Morphological and molecular characterisation of mycelia of ectomycorrhizal fungi in pure culture

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Boletus edulis, *Boletus aestivalis*, *Boletus luridus*, *Amanita muscaria* and *Hebeloma radicosum* mycelia were isolated in pure culture and characterised by morphological and molecular methods. Molecular identification was performed by sequence analyses from the ITS region of nuclear ribosomal RNA genes. The phylogenetic affiliation of the isolated mycelia were evaluated by comparison of their ITS sequences with those deposited in the GenBank database. Pure cultures of isolates of the different fungal genera under investigation showed differences in growth rate, colony morphology and/or hyphal biometric characters. In contrast, the morphological characteristics of the mycelia of the three *Boletus* species were similar, but these species were distinguished by ITS data. Problems remain, however, in the affiliation of these ITS sequences with those of the *B. edulis* group that are currently deposited in public databases.

Key words: *Amanita muscaria*, *Boletus aestivalis*, *Boletus edulis*, *Boletus luridus*, *Hebeloma radicosum*, molecular characterisation, morphological characterisation, pure culture.

Introduction

Mycorrhizal fungi are of great interest for environmental and forestry application due to the advantages that mycorrhizae provide for the host plant. Furthermore, some ectomycorrhizal fungi are also of economic importance in that they are edible, such as truffles, boletes and chanterelles (Watling, 1997; Tibiletti and Zambonelli, 2000). Several species are used in the commercial production of infected plants and the starting point is generally seedling inoculation either with spores or mycelial cultures (Hall and Wang, 1998). The first step in the production of infected plants using pure cultures of mycorrhizal

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fungi is to obtain axenic-mass cultures under controlled conditions (Kuek, 1994). Even when the fruit bodies used to obtain these pure cultures are unambiguously identified based on their morphological characters, the identity of the isolated mycelia should also be confirmed by other methods (Iotti *et al*., 2002). Misidentification of pure cultures can occur, in particular for those fungal isolates that grow very slowly on synthetic media, such as ectomycorrhizal fungi (Mello *et al*., 2001; Bridge *et al*., 2003). Some morphological characters of colonies and hyphae have been used to confirm the identity of ectomycorrhizal fungi in pure culture (Brundrett *et al*., 1996) but these characters are not very distinctive with respect to those of sporulating fungi (Arx, 1980; Gravesen *et al*., 1994). Recently, new molecular methods primarily based on the polymerase chain reaction (PCR) have provided rapid, sensitive, and reliable alternatives for identifying ectomycorrhizal fungi at any phase of their life cycle (Amicucci *et al*., 2001; Horton and Bruns, 2001).

Despite their considerable value, molecular methods have so far been applied for the identification of pure cultures for only a restricted number of ectomycorrhizal fungi, primarily to those of *Tuber* spp. (Rossi *et al*., 1999; Iotti *et al*., 2002). The aim of this study was, therefore, to extend the molecular and morphological characterization of ectomycorrhizal mycelia to basidiomycetous species of ecological or economic importance.

Materials and methods

Isolation of the mycelia

Basidiome of *Amanita muscaria* (L.) Hook., *Boletus edulis* Bull., *B. aestivalis* (Paulet) Fr. (= *B. reticulatus* Schaeff.), *B. luridus* Schaeff. and *Hebeloma radicosum* (Bull.) Ricken were collected in summer-autumn 2000-2001 and August 2002 (*B. luridus*, herbarium number 1968) in three different sites of the Emilia Romagna Apennines (Italy) (Table 1), and dried specimens of each species were deposited in the herbarium of the "Centro di Micologia" of Bologna. The species were identified by morphological methods, according to Breitenbach and Kränzlin (1995).

Blocks of tissue 1-2 mm across, aseptically excised from the inner part of the cap, were cultured on modified Woody Plant Medium (mWPM, Table 2) (Lloyd and McCown, 1980) and incubated in the dark, at 22 ± 1°C, for 2-3 months before the first transplanting. The isolated mycelia were then maintained in culture in half strength Potato Dextrose Agar (20 g/l) (hsPDA) (Difco, Detroit, MI, USA).

Table 1. Provenance, herbarium number (herbarium of the "Centro di Micologia" of Bologna, Italy) and probable host plant of the isolated ectomycorrhizal fungi.

Species	Herb. n.	Collection locality	Probable host plant	Date of isolation	Strain
Boletus aestivalis	1773	Loiano	*Castanea sativa* Mill.	15-06-01	Bre1
Boletus edulis	1640	Castiglione dei Pepoli	*Fagus silvatica* L.	20-10-00	Edu2
Boletus luridus	1807	Zocca	*Castanea sativa* Mill.	10-08-01	Blu3
Boletus luridus	1968	Zocca	*Castanea sativa* Mill.	08-08-02	-
Amanita muscaria	1642	Castiglione dei Pepoli	*Fagus silvatica* L.	20-10-00	Amu1
Hebeloma radicosum	1647	Castiglione dei Pepoli	*Fagus silvatica* L.	20-10-00	Hra1

Table 2. Composition of mWPM.

Compound	Concentration	Compound	Concentration
$MgSO_4 \cdot 7H_2O$	0.37 g/l	H_3BO_3	6.2 mg/l
KH_2PO_4	0.17 g/l	$Na_2MoO_4 \cdot 2H_2O$	0.25 mg/l
$CaCl_2 \cdot 2H_2O$	0.096 g/l	$CuSO_4 \cdot 5H_2O$	0.025 mg/l
NH_4NO_3	0.4 g/l	$FeSO_4 \cdot 7H_2O$	27.8 mg/l
$Ca(NO_3)_2 \cdot 4H_2O$	0.556 g/l	$NaEDTA \cdot 2H_2O$	37.3 mg/l
K_2SO_4	0.9 g/l	Myo-inositol	0.1 g/l
$MnSO_4 \cdot H_2O$	22.3 mg/l	D(+)-glucose	8 g/l
$ZnSO_4 \cdot 7H_2O$	8.6 mg/l	Agar	10 g/l

Morphological characterization

The morphology of the colonies was observed both on mWPM and hsPDA plates and described according to Stalpers (1978). Petri dishes of 5 cm diam. containing 12 ml of mWPM were inoculated with a 0.7 cm disk of mycelial felt taken from the rim of 30 day-old cultures on hsPDA. Plates were kept wrapped in Parafilm "M" ® (American National CanTM, Chicago, IL, USA) to avoid dehydration of the culture media and maintained in the dark, at 22 ± 1°C. The growth of the fungal colonies was recorded every week along two preset diametrical lines. There were five replicate plates in each treatment and the whole experiment was repeated three times.

Micro-morphological features of the cultures were only examined in mWPM since in this medium the fungal colonies are looser than in hsPDA, making microscopic analyses easier. Agar plugs (1 cm^2 × 0.3 cm high) collected from the surface of the 60 day-old fungal colonies were examined under a light microscope Laborlux 12 (Leitz, Wetzlar, Germany); images were captured using a high-resolution colour video camera (JVC, Yokohama, Japan) and 50 measurements per parameter made with the Axio Vision 2.05 image analysis software (Zeiss, Jena, Germany). The hyphal growth unit (total hyphal length / number of hyphal tips) (Trinci, 1973), branching angle, hyphal

diameter, septal distance and percentage of clamps were measured in the peripheral growth zone of the mycelium.

Molecular characterisation

Molecular identification of the isolated mycelia and of the *Boletus luridus* 1968 basidiome was performed using sequence data of the ITS region of the nuclear ribosomal DNA. Total genomic DNA was isolated from 100 µg of fungal tissue (one-month-old mycelial cultures or fresh basidiome) by DNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and then eluted in 50 µl of sterile water. ITS-1, 5.8S and ITS-2 regions were amplified in a 50 µl volume reaction containing 1-10 ng of genomic DNA, using the primers pair ITS1 and ITS4 (White *et al*., 1990) in a T gradient Thermal Cycler (BIOMETRA, Göttingen, Germany) according to Amicucci *et al*. (1996). PCRs were performed using 2.5 units of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania).

The amplified products were purified by Gene Clean II kit (BIO 101, Vista, CA, USA) and sequenced using the two primers mentioned above. Sequence reactions were run in a ABI PRISM 3700 DNA Analyzer (Applied Biosystem, Foster City, CA, USA) with Big Dye Terminator v3.1 chemistry. The ITS sequences of the different species were compared to those available in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTN search (Altschul *et al*., 1997).

Sequences were aligned using PileUp program in the Genetics Computer Group (GCG) Package version 9.1, Madison, Wisconsin. The programs utilised for the phylogenetic analyses of the complete ITS1-5.8S-ITS2 region are included in the PHYLIP software package version 3.5c (Felsenstein, 1993). Distance matrix was inferred using the DNADIST program and the phylogenetic trees were constructed from the evolutionary distance matrix with FITCH and using the DNAML and DNAPAR programs for the maximum likelihood and the parsimony analyses, respectively. The robustness of the tree topologies was evaluated by bootstrap analysis based on 200 re-samplings of the sequence alignment. Bootstraps were performed with DNABOOT and tree branches with a confidence level > 75% were considered significantly robust. The TreeView program was used to plot the treefiles (Page, 1996).

Nucleotide sequence accession numbers

The ITS sequences obtained in this study have been deposited in GenBank with the following accession numbers: *Amanita muscaria* mycelium (Amu1 strain) (AY278768), *Boletus luridus* mycelium (Blu3 strain)

(AY278765), *B. luridus* fruit body (AY278766), *B. aestivalis* mycelium (Bre1 strain) (AY278769), *B. edulis* mycelium (Edu2 strain) (AY278764), *Hebeloma radicosum* mycelium (Hra1 strain) (AY278767). The other sequences included in this study were obtained from the GenBank database (Table 3).

Results

Morphological and growth characteristics

The growth rate of mycelia on mWPM is reported in Fig. 1. During the exponential growth phase, the radial growth of the mycelium of *Hebeloma radicosum* was about twice as high as that in other species. The *Amanita muscaria* mycelium showed the shortest lag phase. The stationary phase did not occur for *Boletus* mycelia within eight weeks of analysis.

The macroscopic characters of mycelial cultures and the biometric characteristics of the hyphae and clamp frequency of the isolated mycelia are reported in tables 4 and 5, respectively.

Amanita muscaria colonies were white and the mycelium developed only at the surface of the media on both mWPM and hsPDA. They did not exude pigments or droplets. The hyphae often grew interlaced (Fig. 2b) and formed frequent anastomoses. Clamp connections were short, large, and slightly curved (Fig. 2d).

Hebeloma radicosum colonies were cream with abundant aerial mycelium on mWPM, and yellowish with crustose brown areas on hsPDA. They did not exude pigments or droplets. The hyphae only rarely formed anastomoses. Clamp connections were short, large, and abrupt (Fig. 2e).

Boletus edulis colonies were white, whereas those of *B. aestivalis* and *B. luridus* were cream and pale-brown, respectively, on both tested media. On hsPDA, mycelia of the three *Boletus* species exuded brown pigments, that coloured the medium from yellowish to brownish. Moreover, on that medium, *Boletus* colonies exuded pigmented droplets from the aerial hyphae. Conversely they did not secrete pigments and droplets in the mWPM. The mycelium of all the studied *Boletus* species developed both on the surface of the media and within them but only the hyphal morphology of *B. luridus* was greatly modified within the medium (Fig. 2a) showing a helical growth as already reported by Iotti *et al.* (2002) for *Tuber* mycelia. Hyphal swelling was common in the three *Boletus* species (Figs. 2c-f). Hyphal septa were generally localised close to the hyphal branches and lacked clamp connections.

Table 3. Species analysed in this study.

Species	GenBank accession no.	Lenght (bp)	References
A. muscaria (L.) Hook.	AF085490	628	Lim and Jung (1998)
	AB081295	695	Oda *et al.* (2002)
	Z54294	669	Nehls (1995) *
	AJ549964	634	Schmid *et al.* (2003) *
A. rubescens (Pers.) Gray	AF085484	643	Lim and Jung (1998)
	AB015682	723	Oda *et al.* (1999)
A. spissa (Fr.) P. Kumm.	AF085486	653	Lim and Jung (1998)
A. citrina (Schaeff.) Pers.	AB015679	734	Oda *et al.* (1999)
	AF085489	660	Lim and Jung (1998)
	AY325846	506	Hallen *et al.* (2003) *
A. phalloides Fr.	AY325836	486	Hallen *et al.* (2003) *
	AJ308097	697	Vasilenko and Rtischeva (2001) *
A. virosa (Fr.) Bertill.	AY325829	480	Hallen *et al.* (2003) *
	AB015676	695	Oda *et al.* (1999)
A. caesarea (Scop.) Pers.	AY486237	515	Bernedo Cornejo *et al.* (2003)*
A. gemmata (Fr.) Gillet	AF335440	1217	Berbee *et al.* (2001) *
A. pantherina (DC.) Krombh.	AB080978	704	Oda *et al.* (2002)
	AB015701	712	Oda *et al.* (1999)
H. radicosum (Bull.) Ricken	AF124700	610	Aanen *et al.* (2000)
H. truncatum (Scaeff.) P. Kumm.	AF124701	616	Aanen *et al.* (2000)
H. sinapizans (Fr.) Sacc.	AF124682	614	Aanen *et al.* (2000)
	AY320380	660	Boyle *et al.* (2003) *
H. mesophaeum (Pers.) Fr.	AY311521	662	Boyle *et al.* (2003) *
	AF124692	615	Aanen *et al.* (2000)
H. senescens (Batsch) Berk. & Broome	AY312987	663	Boyle *et al.* (2003) *
H. edurum Métrod	AF124698	614	Aanen *et al.* (2000)
H. sarcophyllum (Peck) Sacc.	AF124715	610	Aanen *et al.* (2000)
H. sacchariolens Quél.	AY312985	663	Boyle *et al.* (2003) *
	AF124689	612	Aanen *et al.* (2000)
H. pallidoluctuosum Gröger & Zschiesch.	AY311526	664	Boyle *et al.* (2003) *
H. pusillum J.E. Lange	AY312982	659	Boyle *et al.* (2003) *
	AF124702	609	Aanen *et al.* (2000)
H. helodes J. Favre	AY311516	659	Boyle *et al.* (2003) *
	AF124710	606	Aanen *et al.* (2000)
H. crustuliniforme (Bull.) Quél.	AF124708	610	Aanen *et al.* (2000)
B. luridus Scaeff.	AJ419191	644	Martin and Raidl (2002)
Uncultured ectomycorriza	AF465183	648	Selosse *et al.* (2002)
B. rhodoxanthus (Krombh.) Kallenb	AJ419189	732	Martin and Raidl (2002)
B. calopus Pers.	AJ296293	680	Martin and Raidl (2002)
B. fragrans Vittad.	AJ419186	686	Martin and Raidl (2002)
B. impolitus Fr.	AJ419187	653	Martin and Raidl (2002)

Table 3 continued. Species analysed in this study.

Species	GenBank accession no.	Lenght (bp)	References
B. erythropus Fr.	AJ496595	602	Martin and Raidl (2002)
B. aestivalis (Paulet) Fr.	AY130295	957	den Bakker *et al.* (2002) *
B. pinicola (Vittad.) A. Venturi	AJ419190	642	Martin and Raidl (2002)
B. edulis Bull.	AJ419182	539	Martin and Raidl (2002)
	AF074921	601	Grubisha *et al.* (2002)
	AJ416955	218	Moor *et al.* (2002)
Agrocybe praecox (Pers.) Fayod	AF124713	611	Aanen *et al.* (2000)
Pisolithus tinctorius (Pers.) Coker & Couch	AF374710	640	Martin *et al.* (2002)

* published only in GenBank database.

Table 4. Macroscopic characters of mycelial cultures.

Strain	Characters of the mat								Reverse	
	Advancing zone				Aerial mycelium		Colony colour			
	Mycelial growth		Outline		Texture of mat					
	mWPM	hsPDA	mWPM	hsPDA	mWPM	hsPDA	mWPM	hsPDA	mWPM	hsPDA
Amu1	appressed	appressed	even	even	felty	felty	white	white	slightly darkened	slightly darkened
Hra1	appressed	submerged	bayed	fringed	felty	subfelty	cream	yellowish	unchanged	unchanged
Edu2	appressed	appressed	bayed	bayed	felty	felty	white	white	darkened	darkened
Bre1	appressed	appressed	fringed	fringed	felty	felty	cream	cream	darkened	darkened
Blu3	appressed	submerged	even	even	felty	felty	pale brown	pale brown	darkened	darkened

Table 5. Hyphal morphological characteristics of the isolates.

Strain	Hyphal growth unit (μm)	Branching angle (°)	Septal distance (μm)	Hyphal diameter (μm)	Clamp frequency (%)
Bre1	427.90 ± 159 (177.26-947.41)	54.76 ± 14.35 (33.45-81.54)	78.82 ± 20.94 (35.37-130.99)	2.47 ± 0.23 (2.04-2.98)	0
Edu2	473.14 ± 168.12 (201.56-1054.4)	32.40 ± 10.57 (20.71-64.61)	82.71 ± 27.84 (36.67-130)	2.75 ± 0.27 (2.37-3.4)	0
Blu3	466.91 ± 165.01 (259.8-979.17)	40.72 ± 10.72 (27.43-70.77)	85.25 ± 25.30 (30.93-133.39)	2.35 ± 0.24 (1.85-2.84)	0
Amu1	462.20 ± 206.12 (216.86-1102)	59.52 ± 13.46 (36.42-93.12)	40.15 ± 14.07 (20-63.22)	1.99 ± 0.21 (1.62-2.37)	24.72
Hra1	426.75 ± 256.11 (122.88-985.23)	58.67 ± 17.09 (31.42-89.55)	46.19 ± 13.38 (20.92-78.37)	2.22 ± 0.25 (1.74-2.78)	62.50

Data are the mean of 50 measures from three different Petri dishes.

Molecular characterisation

Comparison of the ITS sequences obtained from the isolated mycelia with the sequences available in the GenBank databases allowed us to analyse the phylogenetic affiliation of *Amanita muscaria*, *Boletus luridus*, *B. aestivalis*, *B. edulis*, and *Hebeloma radicosum*.

ITS sequence of Blu3 strain, isolated from a fruit body of *Boletus luridus*, showed the highest levels of similarity (99%, identity = 598/601 nt) with a nearly complete ITS1-5.8S-ITS2 sequence of an uncultured ectomycorrhiza of presumptive *Boletaceae* family (accession number AF465183), described by Selosse *et al*. (2002), and with our sequence of the *B. luridus* basidiome (herbarium number 1968) collected in Italy; the similarity with the sequence of a strain labelled *B. luridus* (accession number AJ419191) by Martin and Raidl (2002) was only 94% (identity = 579/613). The phylogenetic position shown in Fig. 3 confirms that our Blu3 strain corresponds to *B. luridus*.

The molecular data available for *B. edulis* are confusing and the molecular identification of both *B. edulis* and *B. aestivalis* is contradictory. Several sequences with a significantly high level of ITS similarity (> 90%) from *B. edulis* and *B. aestivalis* mycelia were partial sequences containing only the ITS-1 region of about 200 nt.

In this study, the phylogenetic analysis was carried out using only nearly full length ITS1-5.8S-ITS2 sequence available in the databases; however to better analyse the molecular data existing, Fig. 4 shows a pairwise alignment based on the best available sequences of the ITS-1 region of *B. edulis*, in which it is possible to verify the high level of similarity (identity = 161/161 nt) of our *B. aestivalis* sequence with the ITS-1 of a *B. edulis* sample (accession number AJ416955) described as "Chinese king bolete" by Moor *et al*. (2002).The phylogenetic position of the nearly complete ITS1-5.8S-ITS2 sequence from *B. edulis* mycelium shows a monophyletic origin with respect to the other edible boletes but branches separately from the *B. edulis* described by Grubisha *et al*. (2002) (accession number AF074921) and Martin and Raidl (2002) (accession number AJ419182) (Fig. 3). The ITS sequence obtained from our *B. edulis* mycelium showed 99% similarity (identity = 717/719 nt) with a ITS sequences of *B. aestivalis* (accession number AY130295) not published yet and with a partial ITS-1 sequence (accession number AJ416956) described as summer bolete by Moor *et al*. (2002).

Sequence variation among *Amanita muscaria* was found to be generally very low. In particular the ITS1-5.8S-ITS2 sequence our *A. muscaria* mycelium differed by only 1 or 2 bp in the ITS-1 and by 2 or 4 bp in the ITS-2 from *A. muscaria* sequences available in the database and its phylogenetic position is shown in Fig. 5.

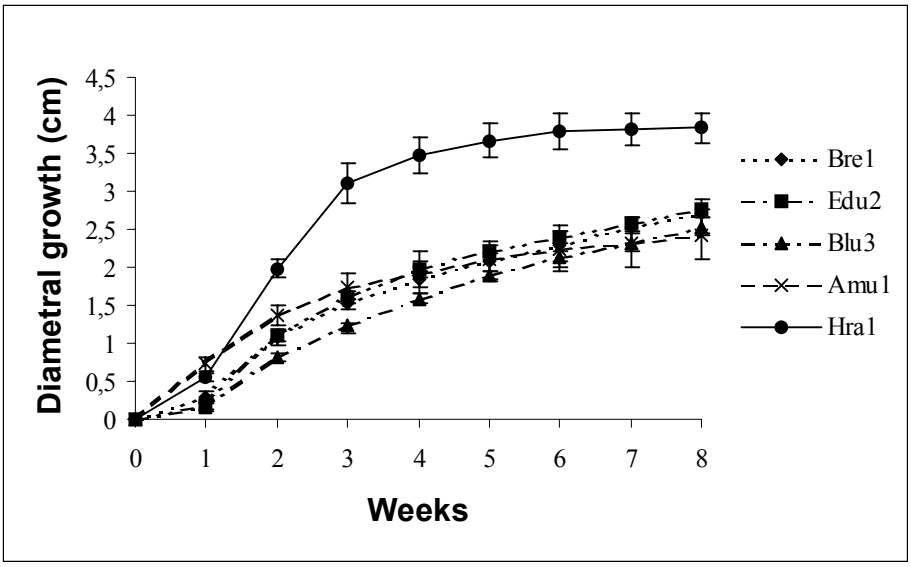

Fig. 1. Growth trend of the isolated strains on mWPM: Bre1 (*B. aestivalis*), Blu3 (*B. luridus*), Edu2 (*B. edulis*), Amu1 (*A. muscaria*) and Hra1 (*H. radicosum*).

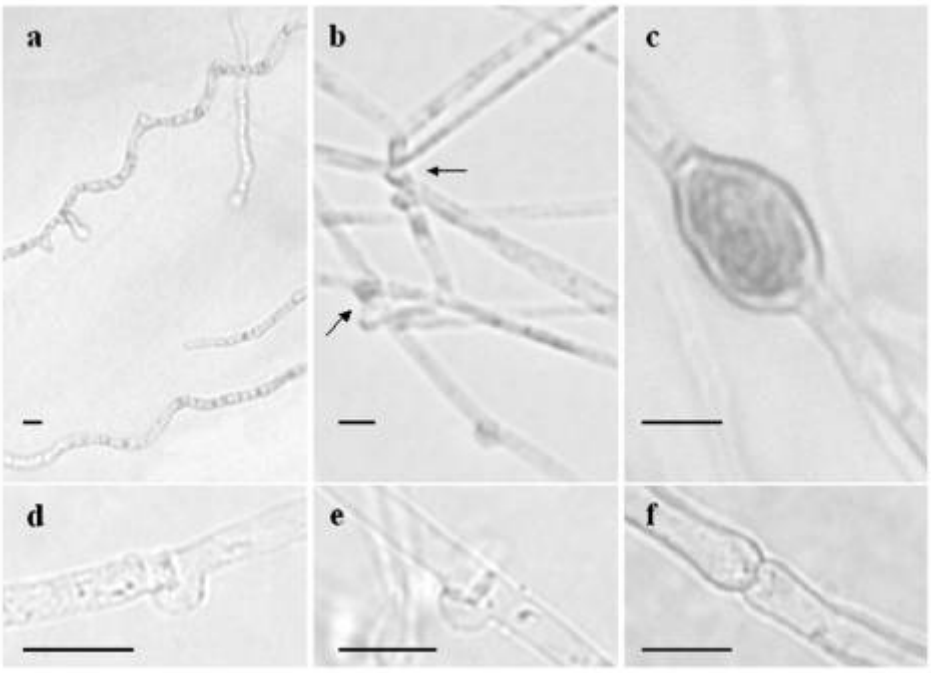

Fig. 2. Morphological characteristics of mycelia. **a.** Hyphal growth of *B. luridus* mycelium into the agar. **b.** Interlacing hyphae of *A. muscaria*. **c.** Hyphal swelling of *B. aestivalis*. **d.** Clamp of *A. muscaria*. **e.** Clamp of *H. radicosum*. **f.** Hyphal swelling of *B. edulis* closely a septum. Bars = 5 µm.

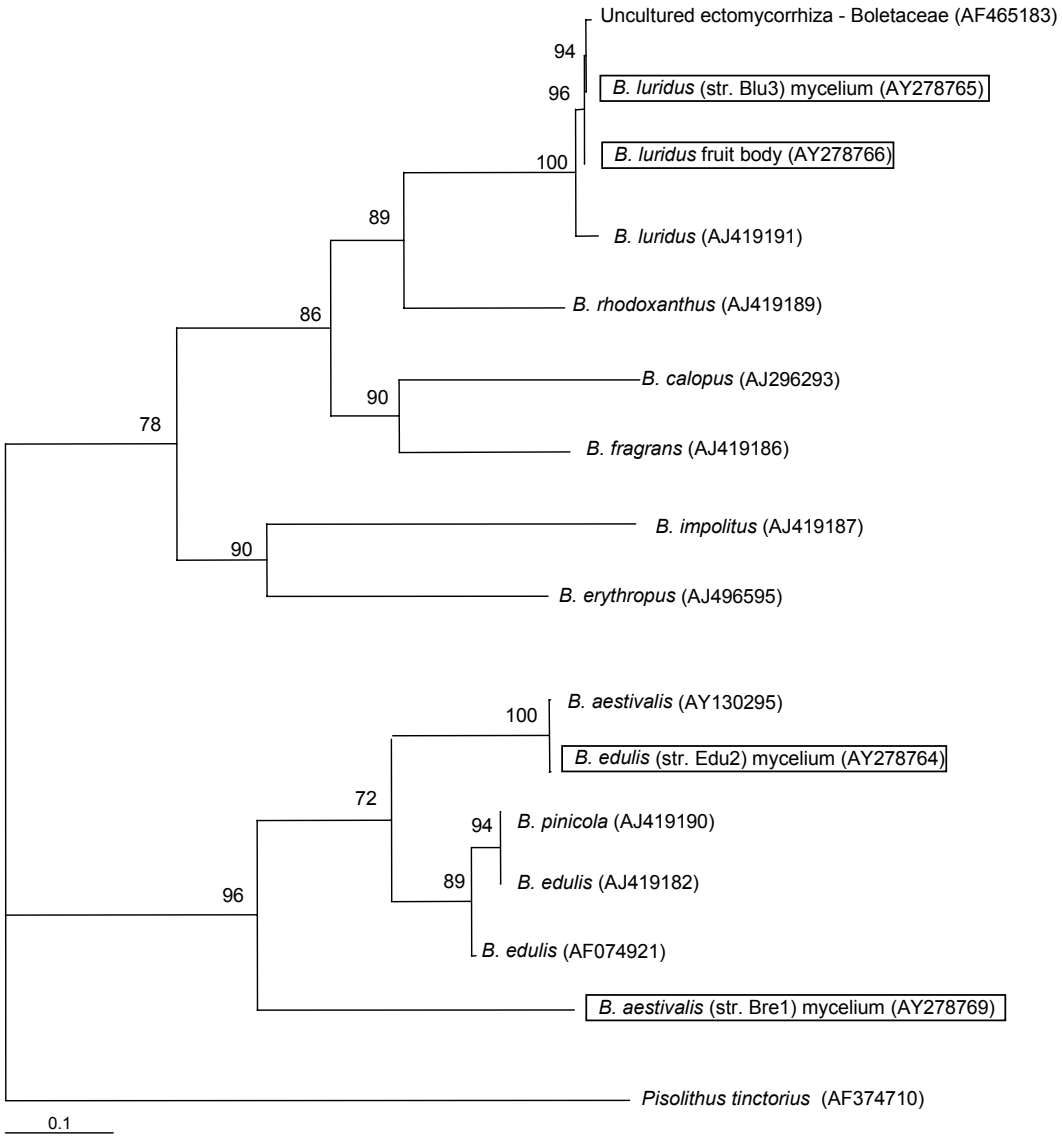

Fig. 3. Phylogenetic position of *B. aestivalis* mycelium (Bre1 strain), *B. edulis* mycelium (Edu2 strain), *B. luridus* mycelium (Blu3 strain). Tree inferred by maximum likelihood analysis based on rDNA sequences, including the ITS-1, 5.8S and ITS-2 regions (Ln likelihood = -7166.58725). *Pisolithus tinctorius* was used as outgroup. The numbers below the branches indicate the percentage at which a given branch was supported in 200 bootstrap replications.

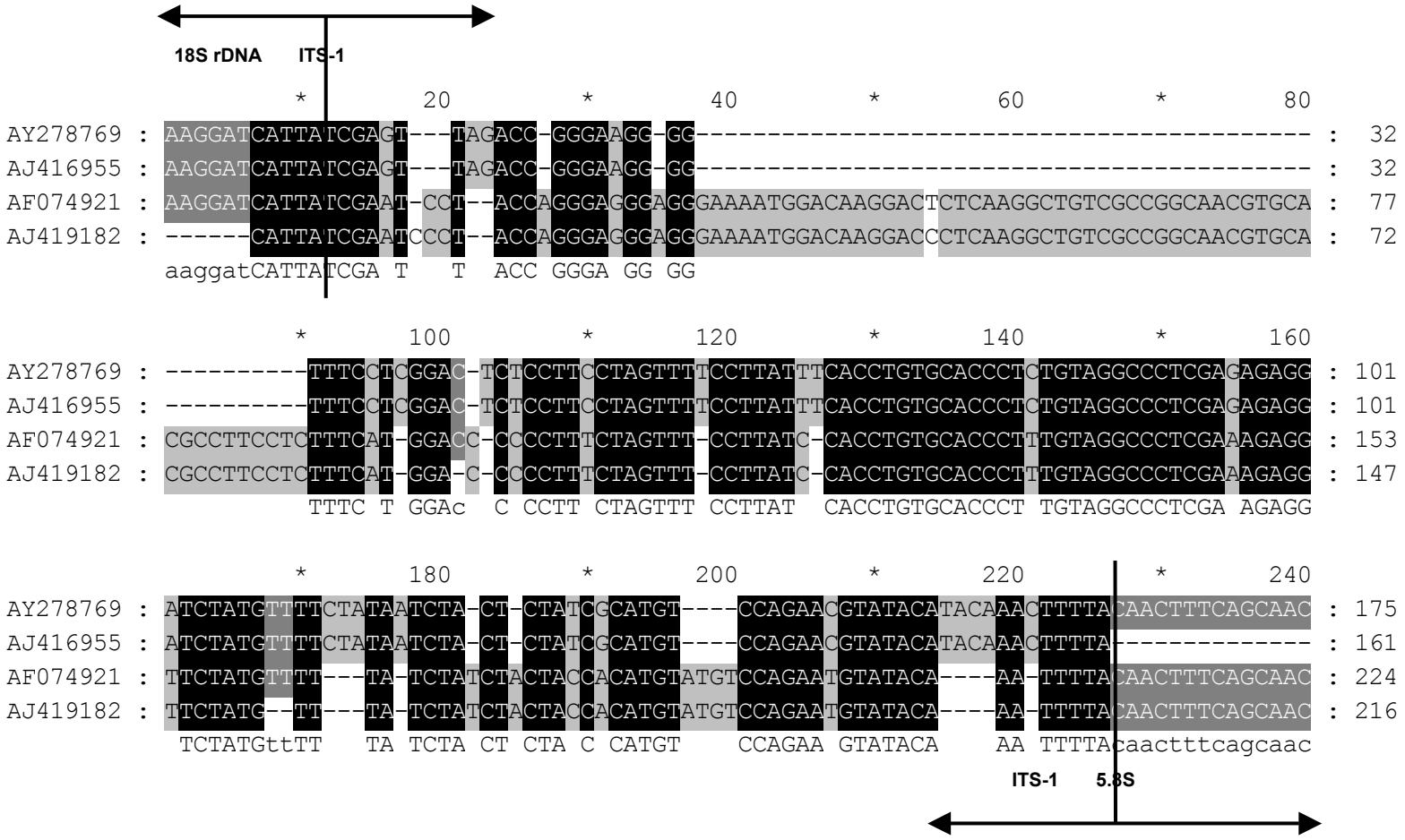

Fig. 4. *Boletus edulis* ITS-1 region sequence alignment. *Boletus aestivalis* mycelium (Bre1 strain) - AY278769; "Chinese" *B. edulis* - AJ416955; *B. edulis* - AF074921; *B. edulis* - AJ419182.

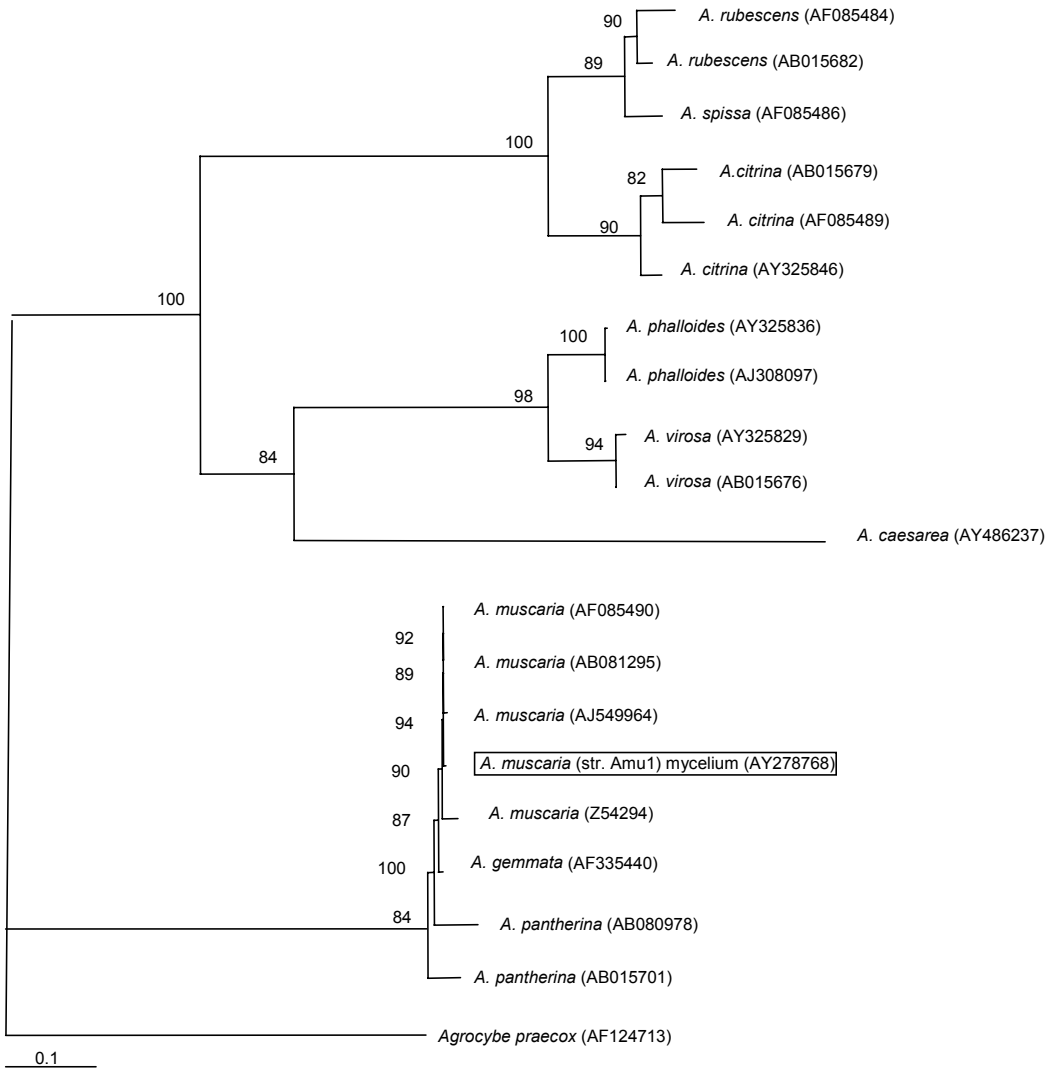

Fig. 5. Phylogenetic position of *A. muscaria* mycelium (Amu1 strain). Tree inferred by maximum likelihood analysis based on rDNA sequences, including the ITS-1, 5.8S and ITS-2 regions (Ln likelihood = -5929.64239). *Agrocybe praecox* was used as outgroup. The numbers below the branches indicate the percentage at which a given branch was supported in 200 bootstrap replications.

There was also little sequence variation among *Hebeloma* spp. In this study the ITSs from *H. radicosum* mycelium differed only by 1 bp in the ITS-1 and by 1 bp in the ITS-2 with the ITS sequences belonging to *H. radicosum* (AF124700) submitted to the Genbank by Aanen *et al*. (2000). The phylogenetic position of the sample of *Hebeloma* collected in Italy is presented in Fig. 6.

The topologies of the phylogenetic trees produced by distance matrix and parsimony criterions based on this data set agreed with that of the maximum likelihood.

The robustness of the different branches of interest was confirmed by significant bootstrap values (> 80%) except for the *B*. *edulis* and *B*. *aestivalis* cluster (< 75%).

Discussion

Ectomycorrhizal fungi have been widely studied in different parts of the world and have considerable economic and ecological importance. For this reason, these species are often used both for experimental and applied purposes (Hall and Wang, 1998; Ohta, 1998; Yamanaka *et al*., 2000). This paper uses both morphological and molecular methods to characterise the mycelia of these ectomycorrhizal fungi.

Although morphological characters have been shown to be able to distinguish the culture of different genera of the isolated fungi, it was difficult to differentiate between the three *Boletus* species examined in this study on the basis of the colony morphology and hyphal characters.

As described by Torres and Honrubia (1991) on PDA and on other media, *Amanita muscaria* culture has a regular edge, white mycelium and without exuded pigments. We also found that *A*. *muscaria* hyphae did not grow into the solid media and its mycelium develop mainly in contact with air on the upper layer of floating inocula on liquid mWPM (data not shown) probably because this species needs an oxygen rich environment.

Hebeloma radicosum and *Amanita muscaria* colonies showed evident clamped septa, a feature rare in *Boletus* spp. (Del Vesco, 1963; Scurti *et al*., 1978-79) and never observed in our *Boletus* strains. However, these characters seem to vary according to the fungus life stage and environmental conditions, since some Authors (Tozzi *et al*., 1980-81; Brunner *et al*., 1992) observed a high clamp frequency in emanating hyphae of mycorrhizae formed by *Boletus* species.

Our *Boletus* mycelia only showed differences in the morphology of the marginal zone and in the colour of the colonies. All the examined species of *Boletus* exuded brown pigments in hsPDA confirming that this is a distinctive character of *Boletus* mycelia (Scurti *et al*., 1978-79).

The analysis of ITS sequences made it possible to confirm the identity of our mycelium cultures (Figs. 3-6). To date, however, there is a limited number of ITS rDNA sequences from *Boletaceae* in public databases, and few of them cover the entire ITS length. In this study, it was therefore not possible to resolve

the exact taxonomic affiliation of our isolate of the *B*. *edulis* species complex. The scarcity of ITS sequences in the database also prevented a taxonomical attribution of a member of the *B*. *edulis* species group growing in New Zealand (Stringer *et al*., 2001). Clearly, molecular data from additional samples from different geographical origin is still needed to resolve the taxonomy of *B*. *edulis* and allied species.

Among the available ITS sequences of the edible bolete, *B*. *edulis* (accession number AJ416955) described by Moor *et al*. (2002) contains the ITS-1 region of a sample from China that shows the highest score of similarity with the ITS sequences we obtained from our mycelium of *B*. *aestivalis* (Fig. 4). Moor describes the molecular difference among the ITS-1 region of some European *Boletus edulis* with a Chinese *Boletus edulis* (61 bp of insertion) to highlight the presence of "Chinese king bolete" on the market and the possible target for fraudulent labelling. Unfortunately in his paper there is some misidentification with the GenBank numbering: the ITS sequence AJ416954 presented as *B*. *edulis* (Europe) is identical (274/274 nt = 100% similarity) to the summer bolete (*B*. *aestivalis* AJ416956) listed in the same paper. Thus the original sequence of European *B*. *edulis* described by Moor is missing and the similarity of the Italian *B*. *aestivalis* sequence to the Chinese *B*. *edulis* leads to further discussion and deeper investigation into the origin and polymorphism among the Italian and Chinese king boletes.

ITS typing is a useful tool for identifying both isolated mycelia and ectomycorrhizae collected in the field. In fact, the comparison of the ITS sequences obtained in our research with the sequences available in the database made it possible to affiliate an uncultured ectomycorrhiza of *Boletaceae* to the *B*. *luridus* species. As suggested by Horton and Bruns (2001), if all researchers deposited ITS sequences from at least the major species found in their studies, this would greatly increase the chance of eventually identifying these species and would also increase the comparability of species lists across studies.

In this paper we have highlighted the need for the reliability of DNA sequences deposited in public databases, as recently also suggested by several Authors (Crous, 2002; Deckert *et al*., 2002).

Our results also indicate that although it is relatively easy to isolate and culture these ectomycorrhyzal fungi, it is necessary to use molecular techniques to avoid misidentification of fungal isolates. It has been shown that misidentification could occur for the ectomycorrhizal fungi growing slowly in pure culture such as truffles, *Amanita* and *Boletus* species (Mello *et al*., 2001; Bridge *et al*., 2003).

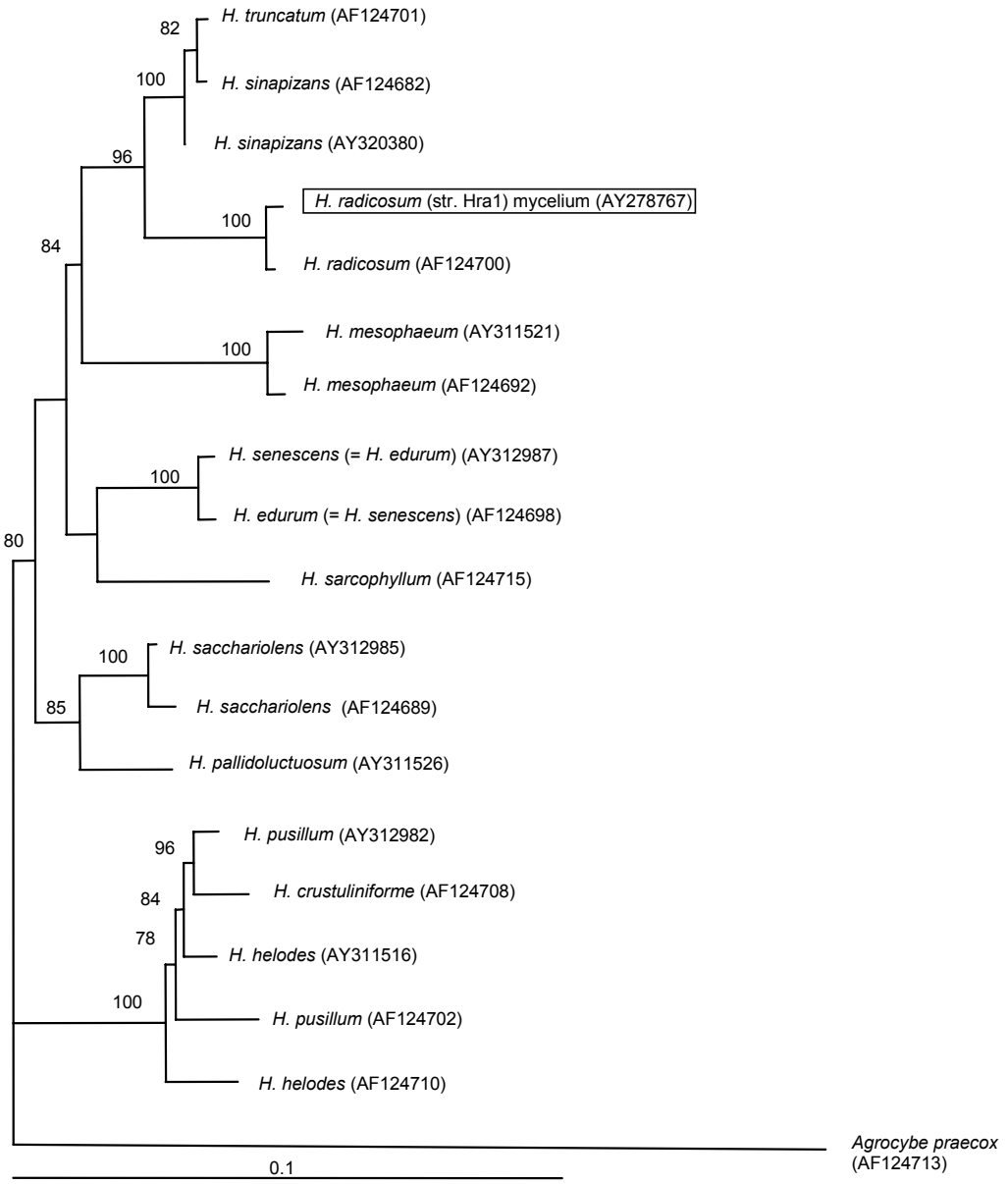

Fig. 6. Phylogenetic position of *H. radicosum* mycelium (Hra1 strain). Tree inferred by maximum likelihood analysis based on rDNA sequences, including the ITS-1, 5.8S and ITS-2 regions (Ln likelihood = -2445.44712). *Agrocybe praecox* was used as outgroup. The numbers below the branches indicate the percentage at which a given branch was supported in 200 bootstrap replications.

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