explained only about 49.95% of the total variance. In few cases due to identical values, some specimens overlapped.

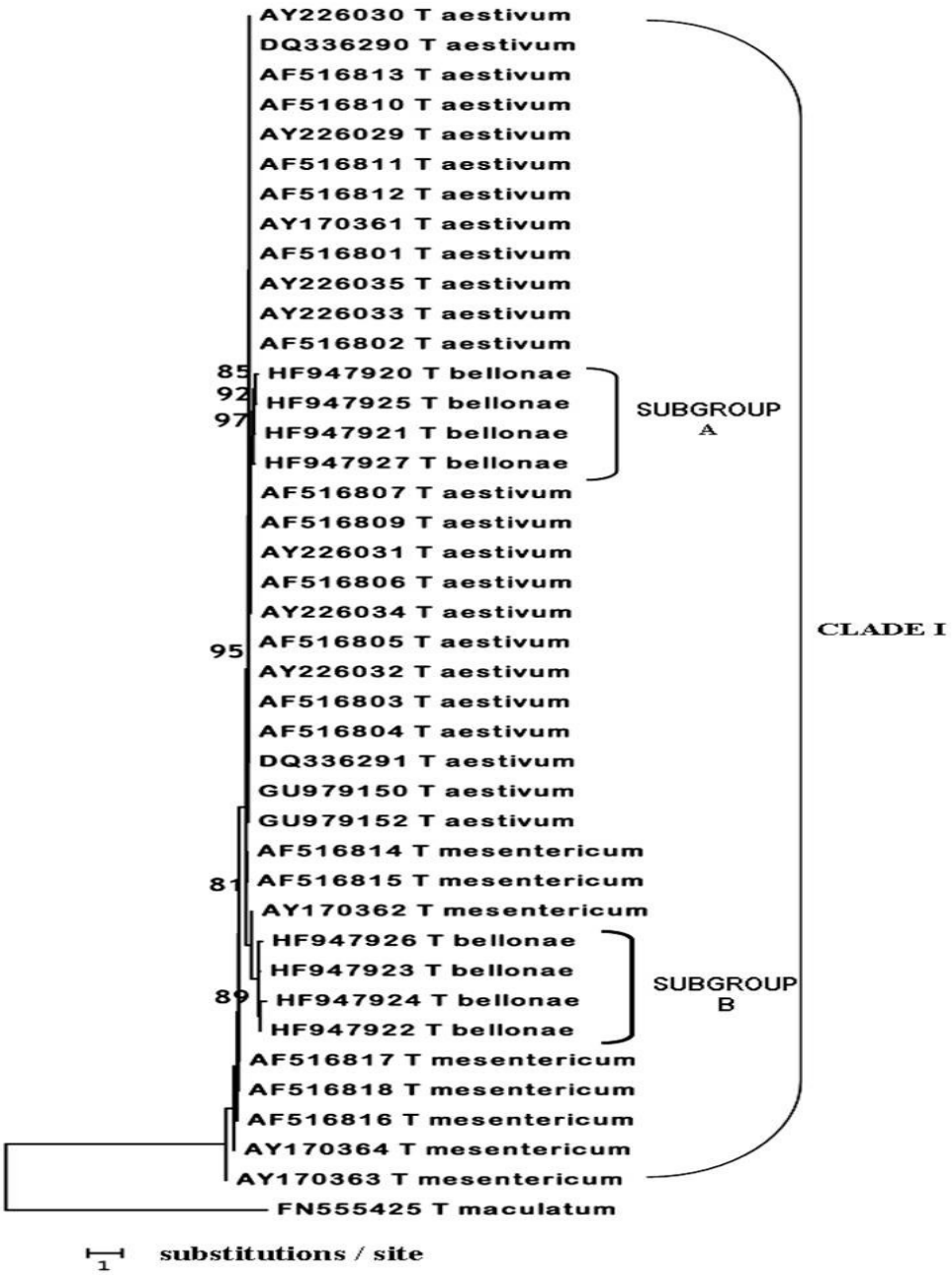

Figure 6. Consensus tree based on Neighbor-Joining analysis of partial β-tubulin gene sequences (361 bp) from 41 specimens of *T. bellonae*, *T. aestivum* and *T. mesentericum*. Taxa are labelled with GenBank numbers followed by the respective scientific names. Numbers on branches indicate bootstrap values from 1000 replicates. Branches correspondimg to partitions reproduced in less than 70 % bootstrap replicates are collapsed. The partial β-tubulin sequence of *T. maculatum* (FN555425) is added as an out-group. Evolutionary analyses were conducted in MEGA v.6.0.

DISCUSSION

Compared to the previous works (Pacioni and Fantini, 1997; Ławrynowicz *et al*., 2008) and treatises (Montecchi and Sarasini, 2000; Riousset *et al*., 2001) on *T. bellonae* in which no precise numerical data (e.g. number of measured ascospores) are reported, a satisfactory large ascospore number (412) was considered in this study. Therefore, the results referring to *T. bellonae* ascospore morphology and dimensions should be considered statistically more effective. The average ascospore volume in 4-spored asci of *T. bellonae*

ascomata from Basilicata was 49,379 μm^3 instead of 15,072 +/- 2.712 μm^3 reported by Pacioni and Fantini (1997). In our study, only the 16.3 % of ascospores in 4-spored asci of *T. bellonae*, had an average volume of 21,781 μm^3, value which is comparable with data of the above authors. Only for these spores, the resulting average diameter, i.e. 35.0 µm, can be considered close to the value of 31.5 µm which can be extrapolated from the results of Pacioni and Fantini (1997) using the formula $4/3\ \pi\ r^3$ reported in materials and methods.

VOCs analysis through SPME-GS-MS of *T. bellonae* ascomata was not accomplished in the past. Among the VOCs detected in this study, dimethylsulphide was found only in one of the examined ascomata whereas it had been detected by Mauriello *et al*. (2001) in 100 % and 80 % of samples of *T. mesentericum* and *T. aestivum*, respectivelly; 1-methoxy-3-methylbenzene, always present in aroma of *T. bellonae*, was found by the same authors in 78.5 % and 25 % of *T. mesentericum* and *T. aestivum* ascomata, respectively. Therefore, the above two VOCs could not be used to differentiate the three species of *Tuber*. Useful to recognize *T. bellonae* resulted, *vice versa*, the 1,4-dimethoxy-2-methylbenzene, which was always absent in aroma of *T. mesentericum* and *T. aestivum.* Another compound, 3,4-dimethoxytoluene, detected in 82 % of *T. bellonae* samples and not present in any of the two other mentioned *Tuber* species, could be also used to discriminate *T. bellonae*. Oddly enough, 3-methyl-anisole, with a phenolic odor, and etil-methyl-chetone, reported as characteristic for less and more mature *T. bellonae* ascomata by Pacioni and Fantini (1997), were never detected in the samples analyzed in this study.

Existence of a marked intraspecific heterogeneity in *T. bellonae* (and the two other related species) was confirmed by these preliminary results of genotypic analyses. In fact, all three markers employed [M13 minisatellite, $(GTG)_5$ microsatellite and β-tubulin] were suitable to prove the existence of high polymorphism in all the three analyzed taxa. First data obtained in this study using partial β-tubulin gene point out an intermediate position of *T. bellonae* between *T. aestivum* and *T. mesentericum* which does not exactly fit outcomes from earlier works based on morphology and isozyme patterns (Pacioni and Pomponi, 1991; Pacioni and Fantini, 1997). Furthermore, findings of this research demonstrate, for the first time, that a high molecular diversity is present in *T. bellonae*, a taxon not well investigated till now at molecular level. Furthermore, differently from Gryndler *et al*. (2011) who studied, among other truffle species, also a single ascoma of *T. bellonae* but did not obtain for it any PCR amplification employing Glass and Donaldson's β-tubulin gene primers, the fungal universal primers Bt2a+Bt2b employed in this study, allowed an effective and straightforward approach to investigate for the first time this gene into *T. bellonae* (Figure 4).

Microsatellite-primed PCR, using $(GTG)_5$ as primer, showed that a clear polymorphism exists among the tested specimens of *T. bellonae* from Basilicata region. Nevertheless, further studies using various molecular markers with different evolution rates and an increased number of ascomata are needed to draw some steady conclusions. A high and a modest genetic diversities were previously reported by other authors in *T. aestivum* and in *T. mesentericum*, respectively (Pacioni and Pomponi, 1991; Mello *et al*., 2002; Wedén *et al*., 2004; Wedén *et al*., 2005; Pomarico *et al*., 2007; Sica *et al*., 2007). The elevate intra and inter-specific polymorphism found in this work well matches these results. The M13 minisatellite marker was also useful to illustrate the presence of an intra- and inter-polymorphism within and among the analyzed *Tuber* taxa. Results of the present investigation confirmed those of Pomarico *et al*. (2007) who showed that analyzing different *Tuber* species, including *T. aestivum* and *T. mesentericum*, M13 marker can be a useful tool to explore intra-specific genetic variability.

The relationships found in this study among specimens belonging to the three *Tuber* taxa are not consistent with the results of Pacioni and Fantini (1997) who showed, by isozyme markers, the neat separation of *T. bellonae* from *T. aestivum* and *T. mesentericum*. In fact, based on minisatelite and microsatellite data, all ten *T. bellonae* specimens were not very clearly separated from the above two closely related taxa despite of a slight tendency of distinct clustering observed for eight of them. This may perhaps mean that M13 and $(GTG)_5$ markers could have a weak statistical power to detect *T. bellonae* into *T. aestivum-T. mesentericum* species-complex.

Although, the outcomes of morphological observations and spores measurement appear useful to distiguish the three species, the preliminary molecular acquirements achieved with this study still suggest an incomplete separation of *T. bellonae* from *T. aestivum* and *T. mesentericum.* In fact, the analysis of the β–tubulin gene variation seems to confirm that the *T. bellonae* is part of the large *T. aestivum-T. mesentericum* complex and is closely related to both species, although, perhaps, due to the small sample size and single gene consideration, the definition of groups into clade I lacks of very high statistical support (bootstrap values less than 70 %). Although, this work demonstrates the existance of a high polymorphism in *T. bellonae* and its proximity to *T. aestivum* and *T. mesentericum*, its exact phylogenetic position within the *T. aestivum-T. mesentericum* species complex cannot be clearly defined. In future, multiple loci analyses (MLA) already applied into truffle species phylogeny (Wang *et al*., 2006; Bonito *et al*., 2010; Bonuso *et al*., 2010) will be essential to confirm whether

this taxon could be considered or not as a separate species.

Disclosure Statement: No potential conflict of interest was reported by the authors.

REFERENCES

Altschul S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25(17): 3893-3402.

Bertault G., F. Rousset, D. Fernandez, A. Berthomieu, M.E. Hochberg, G. Callot and M. Raymond (2001). Population genetics and dynamics of the black truffle in a man-made truffle yield. Heredity 86: 451-458.

Bonito G (2009). Systematics and ecology of truffles (*Tuber*). PhD thesis, presented at Department of Biology Graduate School, Duke University, Durham, North Carolina.

Bonito G., J.M. Trappe, P. Rawlinson and R. Vilgalys (2010). Improved resolution of major clades within *Tuber* and taxonomy of species within the *Tuber gibbosum*-complex. Mycologia 102(5): 1042-1057.

Bonuso E, A. Zambonelli, S.E. Bergemann, M. Iotti and M. Garbelotto (2010). Multilocus phylogenetic and coalescent analyses in the Italian bianchetto truffle, *Tuber borchii* Vittad. Conserv. Gen. 11: 1453-1466.

Božac R., I. Širić and I. Kos (2012). *Tuber decipiens*, a new black Tuber species from Croatia. Period. Biol. 114(1): 125-128.

Buscot F., D. Wipp, C. Di Battista, J.C. Munch, B. Botton and F. Martin (1996). DNA polymorphism in morels: PCR/RFLP analysis of the ribosomal DNA spacers and microsatellite-primed PCR. Mycol. Res. 100: 63-71.

Chen J., S.-X. Guo and P.-G. Liu (2011). Species Recognition and Cryptic Species in the *Tuber indicum* complex. PLoS ONE 6(1): e14625.

D'Auria M., G. Mauriello, R. Racioppi and G.L. Rana (2006). Use of SPME-GC-MS in the study of time evolution of the constituents of saffron aroma: modifications of the composition during storage. J. Chromatogr. Sci. 44(1): 18-21.

Don R.H., P.T. Cox, B.J. Wainright, K. Baker, and J.S. Mattick (1991). 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. 19(14): 4008.

Eck R.V. and M.O. Dayhoff (1966). Atlas of Protein Sequence and Structure. National Biomedical Research Foundation, Silver Springs, Maryland.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39(4): 783-791.

Frank J.L., D. Southworth and J.M. Trappe (2006). NATS truffle and truffle-like fungi 13: *Tuber quercicola* and *T. whetstonense*, new species from Oregon, and *T. candidum* redescribed. Mycotaxon 95: 229-240.

Frizzi G., G. Lalli, M. Miranda and G. Pacioni (2001). Intraspecific isozyme variability in Italian populations of the white truffle *Tuber magnatum*. Mycol. Res. 105(3): 365-369.

Glass N.L. and G.C. Donaldson (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl. Env. Microbiol. 61(4): 1323-1330.

Granetti B., A. De Angelis and G. Materozzi (2005). Umbria terra di tartufi. Regione Umbria, Assessorato Agricoltura, Foreste, Caccia e Pesca (Eds.), Umbria, Italy.

Gryndler M., H. Hršelovà, L. Soukupovà, E. Streiblovà, S. Valda, J. Borovička, H. Gryndlerovà, J. Gažo and M. Miko (2011). Detection of summer truffle (*Tuber aestivum* Vittad.) in ectomycorrhizae and in soil using specific primers. FEMS Microbiol. Lett. 318(1): 84-91.

Guenaoui C., S.M. Mang, G. Figliuolo and M. Neffati (2013). Diversity in *Allium ampeloprasum*: From small and wild to large and cultivated. Gen. Res. Crop Evol. 60(1): 97-114.

Halász K., Z. Bratek, D. Szeg, S. Rudnóy, I. Rácz, D. Lásztity and J.M. Trappe (2005). Tests of species concepts of the small, white, European group of *Tuber* species based on morphology and rDNA sequences with special reference to *Tuber rapaeodorum*. Mycol. Progr. 4: 291-298.

Harkness H.W. (1899). Californian hypogeous fungi. Proc. Californian Acad. Sci. 3(1): 241-292.

Hobart C. (2011). *Tuber mesentericum* recorded in Britain. Field Mycol. 12(1): 23–25.

Jeandroz S., C. Murat, Y. Wang, P. Bonfante and F. Le Tacon (2008). Molecular phylogeny and historical biogeography of the genus *Tuber*, the 'true ruffles'. J. Biogeogr. 35: 815-829.

Latouche G.N., H.M. Daniel, O.C. Lee, T.G. Mitchell, T.C. Sorrel and W. Meyer (1997). Comparison of use of phenotypic and genotypic characteristics for identification of species of the amorph genus *Candida* and related teleomorph yeast species. J. Clin. Microbiol. 35: 3171-3180.

Ławrynowicz M . (2009). Four *Tuber* species accompanying *T. mesentericum* in natural sites in Poland. An. Jardin Bot. de Madrid 66 (Suppl. 1): 145-149.

Ławrynowicz M., T. Krzyszczyk and M. Fałdziński (2008). Occurrence of black truffles in Poland. Acta Mycol. 43(2): 143-151.

Lazzari B., A. Caprera ,C. Cosentino A., Stella , L. Milanesi and A. Viotti (2007). ESTuber db: An online database for *Tuber borchii* EST sequenze. BMC Bioinf. 8 (Suppl 1): S13.

Mang S.M. and G. Figliuolo (2010). Species delimitation in *Pleurotus eryngii* species complex inferred from ITS and EF-1α gene sequences. Mycology 1(4): 269-280.

Mauriello G., R. Marino, M. D'Auria, G. Cerone and G.L. Rana (2001). Determinazione dei componenti volatili dei tartufi lucani. Micol. Ital. 30(3): 11-29.

Mello A., A. Cantisani, A. Vizzini and P. Bonfante (2002). Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Env. Microbiol. 4(10): 584-594.

Mello A., C. Napoli , C. Murat, E. Morin, G. Marceddu and P. Bonfante (2011). ITS-1 versus ITS-2 pyrosequencing a comparison of fungal populations in truffle grounds. Mycologia 103(6): 1184-1193.

Montecchi A. and M. Borelli (1990). Funghi ipogei raccolti nelle località vittadiniane. Riv. di Micol. 33(3): 278-280.

Montecchi A. and M. Sarasini (2000). Funghi ipogei d'Europa. A.M.B., Trento (Ed.); a cura della Fondazione Centro Studi Micologici A. M. B. Vicenza, Italy.

Murat C., E. Zampieri, A. Vizzini and P. Bonfante (2008). Is the Perigord black truffle threatened by an invasive species? We dreaded it and it has happened. New Phytologist 178(4): 699-702.

Nei M. and W.H. Li (1979). Mathematical model studying genetic variation in terms of restriction endonucleases. Proc. Nat. Acad. Sci. U.S.A. 76(10): 5269-5273.

Nei M. and Kumar S. (2000). Molec. Evol. and Phylogen. Oxford University Press, New York, U.S.A..

Pacioni G. and O. Comandini (1999). *Tuber*. In: Ectomycorrhizal Fungi. Key genera in Profile. J.W.G. Cairney and S.M. Chambers (Eds). Chap. 6, pp. 163-186. Springer. Berlin Heidelberg, Germany.

Pacioni G. and P. Fantini (1997). *Tuber bellonae* un tartufo mediterraneo del complesso *Tuber aestivum-T. mesentericum*. Micol. Veg. Medit.12(1): 15-20.

Pacioni G. and G. Pomponi (1991). Genotypic patterns of some Italian populations of the *Tuber aestivum-T. mesentericum* complex. Mycotaxon XLII: 171-179.

Paolocci F., A. Rubini, C. Riccioni, F. Topini and S. Arcioni (2004). *Tuber aestivum* and *Tuber uncinatum*: two morphotypes or two species? FEMS Microbiol. Lett. 235(1): 109-115.

Patouillard N. (1903). Additions au catalogue des champignons de la Tunisie. BSMF 19: 245-261.

Peakall R. and P.E. Smouse (2006). GENALEX 6: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research. Mol. Ecol. Notes 6(1): 288-295.

Peakall R. and P.E. Smouse (2012). GenAlex 6.5: Genetic Analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28(19): 2537-2539.

Pico V. (1788). *Melethemata inauguralia: De Fungorum Generatione et Propagatione*. F. Rusconi (Eds.), Turin, Italy.

Pomarico M., G. Figliuolo and G.L. Rana (2007). *Tuber* spp. biodiversity in one of the southernmost European distribution areas. Biodiv. Conserv. 16(12): 3447-3461.

Quelét L. (1887). *Les champignons du Jura et des Vosges*. Suppl. 16 Assoc. Franc. Pour l'avanc. Des Sci. Tolouse, France.

Rana G.L., Mang S.M., Camele I., 2015. Biodiversity of Hypogeous Fungi in Basilicata. Chapter 8 in: The Sustainability of Agro-food and Natural Resource Systems in the Mediterranean Basin. A. Vastola (Ed.), pp. 305-318.

Rana G.L., A. Baglivo, R. Signore, R. Bochicchio, I. Camele. and S.M. Mang (2012). Studi recenti sui funghi ipogei lucani e pugliesi. Atti IX Conv. Naz. Biodiversità, 5-7 sett. 2012, I.A.M. Bari (Valenzano), Italia (1): 411–417.

Rana G.L., R. Marino, I. Camele and L. Altieri (2008). Nuove acquisizioni sui funghi ipogei della Basilicata. Micol. Ital. anno XXXVII 37(3): 52-64.

Riccioni C., B. Belfiori, A.Rubini, V. Passeri, S. Arcioni, F. Paolocci (2008). *Tuber melanosporum* outcrosses: analysis of the genetic diversity within and among its natural populations under this new scenario. New Phytologist 180(2): 466-478.

Riousset L. G., G. Chevalier and M. C. Bardet (2001). Truffles d'Europe et de Chine. Pubbl. Ctfl. I.N.R.A. (Ed.), Paris.

Rossi I., B. Bartolacci, L. Potenza, L. Bertini, E. Barbieri, and Stocchi V. (2000). Identification of white truffle species using RAPD markers. Plant and Soil 219(1-2): 127-133.

Rubini A., F. Topini, F. Riccioni, F. Paolocci and S. Arcioni (2004). Isolation and characterization of polymorphic microsatellite loci in white truffle (*Tuber magnatum*). Mol. Ecol. Notes 4(1): 116-118.

Rzhetsky A. and M. Nei (1992). A simple method for estimating and testing minimum evolution trees. Mol. Biol. Evol. 9(5): 945-967.

Saitou N. and M. Nei (1987). The neighbor joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4)**:** 406-425.

Schoch C.L., K.A. Seifert, S. Huhndorf, V. Robert, J.L. Spouge, C.A. Levesque, and W. Chen (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc. Nat. Acad. Sci. 109(16): 6241-6246.

Sica M., L. Gaudio and S. Aceto (2007). Genetic structure of *Tuber mesentericum* Vitt. Based on polymorphism at the ribosomal DNA ITS. Mycorrhiza 17(5): 405-414.

Sneath P.H.A. and R.R. Sokal (1973). Numerical Taxonomy. Freeman (Ed.), San Francisco, USA.

Stobbe U., U. Büntgen, L. Sproll , W. Tegel, S. Egli and S. Fink (2012). Spatial distribution and ecological variation of re-discovered German truffle habitats**.** Fungal Ecol. 5(5): 591-599.

Tamura K., M. Nei and S. Kumar (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Nat. Acad. Sci. 101(30): 11030-11035.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 30(12): 2725-2779.

Urbanelli S., P. Sallicandro, E. De Vito, L. Bullini and E. Biocca **(**1998). Biochemical systematics of some species in the genus Tuber. Mycologia 90(3): 537-546.

Vittadini C. (1831). *Monographia Tuberacearum*. Felicis Rusconi (Ed.), Milan, Italy.

Wang Tan Z.M., D.C. Zhang, C. Murat, S. Jeandroz and F. Le Tacon (2006). Phylogenetic relationships between *Tuber pseudoexcavatum*, a Chinese truffle, and other *Tuber* species based on parsimony and distance analysis of four different gene sequences. FEMS Microbiol. Lett. 259: 269-281.

Wedén C., E. Danell, F.J. Camacho and A. Backlund (2004). The population of the hypogeous fungus *Tuber aestivum* syn. *T. uncinatum* on the island of Gotland. Mycorrhiza 14(1): 19-23.

Wedén C., E. Danell and L. Tibell (2005). Species recognition in the truffle genus *Tuber* - the synonyms *Tuber aestivum* and *Tuber uncinatum*. Env. Microbiol. 7(10): 1535-1546.

Williams J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18(22): 6531-6535.

Zambonelli A., M. Iotti and F. Piattoni (2012). Chinese *Tuber aestivum sensu lato* in Europe. Open Mycol. J. 6: 22-26.

Zhang J.P., P.G. Liu and J. Chen (2012). *Tuber sinoaestivum* sp. nov., an edible truffle from southwestern China. Mycotaxon 122: 73-82.