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Assessment of inter- and intra-specific variability in the main species of *Boletus edulis* complex by ITS analysis

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Abstract

The *Boletus edulis* species complex includes ectomycorrhizal fungi producing edible mushrooms appreciated worldwide. However, species delineation is very difficult in these fungi, because it is based exclusively on a few, highly variable morphological features. As a consequence, a high number of *taxa* – including several varieties, subspecies and/or species *sensu stricto* – have been described in this species complex. In this paper we report on an extensive analysis of internal transcribed spacer of the nuclear rDNA region on a large sample of species of the *B. edulis* complex, mainly harvested in Italy, and representative of the European variability of this group. The molecular analysis allowed us to discriminate among and within *B. edulis*, *B. aestivalis*, *B. pinophilus* and *B. aereus* spp. and resolve their phylogenetic relationship.

Keywords: *Boletus edulis* complex; Specific variability; ITS; RFLP; Sequences analysis

1. Introduction

More than 5000 species of fungi form symbiotic associations, called ectomycorrhizae, with the apical root-tips of most shrubs and trees. Several ectomycorrhizal species produce edible fruiting bodies for which a flourishing market exists [1]. Among these, there are mushrooms that belong to the genus *Boletus* Bull.: Fr. with white, sweet flesh, which are commonly referred to as porcini, cèpes, boletes or steinpilze. These mushrooms have been well known since ancient times; Pliny the Elder (23–79 DC) praised their qualities and reported that they were consumed and commercialised widely during his age. The Italian market of boletes alone involves almost 500 companies and accounts for about two million Euros, although the demand for these fungi is only very partially (approximately 10%) satisfied by internal production [2]. These mushrooms are harvested and marketed in different countries worldwide (France, Spain, Eastern European countries but also South Africa, Mozambique, China, the Philippines, to name a few). Accordingly, boletes represent a valuable economical resource for developing countries and for marginal and inland areas of developed countries. At present, the identification of

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the different European species of *Boletus*, is very difficult because it is based exclusively on a few, highly variable morphological characters [3–8].

Therefore, marketing strategies aimed at protecting the peculiarity of each *Boletus* species, through price differentiation and labelling according to the species, strain and/or provenance, need molecular markers to ensure a reliable species and strain characterization. These markers will also be particularly useful in typing these fungi throughout their life cycle and would provide mycologists with reliable tools for the correct identification of their mycelia and mycorrhizal roots. These are the preliminary steps towards the production, under controlled conditions, of mycorrhizal plants to be used in programmes of boletes cultivation. The cultivation of such symbiotic fungi will contribute to the economic and social growth of marginal areas and increase plant nutrient uptake and fitness in agroforestry systems. The aim of the present research was to find out whether the distinction among and within *Boletus taxa* based on morphological features is supported by a phylogenetically and taxonomically informative marker such as the internal transcribed spacer (ITS) of the rDNA region.

Setting up a reliable method for typing these *Boletus* species during the various phases of their life cycle is also a preliminary condition for their possible future cultivation.

2. Materials and methods

2.1. Samples collection

The criteria used for sampling *Boletus* fruiting bodies were: species, geographical origin, pedological substrates and host-plant association. Specimens deposited in the herbaria of the University of L'Aquila (AQUI) and of the Royal Botanic Gardens (Kew) as well as from the authors' private collections were also considered. Each basidioma was first accurately cleaned and then identified through a macro- and microscopic analysis [7,9]. More than 120 samples were examined, and among these the specimens showing the typical morphological and anatomical traits of *Boletus edulis* Bull.: Fr., *B. aestivalis* (Paulet) Fr., *B. pinophilus* Pilát et Dermek and *B. aereus* Bull.: Fr., those exhibiting deviating (morphological and/or ecological) features in comparison with the typical form and finally those of uncertain identification were subjected to molecular analysis. The samples for which molecular analyses were carried out are listed in Table 1.

Vouchers of all the samples collected in the field and used in this study are deposited in the *Herbarium Mycologicum Aquilanum* (AQUI).

2.2. Molecular analyses

DNA from fresh and dried basidiomata (0.35–200 mg) was isolated as described by Paolocci et al. [10]. The DNA quality and concentration was evaluated on 1% agarose gel in presence of High DNA Mass Ladder (Invitrogen). Primers used for PCR amplification and sequencing of the ITS region were ITS1F [11], ITS4 [12], 5.8SF and 5.8SB [13]. The amplifications were performed in a Gene Amp PCR system 2400 and 9700 (Applied Biosystems) according to Rubini et al. [13] using the following cycling parameters: an initial denaturation at 94 °C for 2 min and 30 s; 25 cycles consisting of 30 s at 94 °C, 30 s at 55 °C and 45 s at 72 °C; a final extension at 72 °C for 7 min. The DNA isolated from herbarium specimens was amplified using a higher number of cycles (35–40) according to Bruns et al. [14], Taylor and Swan [15]; in this case the PCR reaction mix was enriched by 5 μl of 70 mg/ml bovine serum albumin (BSA; Sigma–Aldrich), as reported in Paolocci et al. [10].

The PCR products were purified using GenElute™ PCR DNA Purification Kit (Sigma–Aldrich) before sequencing. Sequence analysis was carried out using the BigDye™ Terminator Cycle Sequencing Reaction Kit (Applied Biosystems) according to the manufacturer's protocol on ABI 310 and ABI 377 DNA sequencers (Applied Biosystems). The amplified ITS from *B. aestivalis* were cloned in pGEM-T-Easy Vector system (Promega) and sequenced using T7 and SP6 primers. The sequences are deposited in GenBank and the corresponding accession numbers are reported in Table 1.

Sequences were aligned in Clustal X version 1.81 [16] and the alignment was manually optimized. Phylogenetic analyses were performed using MEGA version 2.1 [17]. The phylogenetic tree was obtained using the neighbour-joining method [18] based on the two parameter distance model of Kimura [19]. *Boletus pseudoregius* (Bps1; GenBank accession no. AY680996) and *Boletus luridus* (GenBank accession no. AY278765), were chosen as the outgroup. A bootstrap test was performed using 1000 replicates. Gaps were considered by selecting the pairwise deletion option in MEGA.

The PCR/RFLP analysis of ITS1F/ITS4 amplicons was carried out for 2–4 h with 1–2 units of the *Alu*I restriction enzyme according to manufacturer's instructions (Sigma–Aldrich). The products resulting from endonuclease digestions were size-fractioned on 2% agarose gel.

3. Results and discussion

As a result of the macroscopic and microscopic observation, samples were divided into two classes: those showing the most typical and representative characters of *B. edulis*, *B. aestivalis*, *B. pinophilus* and

Table 1
List of basidiomata of the *Boletus edulis* species complex considered in this study

Species	Samples	GenBank accession nos.	Collection sites	Morphological and ecological notes
B. edulis	Be1F	AY680982	La Morricana – Teramo (I)	Typical; *Fagus silvatica*
B. edulis	Be2F	AY680983	La Morricana – Teramo (I)	Typical; *Fagus silvatica*
B. edulis	Be3F	AY680984	La Morricana – Teramo (I)	Ecru-coloured cap
B. edulis var. *pusteriensis*	Bepust 6	AY680985	Montminal (F)	–
B. personii	Be2297	AY680986	Baselica – Parma (I)	*Castanea sativa*
B. personii	BpersBxF	AY680981	Val Fondillo – L'Aquila (I)	*Fagus silvatica*
B. venturii	Bvent2226	AY680989	Kardich (A)	–
B. edulis	Be6F	AY680994	La Morricana – Teramo (I)	Typical; *Fagus silvatica*/*Abies alba*
B. edulis	Be7F	AY680993	Ceppo – Teramo (I)	Typical; *Fagus silvatica*/*Abies alba*
B. edulis	Be8F	AY680992	Ceppo – Teramo (I)	Large and deep network on stipe
B. edulis	Be9F	AY680991	La Morricana – Teramo (I)	Ash-coloured cap
B. edulis	Be821	AY680995	Lago Scuro – Parma (I)	Typical
B. edulis	Be953	AY680988	Schiena dell'Asino – Reggio Emilia (I)	Typical; very close white network on stipe
B. edulis	Be1983	AY680990	Brunico – Bolzano (I)	Olivaceous; coloured stipe
B. edulis	Be2173	AY680987	Pattanella – Grosseto (I)	Rooting stipe; brown-orange cap; on sea sands
B. pinophilus	BpiA	AY680972	Ceppo – Teramo (I)	Typical; *Abies alba*
B. pinophilus	Bpi2F	AY680974	Chiarino – Teramo (I)	Typical; *Fagus silvatica*
B. pinophilus	Bpi3F	AY680975	M. Ernici – L'Aquila (I)	Chestnut-brown; on conglomerates
B. pinophilus	Bpi4F	AY680976	Chiarino – Teramo (I)	Decolourized
B. pinophilus	Bpi7F	AY680977	Chiarino – Teramo (I)	Small network on stipe
B. pinophilus	Bpi9A	AY680973	La Morricana – Teramo (I)	Typical; *Abies alba*
B. pinophilus	Bpi1622	AY680978	Botte Donato – Cosenza (I)	On granitic sands; *Pinus calabrica*
B. pinophilus	Bpi1623	AY680979	Cugnale di Falcone – Cosenza (I)	On granitic sands; *Pinus calabrica*
B. pinophilus	Bpi1447	AY680980	Lago Gora – Reggio Emilia (I)	Dark; *B. aereus* like
B. aestivalis	Baest7Q	AY680963	Le Felciare – L'Aquila (I)	Dark-brown; finely cracked cap edge; *Quercus pubescens*
B. aestivalis	Baest1379	AY680964	Monteorsaro – Reggio Emilia (I)	Dark-brown cap cracked on the edge; *Fagus silvatica*
B. aestivalis[a]	Baest2133	a: AY680967 b: AY680968	Waldshenke – Bolzano (I)	Pale orange pores; infertile
B. aestivalis[a]	Baest1914	a: AY680969 b: AY680970	Lago Scuro – Parma (I)	Fawn-coloured cap; *Abies alba*/*Picea abies*/*Pinus silvestris*
B. aestivalis[a]	Baest6F	a: AY680965 b: AY680966	Cascine – L'Aquila (I)	Bright with very cracked cuticle; on rendzine soil; *Fagus silvatica*
B. aestivalis	BaestAR	AY680971	Città di Castello – Perugia (I)	*B. edulis* like; *Castanea sativa*
B. aereus	Baer2100	AY680961	Pulpiano – Reggio Emilia (I)	Ocraceous with very cracked cuticle
B. aereus	Baer76	AY680960	Calizzo – Reggio Emilia (I)	Typical with dark network
B. aereus	BaerZac1C	AY680954	Civitella Roveto – L'Aquila (I)	Bleached
B. aereus	BaerZac2C	AY680955	Civitella Roveto – L'Aquila (I)	Bleached
B. aereus	BaerZac3C	AY680956	Canistro – L'Aquila (I)	*B. aestivalis* like
B. aereus	BaerZac4C	AY680957	Canistro – L'Aquila (I)	*B. aestivalis* like
B. aereus	BaerZac5C	AY680958	Canistro – L'Aquila (I)	*B. aestivalis* like
B. aereus	BaerZac6C	AY680959	Pian di mele – Viterbo (I)	Typical, very dark; on vulcanic ashes
B. aereus	Baer1585	AY680962	Gallice – Cosenza (I)	Silver fibrillous cap edge; *B. edulis* like

[a] Indicates ITS sequences from cloned PCR fragments.

B. aereus and those exhibiting morphological features of uncertain classification. The ITS region of the nuclear ribosomal repeat DNA was chosen as a suitable molecular marker to investigate inter- and intra-specific relationships within and among various fungal species [20]. The amplicons resulting from the ITS1F/ITS4 amplification showed length polymorphisms across the samples with the morphological traits typical to the four species considered, with products ranging from 750 to 898 bp (Fig. 1(a)). A further picture of ITS length polymorphisms among these species was obtained by amplifying the two internal transcribed spacers, ITS1 and ITS2, separately. This analysis revealed that the greater size polymorphism was relative to the ITS2 rather than ITS1 spacer. *B. pinophilus* and *B. edulis* as well as *B. aereus* and *B aestivalis* showed, in fact, ITS1 with similar size, while all four species were clearly differentiated on the basis of ITS2 length (Fig. 1(b)–(c)). Thus, the ITS2 appears to be the most informative rDNA region for achieving species differentiation in the *B. edulis* species complex. However, all the samples of uncertain classification did not show clear differences in the ITS length with respect to *B. edulis*, *B. aestivalis, B. pinophilus* or *B. aereus sensu stricto*.

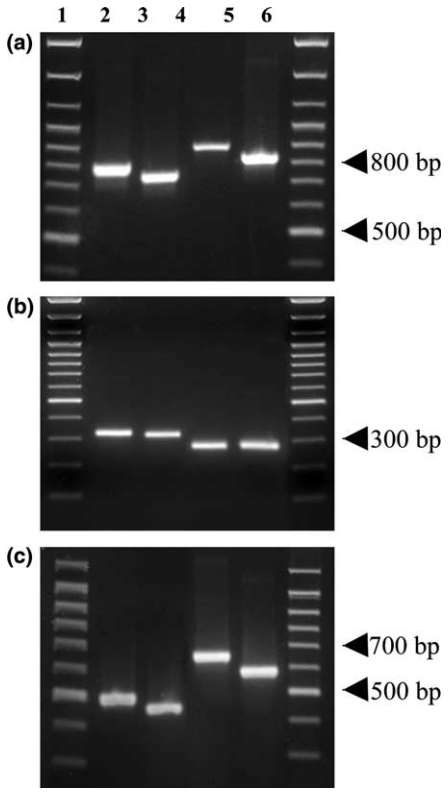

Fig. 1. PCR amplification of ITS region from *Boletus* spp. fruit bodies using the primers ITS1F/ITS4 (a), ITS1F/5.8SB (b) and 5.8SF/ITS4 (c). Lanes 1 and 6: Gene Ruler 100 bp DNA Ladder (Fermentas); lane 2: *B. edulis*, sample Be1F; lane 3: *B. pinophilus*, sample BpiA; lane 4: *B. aereus*, sample BaerZac6C; lane 5: *B. aestivalis*, sample Baest6F.

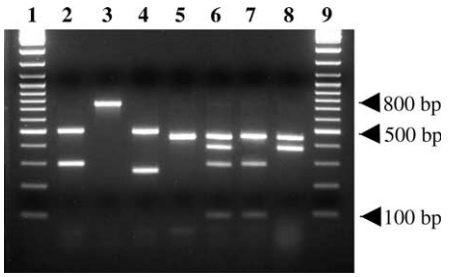

Fig. 2. *Alu*I RFLP analysis of *Boletus* spp. ITS region amplified with ITS1F/ITS4 primers pair. Lanes 1 and 9: Gene Ruler 100 bp DNA Ladder (Fermentas); lane 2: *B. edulis*, sample Be1f; lane 3: *B. pinophilus*, sample BpiA; lane 4: *B. pinophilus*, sample Bpi3F; lane 5: *B. aereus*, sample BaerZac6C; lane 6: *B. aestivalis*, sample Baest6F; lane 7: *B. aestivalis*, sample Baest7Q; lane 8: *B. aestivalis*, sample Baest1914.

Therefore, to better assess the degree of inter- and intra-specific polymorphism as well as the phylogenetic relationship within *B. edulis* complex, ITS sequencing was performed.

When ITS1F/ITS4 amplicons were directly sequenced, clean sequences were obtained in all samples with the exception of few *B. aestivalis* individuals. On these last samples a cloning step was performed before sequencing. The sequence analysis of multiple ITS clones revealed the simultaneous presence in each of these *B. aestivalis* samples of at least two ITS spacers that differ for a single nucleotide insertion/deletion (indel). This indel was the cause of the unsuccessful direct sequence analysis in these samples.

The extensive ITS sequencing analysis allowed us to select a number of restriction enzymes to evaluate using RFLP the genetic polymorphism, within and among the four species considered [21]. The *Alu*I enzyme turned out to be the most interesting one allowing us to distinguish among the different *Boletus* spp. as well as strains within *B. pinophilus* and *B. aestivalis* (Fig. 2). More specifically, the *Alu*I RFLP map shows the presence of only one recognition sequence in *B. edulis* that produces two fragments of 296 and 498 bp, respectively. We found a similar situation in *B. aereus* where two bands, each one of about 450 bp, resulted from the digestion. Conversely, two RFLP patterns were displayed after *Alu*I digestion in *B. pinophilus*, with most (seven out of nine) samples showing an *Alu*I restriction site, while the other two did not. Interestingly, samples showing either RFLP pattern were harvested in the same collection site. Similarly, within *B. aestivalis*, two ITS alleles were detected, differing for the number of *Alu*I recognition sites. Because some samples showed the presence of both ITS forms, a total of three *Alu*I RFLP patterns were revealed within *B. aestivalis* (Fig. 2).

The phylogenetic analysis showed that the 39 *Boletus* specimens under investigation are grouped into four, clearly distinguished clusters, as supported by the high bootstrap values (Fig. 3).

The similarity observed among the *B. edulis–B. pinophilus* and *B. aestivalis–B. aereus* pairs on the basis of the ITS1 length, was also confirmed by the ITS phylogenetic tree: each pair probably clusters sister species. Interestingly, molecular data are in agreement with morphological traits (i.e., slimy or dry cuticle, flesh colour under the cuticle, different structure and organization of the pileipellis, spore size) that showed high similarity between *B. edulis* and *B. pinophilus* as well as between *B. aestivalis* and *B. aereus* species, respectively [6–8].

Inside each group, the analysis shows a low variability in ITS sequences with an average nucleotide diversity of 0.01%, 0.13%, 0.33% and 0.25% in *B. edulis*, *B. pinophilus*, *B. aereus* and *B. aestivalis*, respectively.

Notably, the samples classified as varieties (*B. edulis* var. *pusteriensis*) or those exhibiting peculiar characters compared to the typical specimens (i.e., bleached forms, colour variation of cuticle and pores, etc.) did not show distinctive ITS sequences. Other molecular markers such as AFLP, RAPDS or SSR might help to disclose better than ITS any possible genetic polymorphism within these *B. taxa*. However, the ITS sequencing analysis does not support the classification of *B. personii* and

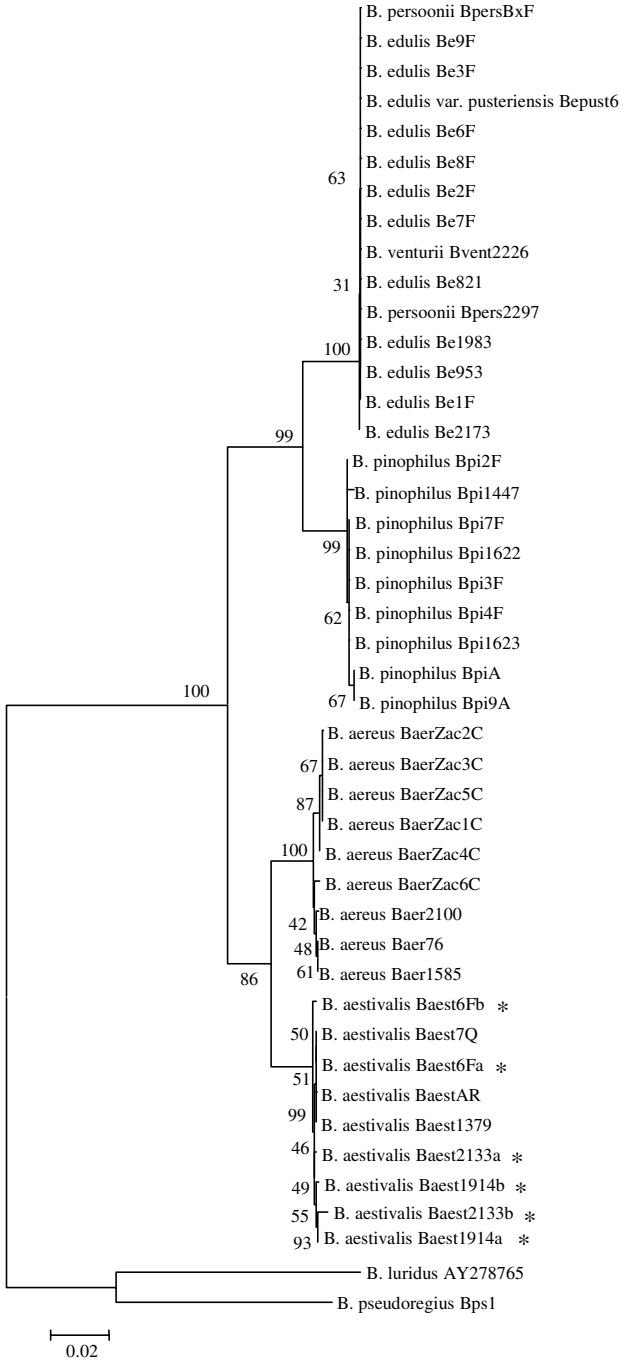

Fig. 3. Neighbour-joining tree obtained from ITS1-5.8s-ITS2 sequences (* indicated sequences derived from cloned PCR fragments).

B. venturii samples at the rank of species but, conversely, it proves that they clearly belong to *B. edulis*.

Herein we provide molecular markers for the successful discrimination among closely related *Boletus* species. The availability of such markers is a pre-requisite for the reliable identification of these mushrooms in order to foster their marketing, cultivation and studies on their ecology. The sole ITS2 length polymorphism turned out to be an informative tool in order to differentiate among mycelia and fresh or preserved mushrooms of the four species of commercial interest belonging to the *B. edulis* complex, whereas the ITS/RFLP approach allowed us to characterize strains at least within *B. pinophilus* and *B. aestivalis*. In addition, extensive ITS sequencing allowed us to assess the phylogenetic relationship among these taxa while showing informative sites where ITS primers specific to each *Boletus* species could be designed. Work is in progress to design such primers and standardize the relative PCR conditions to provide mycologists with a very efficient tool to start in-depth, specific studies on the ecology and biodiversity of these symbiotic fungi as well as to type mycorrhizal plants that could be nursery inoculated with *Boletus* spp. to promote their cultivation.

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