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Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation

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Summary

***Tuber magnatum*, an ascomycetous fungus and obligate ectomycorrhizal symbiont, forms hypogeous fruit bodies, commonly called Italian white truffles. The diversity of bacterial communities associated with *T. magnatum* truffles was investigated using culture-independent and -dependent 16S rRNA gene-based approaches. Eighteen truffles were classified in three groups, representing different degrees of ascocarp maturation, based on the percentage of asci containing mature spores. The culturable bacterial fraction was $(4.17 \pm 1.61) \times 10^7$, $(2.60 \pm 1.22) \times 10^7$ and $(1.86 \pm 1.32) \times 10^6$ cfu g^{-1} for immature, intermediate and mature ascocarps respectively. The total of bacteria count was two orders of magnitude higher than the cfu g^{-1} count. Sequencing results from the clone library showed a significant presence of α-*Proteobacteria* (634 of the 771 total clones screened, *c.* 82%) affiliated with *Sinorhizobium*, *Rhizobium* and *Bradyrhizobium* spp. The bacterial culturable fraction was generally represented by γ-*Proteobacteria* (210 of the 384 total strains isolated, *c.* 55%), which were mostly fluorescent pseudomonads. Fluorescent *in situ* hybridization confirmed that α-*Proteobacteria* (85.8%) were the predominant components of truffle bacterial communities with β-*Proteobacteria* (1.5%), γ-*Proteobacteria* (1.9%), *Bacteroidetes* (2.1%), *Firmicutes* (2.4%) and *Actinobacteria* (3%) only poorly represented. Molecular approaches made it possible to identify α-*Proteobacteria* as major constituents of a bacterial component associated with *T. magnatum* ascoma, independently from the degree of maturation.**

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Introduction

Truffles are ascomycetous hypogeous fungi belonging to the *Pezizales* order, which establish ectomycorrhizal (ECM) relationships with the roots of gymnosperms and angiosperms (Pegler *et al*., 1993). ECM fungi are of great importance not only because of the beneficial effect they have on the growth of their host trees and in soil nutrient cycling, but also because they form more than 900 edible mushrooms species (I.R. Hall, pers. comm.; Hall *et al*., 1998; 2005). *Tuber magnatum* Pico, well-known as the Italian white truffle, is highly regarded by chefs and gourmets because of the flavours it imparts to foods. This species is primarily found in Italy, Croatia, and less frequently in Switzerland, Slovenia (Ceruti *et al*., 2003), Serbia (Glamočlija *et al*., 1997) and Hungary (Bratek *et al*., 2004). Hence, *T. magnatum* truffles are in great demand and command high prices (Hall *et al*., 2003; Mello *et al*., 2006). The flourishing *T. magnatum* market has encouraged large-scale programmes for cultivating this truffle through the planting of nursery-produced mycorrhizal trees in suitable sites. However, despite the success achieved with other species of edible truffles, such as *Tuber melanosporum*, *Tuber aestivum* and *Tuber borchii*, the cultivation of *T. magnatum* has generally been unsuccessful (Gregori, 2002; Murat *et al*., 2005). Although there have been several advances in truffle biology, many aspects remain unclear (Paolocci *et al*., 2006) and the factors which may control the *T. magnatum* life cycle, such as spore germination, mycorrhiza and fruit body (also called ascoma/ta, sporocarps/s or ascocarp/s) formation, are still unknown (Mello *et al*., 2006).

It has been shown that bacteria can promote the establishment of ectomycorrhizal symbiosis (Frey-Klett *et al*., 1997; 2005; Aspray *et al*., 2006), and it is known that some bacteria influence fruit body formation of saprobic or pathogenic mushrooms such as *Fomitopsis pinicola*, *Fomes fomentarius*, *Echinodontium tinctorium* and

Table 1. List of *T. magnatum* Pico ascocarps used in this study and the corresponding numbers of isolates and clones in the culture collection and the clone library respectively.

Degree of ascoma maturation	Samples	Herbarium numbering	No. isolates	OTUs	No. clones	OTUs	Coverage[a]
I	N1	2223	33	5	35	8	77
I	N6	2188	24	4	36	8	81
I	N10	2515	20	5	48	7	85
I	N11	2519	18	4	42	7	83
I	N13	2521	19	3	47	7	81
I	N14	2522	17	3	44	9	84
I	N18	2532	15	3	47	7	77
II	N2	2224	32	6	31	10	68
II	N4	2227	19	4	28	0	79
II	N7	2210	31	6	40	6	78
II	N8	2512	25	4	50	9	88
II	N15	2523	22	6	49	6	88
II	N17	2531	18	2	50	6	88
II	N19	2533	13	3	53	6	89
III	N3	2226	27	4	36	5	86
III	N5	2228	22	3	39	6	85
III	N9	2513	16	3	45	6	87
III	N16	2530	13	4	51	6	88
Total			384		771		

a. The coverage for the 16S rRNA gene libraries generated from each *T. magnatum* ascocarp was determined according to the formula $C = [1 - (n_1 \times N^{-1})] \times 100\%$, with n_1 being the number of OTUs containing only one sequence and N being the total number of 16S rRNA gene clones analysed as described in Loy and colleagues (2005).

Agaricus bisporus (Larsen *et al*., 1978; Spano *et al*., 1982; Rainey, 1991).

In the case of *T. borchii*, several studies have examined the culturable fractions of the ascoma-associated bacterial communities and have suggested various hypothetical roles for these communities in the fungal growth or nutrition during ascocarp development and maturation (Bedini *et al*., 1999; Gazzanelli *et al*., 1999; Citterio *et al*., 2001; Sbrana *et al*., 2000; 2002). Recently, a molecular approach based on comparative analysis of 16S rRNA gene (rDNA) sequences has shown new rRNA gene sequences in the ascocarp of *T. borchii* (Barbieri *et al*., 2005a). These sequences had never been found in truffles before, raising the question of the role of these non-culturable bacterial communities in *T. borchii* fructification.

The aim of this study was to characterize the bacterial community associated with *T. magnatum* ascocarps and to identify potential symbiotic bacterial partners that may be important during the establishment of ectomycorrhizas and the production of fruit bodies. More specifically, the study aimed to expand and/or confirm the published taxonomic information on *Tuber*-associated bacteria, to provide an estimate of the relative quantity of different bacterial species occurring in *T. magnatum* truffles and to investigate potential dynamic changes in bacterial populations during ascoma maturation.

Two approaches were used to achieve these objectives: (i) culture-independent full-cycle rRNA gene methods, involving the direct 16S rDNA sequence retrieval and clone libraries from DNA extracted from truffles, to clearly identify the *Tuber*-associated bacteria in *T. magnatum*, fluorescence *in situ* hybridization (FISH) with domain/group-specific oligonucleotide probes and confocal laser scanning microscopy, to confirm the results of the clone libraries and relatively quantify the active bacterial communities (Amann *et al*., 1995; Loy *et al*., 2005); (ii) classical culture-dependent method to isolate bacterial strains that may be useful for further study on bacteria–truffle interactions.

Results

T. magnatum *ascoma*

Eighteen freshly collected *T. magnatum* truffles were placed in three groups of maturation based on the percentage of asci-containing mature spores (Zeppa *et al*., 2002). Seven were considered immature because only 0–5% of the asci contained mature spores (maturation stage I), seven fell into an intermediate group where 25–75% of the asci contained mature spores (maturation stage II), and four were fully mature because more than 75% of the asci contained mature spores (maturation stage III) (Table 1 and Fig. S1).

Size of the culturable bacterial communities

Bacteria in the truffles were grown on Tryptone Soy Agar (TSA). The culturable bacterial fraction corresponded to

$(4.17 \pm 1.61) \times 10^7$ (mean ± SE) cfu g^{-1} dry weight for the immature ascocarps (I), $(2.60 \pm 1.22) \times 10^7$ cfu g^{-1} for the maturing truffles (II) and $(1.86 \pm 1.32) \times 10^6$ cfu g^{-1} for the mature truffles (III) (Fig. 1A). There were no significant differences between the bacterial densities in the three different groups of ascocarps according to a one-factor ANOVA (maturation stage) ($P = 0.098$) performed on the cfu g^{-1} dried weight data transformed with the logarithm function (Loper *et al*., 1984). A total of 384 bacterial strains were purified to construct a bacterial collection (Table 1).

The composition of the microbial community

In order to assess the richness and relative abundance of bacteria associated with the *T. magnatum* ascocarp, 18 16S rRNA gene libraries were established for each *T. magnatum* ascocarp analysed. A range of 35–50 clones from each library were randomly selected and in all, 771 individual clones from all the libraries, with the insert near full-length 16S rDNA gene size, were obtained (Table 1). In order to assess the bacterial diversity in the 16S rDNA clone libraries and bacterial culture collection, the phylotypes were determined by a preliminary analysis of sequence similarity. Similarities were calculated with partial 16S rRNA sequences (*c.* 500 nt) obtained from two to four bacterial clones/strains, which are representatives from each restriction pattern obtained from the Amplified rDNA restriction analysis (ARDRA) analysis (data not shown), without application of a conservation filter and after exclusion of columns with insertions or deletions. A similarity threshold up to 98% was applied to define an operational taxonomic unit (OTU) (Table S1). The homologous coverage indicated that the 35–50 clones analysed covered > 75% of the expected richness in the sequences in pooled libraries with the OTUs delineated at high taxonomic level (Singleton *et al*., 2001; Loy *et al*., 2002) (Table 1).

One hundred and eighty-two unique, nearly full-length 16S rDNA (*c.* 1450 nt) clone and isolate sequences were analysed and compared with sequences present in the DDBJ/EMBL/GenBank databases. Of the 182 sequences analysed, 171 (94%) had similarity values higher than 97% compared with the sequences available from the databases, while 11 (6%) showed similarity values between 94% and 97%. Most of the sequences from both clone libraries and bacterial isolates had many of the *T. borchii*-associated bacteria recently described by Barbieri and colleagues (2005a) as nearest neighbours. Sequence analysis identified six major groups of the eubacterial domain, which included members of *Proteobacteria* comprising the α, β and γ subdivisions; the *Bacteroidetes* group; and the *Actinobacteria* and *Firmicutes* phyla. In the clone libraries most of the clones belonged to the α-*Proteobacteria* (634 clones of the 771 total clones screened in the library, *c.* 82%). They were significantly more abundant than the clones belonging to the other bacterial groups, according to a one-factor (identity of bacteria) ANOVA and Bonferroni–Dunn test ($P = 0.001$) (Fig. 1B). In the bacterial collection, *c.* 55% of the bacterial isolates belonged to the γ-*Proteobacteria* group (210 of the 384 total strains isolated). They were significantly more numerous than the bacterial isolates from the other bacterial groups, according to a one-factor (identity of bacteria) ANOVA and Bonferroni–Dunn test ($P = 0.001$) (Fig. 1C).

Members of the Proteobacteria *division*

According to clone analyses, members belonging to α-*Proteobacteria* represented the prevalent bacterial group within the *Proteobacteria*. The majority of them clustered with the *Bradyrhizobium* group.

In particular, for each ascoma analysed, regardless of its maturation stage, a constant presence of bacteria which clustered with *Bradyrhizobium elkanii* (GenBank accession number AB231916) and *Bradyrhizobium* sp. N (GenBank accession number Z94822) was detected. The occurrence of these bacteria was consistent comparing the results obtained from different replicates. *Bradyrhizobium japonicum* clustered as a separate branch (Fig. 2). Some clones and one representative of the bacterial isolates formed a single cluster closely related to *Rhizobium giardinii*. Few clones affiliated with *Agrobacterium tumefaciens* and other clones grouped within the *Mesorhizobium* and *Sinorhizobium/Ensifer* groups. In contrast with the high number of α-*Proteobacteria* clones, few strains belonging to the α-*Proteobacteria* (17 of the 384 isolates in the culture collection, 4.42%) were isolated from TSA medium. These isolates belonged to the *Sinorhizobium*/*Ensifer* group.

Members of the β-*Proteobacteria* were represented by 27 isolates of the 384 isolates analysed (7%) and 26 clones of the 771 clones (3%). The 16S rDNA sequences showed a high similarity (> 98%) with *Variovorax* sp. and different members of the *Burkholderia* group. No significant similarity has been revealed with the sequences of *Burkholderia* endosymbiont of *Gigaspora margarita* (Bianciotto *et al*., 2003) not included in the phylogenetic tree of β-*Proteobacteria* shown in Fig. 2.

Many of the *T. magnatum*-associated bacteria were γ-*Proteobacteria*. They were mostly represented by isolates (210 of the 384 isolates analysed, i.e. 55%) rather than clones (39 of the 771 clones, i.e. 5%). The majority had a significant similarity with fluorescent pseudomonads and more particularly with the species *Pseudomonas costantinii*, *Pseudomonas syringae, Pseudomonas linii* and *Pseudomonas jessenii* (Fig. 3). Few clustered with the *Xanthomonadaceae*, *Moraxellaceae* and *Enterobacteriaceae*. Some of the isolates, as well as clones, grouped

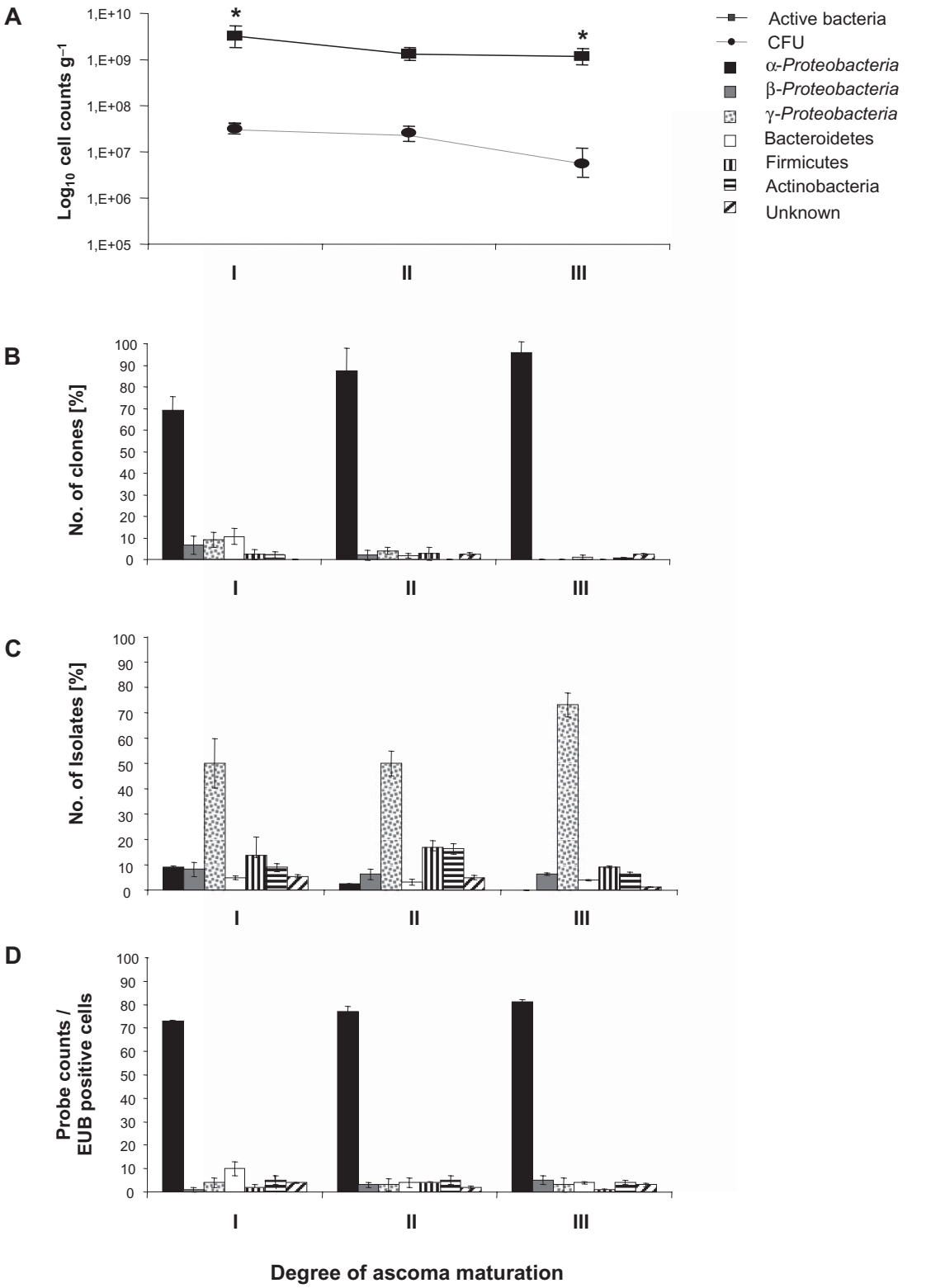

Fig. 1. The microbial community composition of *T. magnatum* ascocarps during maturation (immature ascocarp I, intermediate ascocarp II, mature ascocarp III). Bars represent the standard deviations. $*P = 0.0073$.
A. The bacterial cell count and the culturable bacteria (cfu) fraction expressed as $\log_{10}$ *per* gram of dry weight, calculated for each degree of ascoma maturation as average ± standard deviations.
B and C. Relative abundance of environmental clones (B) and bacterial isolates (C).
D. The fraction of bacterial cells detected by group-specific oligonucleotide probes.

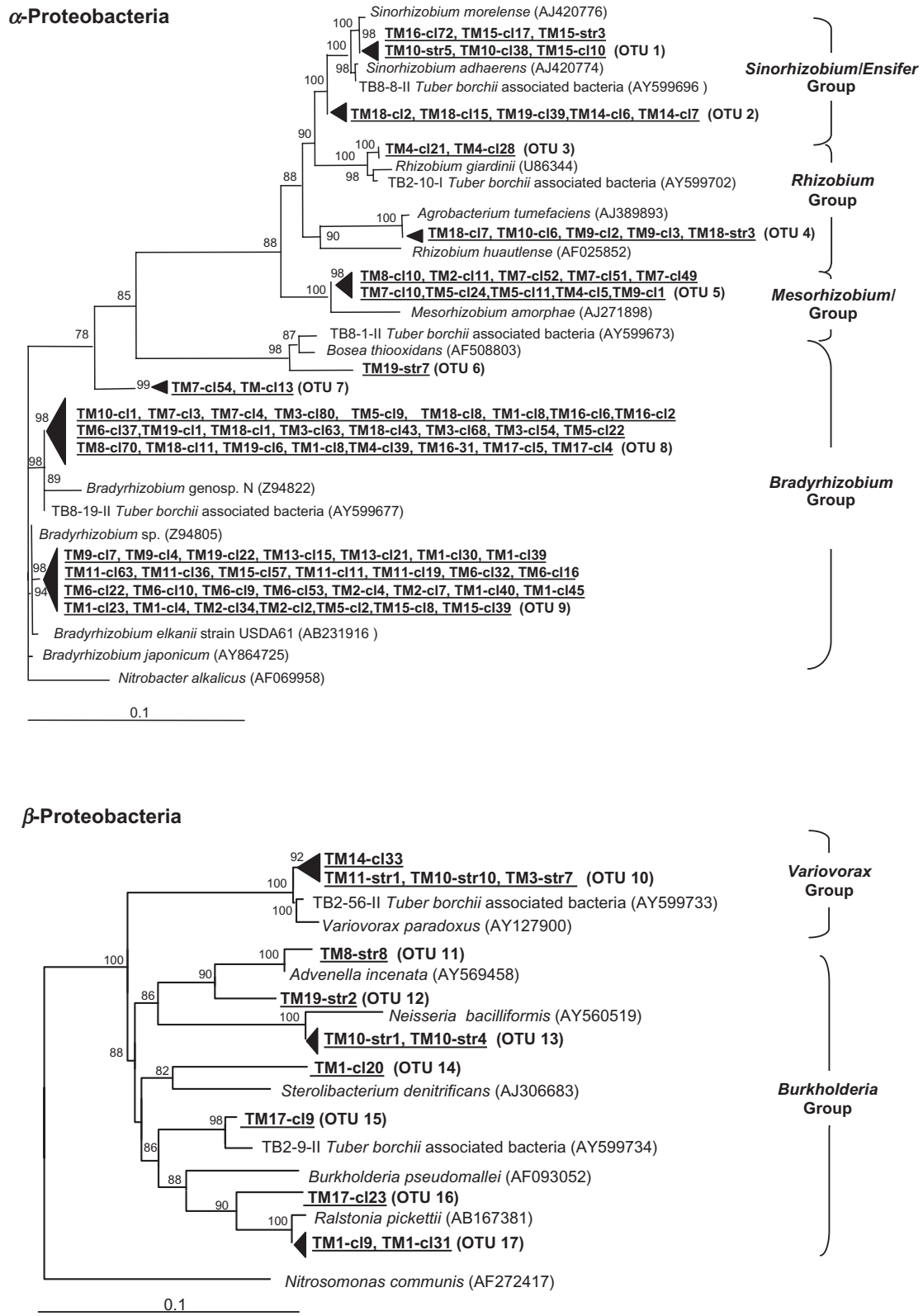

Fig. 2. Neighbour-joining phylogenetic tree for representative 16S rRNA gene sequences of α- and β-*Proteobacteria* from *T. magnatum* ascocarp based on nearly complete 16S rRNA gene sequences (Highlighted in bold are the sequences analysed in this study). The number at each branch node are the bootstrap numbers from 1000 re-samplings. The sequence of *Nitrobacter alkalicus* and *Nitrosomonas communis* were included as the out group. Str, strains; Cl, clones; OTU, operational taxonomic units that are described in Table S1.

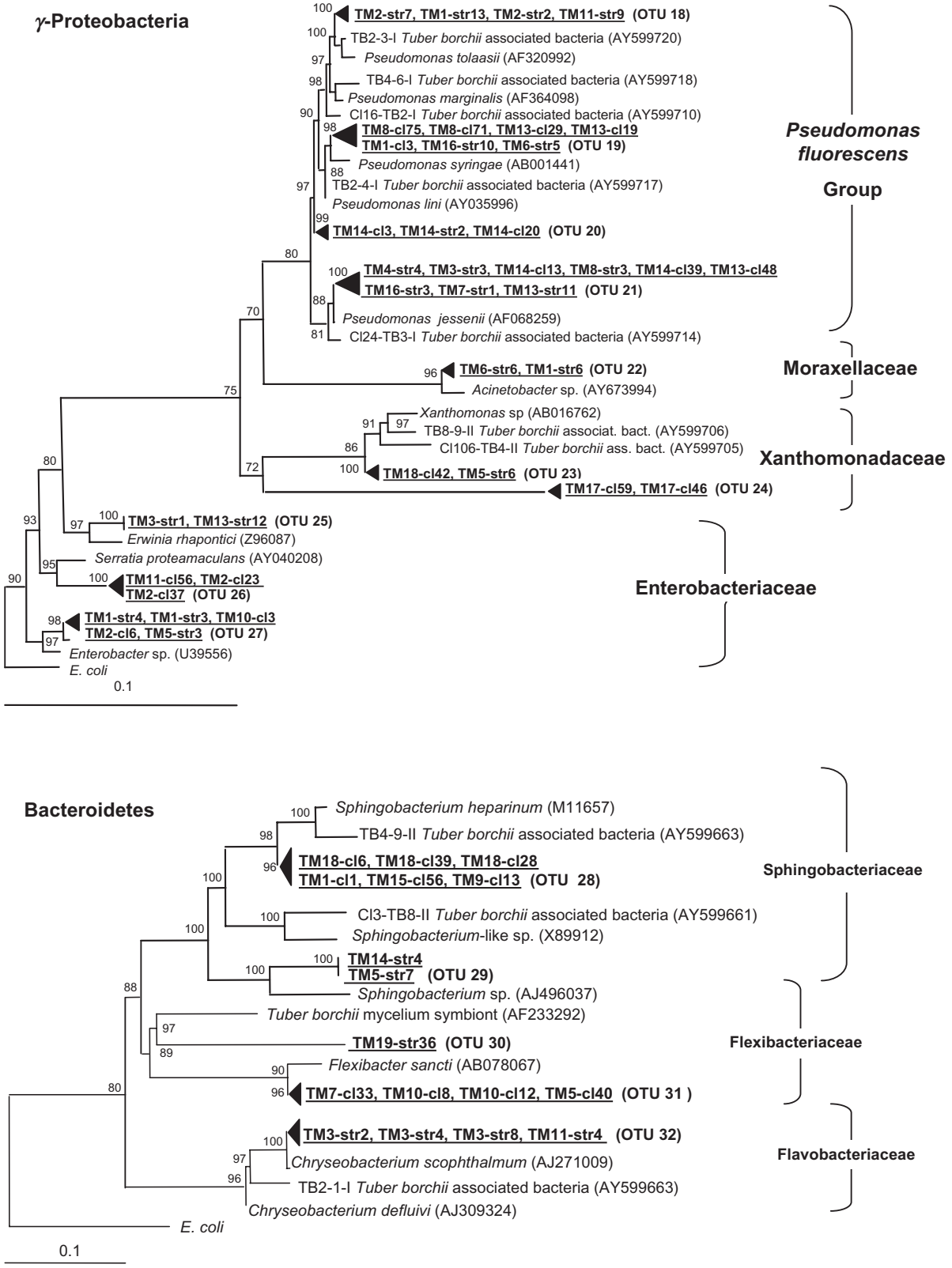

Fig. 3. Neighbour-joining phylogenetic tree for representative 16S rRNA gene sequences of γ-*Proteobacteria* and *Bacteroidetes* from *T. magnatum* ascocarp based on nearly complete 16S rRNA gene sequences (Highlighted in bold are the sequences analysed in this study). The number at each branch node are the bootstrap numbers from 1000 re-samplings. The sequence of *Escherichia coli* was included as the out group. Str, strains; Cl, clones. OTU, operational taxonomic units that are described in Table S1.

with yet to be described *Pseudomonas* species and uncultured bacteria. Although phylogenetic relationships of the genus *Pseudomonas* were analysed reliably through 16S rDNA, sequence delineation within the *Pseudomonas* species *sensu strictu* cannot be assessed exclusively on the basis of 16S rDNA sequence analysis (Kuske *et al*., 1999).

Members of the Bacteroidetes *division*

A significant group of OTUs occurring within the *T. magnatum* ascocarp was also represented by members of the *Bacteroidetes* phylum. Sequences from both isolates (15 isolates, 4%) and clones (39 clones, 5%) belonged to the *Sphingobacteriaceae*, *Flexibacteriaceae* and *Flavobacteriaceae*. The tree topology that resulted from the phylogenetic analysis of the *Bacteroidetes* members associated with *T. magnatum* was comparable to the one obtained from *T. borchii*-associated bacteria (Barbieri *et al*., 2005a). However, no significant similarities were observed either among clones or isolates with the uncultured *Flexibacter* as it was found to be associated with hyphae of *T. borchii* (GenBank accession number AF233292, Barbieri *et al*., 2000; 2002) (Fig. 3).

Members of the Firmicutes *and* Actinobacteria

Most of the members of the *Firmicutes* were represented by isolates (54 isolates, 14%) rather than clones (15 clones, 2%), and belonged to the *Staphylococcaceae*, *Listeriaceae* and *Bacillaceae* (Fig. 4). *Staphylococcus pasteuri* strains, isolated from the *T. magnatum* ascoma, were also found on the roots of micropropagated plantlets and were characterised for their antimycotic activity against the growth of *T. borchii* mycelium (Barbieri *et al*., 2005b). Only one clone TM2-12 of the *Clostridiaceae* was detected on a single branch related to *Clostridium botulinum* with a similarity level of 86% on 1450 nt.

Four principal clusters were generated by the *Actinobacteria* phylum isolates (17, 4%) and clones (7, 1%) and were representatives of the *Microbacteriaceae*, *Micrococcaceae*, *Nocardiaceae* and *Propoionibacteriaceae* (Fig. 4).

Comparison between isolates and clones

The 16S rDNA sequencing comparison between isolates and environmental clones was only possible in few samples. It was feasible to compare the sequences among the *Sinorhizobium* species (in the ascocarp TM15, strain N. 3 was represented by clones N. 17 and N. 10; and in the ascocarp TM10, strain N. 5 was represented by clone N. 38), the *Rhizobium* species (in the ascoma TM18, strain N. 3 was represented by clone N. 7), and the *Pseudomonas fluorescens* species (in the ascocarp TM14, strain N. 2 was represented by clones N. 3, N. 20 and N. 13). No sequence comparison between isolates and clones was possible from bacteria identified within the *Bacteroidetes* phylum or from Gram-positive bacteria (Table S1).

Colonization pattern at the fungal tissue level

The FISH protocol was applied to all eighteen *T. magnatum* ascocarps in different maturation stages. Both fixation methods with ethanol or paraformaldehyde (Amann *et al*., 1995) were tested. Although no differences in terms of hybridization for Gram-positive bacteria were observed, the ethanol fixation gave more autofluorescence from the fungal matrix, thus it was preferable to the paraformaldehyde fixation. rDNA probes were chosen to cover all the bacterial groups previously characterized within the ascoma of *T. borchii* and *T. magnatum* using direct 16S rDNA sequence retrieval and cultivation approach. The total number of bacterial cells *per* gram of dry-weight ascocarp (Fig. 1A), and the relative percentages of the different bacterial groups *per* total number of bacteria were calculated for four immature ascocarps, five for intermediate and three for mature ascocarps (Fig. 1D). On average, the bacteria detected by *in situ* hybridization belonged to the following bacterial subdivisions: 85.84% of α-*Proteobacteria*, 1.52% of β-*Proteobacteria*, 1.90% of γ-*Proteobacteria*, 2.13% of *Bacteroidetes*, 2.45% of *Firmicutes* and 3.06% *Actinobacteria*. Only 3.33% of the diversity of the bacteria detected with the EUB probes could not be identified with the group-specific probes used. There was a small but significant effect of the maturation stage of the ascocarps on the number of the bacterial cells detected with the EUB probes *per* gram of dry-weight ascocarp, according to a one-factor ANOVA (degree of maturation) ($P = 0.0073$) performed on the logarithm of the data (Loper *et al*., 1984). Maturation stages I and III significantly differed from maturation stage II according to the Bonferroni–Dunn test. On the contrary, there was no significant effect of maturation stage on the number of bacterial cells *per* gram of dry-weight ascocarp detected by DAPI staining, according to a one-factor ANOVA (degree of maturation) ($P = 0.82$) performed on the logarithm of the data. In all the ascomata analysed, the amount of α-*Proteobacteria* was significantly higher than the amount of the other groups of bacteria when considering the proportions of the detected bacteria *per* EUB probe-positive cells ($P = 0.001$) and the number of bacteria *per* gram of dry-weight ascoma ($P = 0.001$), according to a two-factor ANOVA (bacterial group and maturation stage) and the Bonferroni–Dunn test. Likewise, the amounts of *Bacteroidetes* and Gram-positive (*Firmicutes* and *Actinobacteria*) bacterial cells were significantly higher than the amount of β- and

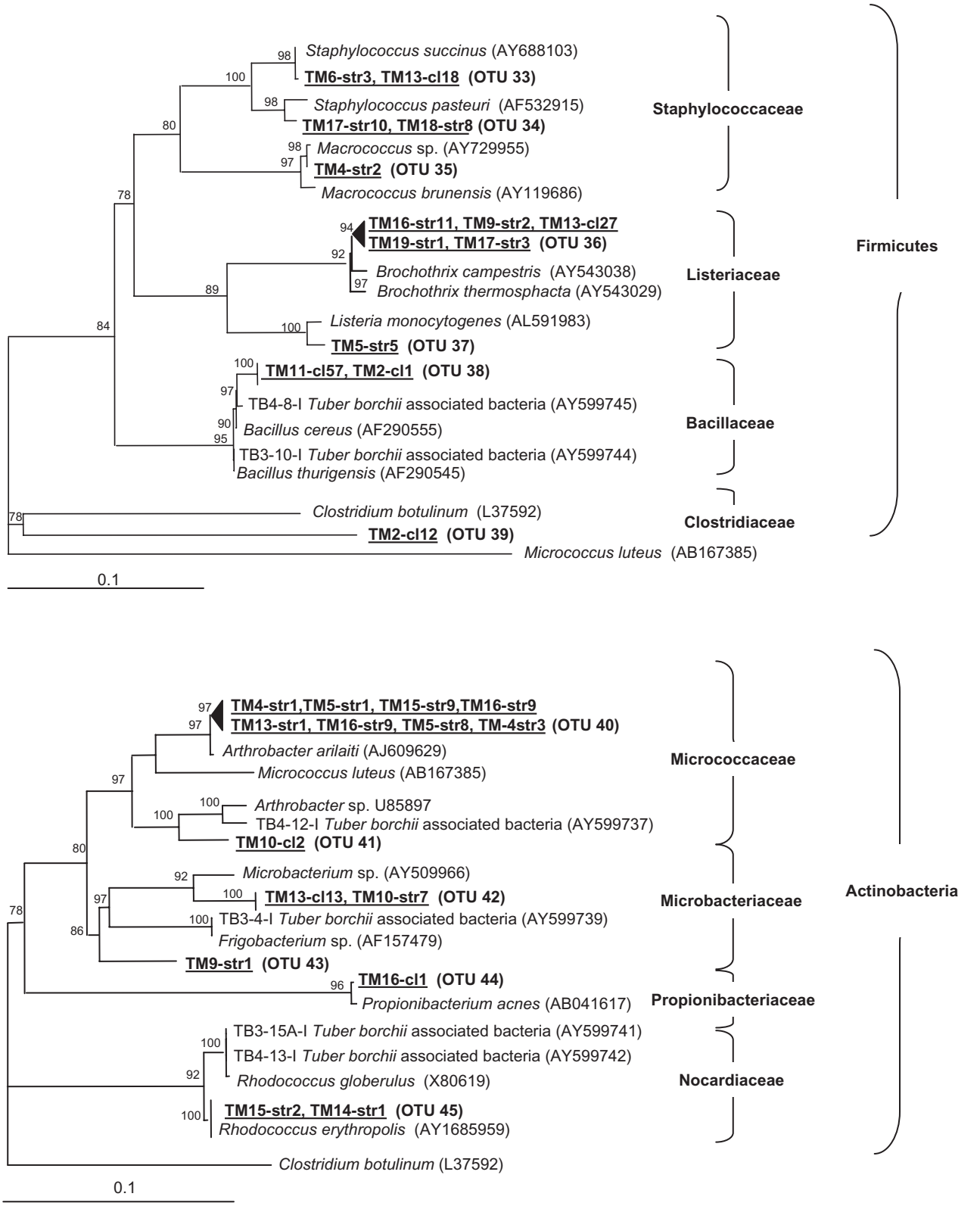

Fig. 4. Neighbour-joining phylogenetic tree for representative 16S rRNA gene sequences of *Firmicutes* and *Actinobacteria* from *T. magnatum* ascocarp based on nearly complete 16S rRNA gene sequences (Highlighted in bold are the sequences analysed in this study). The number at each branch node are the bootstrap numbers from 1000 re-samplings. The sequence of *Micrococcus luteus* and *Clostridium botulinum* were included as the out group. Str, strains; Cl, clones. OTU, operational taxonomic units that are described in Table S1.

γ-*Proteobacteria* when considering the proportions of the detected bacteria *per* EUB probe-positive cells ($P = 0.001$) and the numbers of bacteria *per* gram dry-weight ascoma ($P = 0.001$), according to a two-factor ANOVA (bacterial group and maturation stage) and the Bonferroni–Dunn test. There was no significant effect of the maturation stage on the amount of α-, β-, γ-*Proteobacteria*, *Bacteroidetes*, *Firmicutes* or *Actinobacteria* when the proportions of detected bacterial cells *per* EUB probe-positive cells ($P = 0.98$) or the numbers of bacteria *per* gram dry-weight ascocarp ($P = 0.85$) were calculated.

Preliminary qualitative analyses of the α-*Proteobacteria* with specific probes were carried out in nine *T. magnatum* samples representative of the three degrees of ascoma maturation (N10, N11, N18 among the immature ascocarps; N8, N17, N19 among the ascocarps at the intermediate level of maturation and N3, N9, N16 among mature ascocarps). The *Rhizobium–Agrobacterium*-specific probe RHI1247 provided constant hybridization signals in all the ascomata analysed. Positive hybridization signals were also obtained with the GRb probe for most of the samples analysed. This probe was designed for the *Rhodobacter–Roseobacter* group, but perfectly matched with *Mesorhizobium* spp. as well. Using the 16S rDNA cloning approach, it was obtained a significant number of *Mesorhizobium* spp. clones that could justify the GRb signals from the samples. Few cell signals were obtained for the SPH120 probe specific for the *Sphingomonas* group (Fig. S2).

Furthermore, rare intracellular bacteria were observed by FISH experiments (data not shown) suggesting the existence of potentially endosymbiotic bacteria within another ectomycorrhizal fungus, in addition to those studied in *T. borchii* (Barbieri *et al*., 2000); *Laccaria bicolor* (Bertaux *et al*., 2003; 2005) and *Gigaspora margarita* (Bianciotto *et al*., 2003).

Discussion

This work presents a molecular study of the microbial diversity within *T. magnatum* ascocarps and provides a relative estimate of the bacterial composition of truffles during their maturation. The truffle maturation process includes the formation of spores and production of volatile compounds that characterize the typical truffle aroma (Gioacchini *et al*., 2005). The truffles examined in this study cover different stages in truffle maturation when ascoporogenesis occurs leading to the formation of the spores (Parguey-Leduc *et al*., 1987). In this process spore formation begins with the compartmentalization of the ascus nuclei by the invagination of the ascus plasmalemma. It is completed by the formation of the wall, which progressively assume the characteristic morphological features with reticulate ornamentation and wide meshes typical of the mature ascoma of *T. magnatum* (Zambonelli and Iotti, 2005).

Using cultivation-independent 16S rRNA gene-based molecular techniques it was possible: (i) to identify most of the bacteria occurring during truffle maturation and (ii) to show their relative contribution to the overall cell number.

A cultivation-dependent approach was used, in parallel, to identify the culturable bacterial fraction of *T. magnatum*. The comparison of the quantitative and qualitative information on bacterial diversity in *T. magnatum,* collected with the above-mentioned methods, showed that only a few 16S rDNA sequences of the isolates were comparable to those of environmental clones. This confirmed the importance of non-cultivation approaches in studying bacterial diversity in truffle (Barbieri *et al*., 2005a), notably because cultivation-based diversity surveys are rather unlikely to reflect the true microbial community structure present *in situ* because of inherent qualitative and quantitative biases (Wagner *et al*., 1993; Hugenholtz *et al*., 1998; Ellis *et al*., 2003). Nevertheless, the 16S rDNA sequences obtained from the bacterial isolates extended the taxonomic databases of *Tuber*-associated bacteria useful for comparative systematic studies. Moreover, the bacterial isolates obtained can be considered as representative of the natural truffle bacterial community according to the results of our culture-independent approach. These bacterial strains may be useful for further studies on the mechanisms of the establishment of the natural multitrophic plant–bacteria–fungus complex and for possible biotechnological applications.

The 16S rDNA gene library analyses allowed the identification of different species (measured as OTU numbers) for each maturation level of the *T. magnatum* ascocarp. Of particular interest was the constant and significant presence of α-*Proteobacteria*, mainly represented by members of the *Sinorhizobium*/*Ensifer* and *Rhizobium*/*Agrobacterium* groups, and *Bradyrhizobium* spp. (Fig. 2), well known for their ability to fix nitrogen. The prevalence of α-*Proteobacteria* associated with *T. magnatum* ascocarps, regardless of their stage of maturation, was confirmed by the quantitative FISH approach (85% of α-*Proteobacteria* among all FISH-positive cells). This method demonstrated the occurrence of rhizobia-like bacterial cells, detected by hybridization of the specific *Rhizobium* probe RHI1247. The most representative clones among the α-*Proteobacteria* were closely related to *B. elkanii*. This OTU was consistently present within each ascoma analysed. *Bradyrhizobium elkanii* was previously described as an inoculant for common soybean, which is capable of fixing atmospheric nitrogen in symbiotic interactions with leguminous plants (Rumjanek *et al*., 1993). No information is currently available on the specific interaction of this species with ectomycorrhizal fungi.

The dominance of α-*Proteobacteria* in clone libraries has been reported in many studies (Felske *et al.*, 1999; Kaiser *et al*., 2001; Ellis *et al*., 2003). This group of bacteria may be underestimated in culture collections, because it is mainly composed of slow growing bacterial species (Saito *et al*., 1998). In addition, many of the members of the α-*Proteobacteria* group respond to changes in environmental conditions by entering a viable but non-culturable state which could account for the difficulties encountered in isolating rhizobia-like strains (Toffanin *et al*., 2000; Feng *et al*., 2002). Moreover, the α-*Proteobacteria* harbour several symbiotic species that have a very close relationship with their hosts and may require specific physicochemical conditions not found in simple growth media. This would explain why in our study, few isolates of α-*Proteobacteria*, exclusively from *Sinorhizobium,* were obtained using the cultivation approach.

Unlike the clone libraries, which were dominated by OTUs of α-*Proteobacteria*, the collection of isolates was dominated by members of the fluorescent pseudomonads group among the γ-*Proteobacteria*, which is in agreement with previous studies that have only taken into account the culturable fraction of the *Tuber*-associated bacteria (Bedini *et al*., 1999; Gazzanelli *et al*., 1999; Citterio *et al*., 2001; Sbrana *et al*., 2002). On the contrary, our FISH observations showed a maximum of only 3.5% of γ-*Proteobacteria* using oligonucleotide probe GAM42a. Therefore, although fluorescent pseudomonads were described as being by far the most important members of truffle ascocarps, our results confirmed the data obtained by the 16S rDNA sequencing survey in *T. borchii* ascocarps, in which few clones of γ-*Proteobacteria* were obtained compared with the high number of pseudomonads isolated (Barbieri *et al*., 2005a).

The 16S rDNA sequences retrieval and FISH analysis showed that the diversity of the bacterial communities did not vary with maturation. The presence of well-defined bacterial communities in completely immature ascomata constitutes new evidence which could help us to investigate their role in the first steps of fruiting body formation in the soil. The potential bacterial ability to modify soil nutrient availability during the initial fructification phase could become particularly important in *Tuber*, because, probably after the primordium formation, the fruit body becomes nutritionally independent from the host plant (Barry *et al*., 1994).

Although in this study a significant evidence of microbial community temporal development does not emerge, it is quite apparent that potential nitrogen-fixing bacteria constantly occur in truffles, regardless of the fruit body maturation stage. Thus, potential nitrogen-fixing bacteria may play an important role in the development and maturation of truffle ascomata.

Experimental procedures

Biological materials

Similar-sized (2 cm of diameter) and regular round-shaped *T. magnatum* ascomata were collected in a votive area in north-central Italy. The freshly collected intact ascocarps were transported to the laboratory on ice and processed within few hours. Dried samples of each specimen are preserved in the Herbarium of the Mycology Center of Bologna, University of Bologna (Italy). The ascomata were classified in three maturation stages on the basis of the percentage of asci containing mature spores (Zeppa *et al*., 2002): immature ascocarps (0–5% of asci containing mature spores, stage I); intermediate ascocarps (25–75% of asci containing mature spores, stage II); mature ascocarps (> 75% of asci containing mature spores, stage III). The ascomata were washed with sterile water, gently brushed and their surfaces were briefly flamed.

Some tissues were removed from the inner part of each ascocarp: about 1 g was homogenated in 1 ml of filter-sterilized physiologic solution (0.85% NaCl) to isolate culturable bacterial strains in TSA as previously described (Gazzanelli *et al*., 1999; Sbrana *et al*., 2002; Barbieri *et al*., 2005a) and to extract the DNA directly from the ascoma for the 16S rDNA sequencing retrieval, as described in Barbieri and colleagues (2005a). After processing the samples, the same quantity of homogenated ascocarp was dried at 60°C in order to determine its dry weight. A cubic piece of 2–3 mm thick was sampled for fixation before the FISH analyses. All the procedures regarding the DNA extraction, PCR amplification of bacterial 16S rDNA, clone library construction and screening, assignment of operational taxonomic units by ARDRA analysis, 16S rDNA sequences and the phylogenetic analysis applied to this study were carried out as described in Barbieri and colleagues (2000; 2005a).

FISH procedures

The whole FISH protocol was carried out according to Bertaux and colleagues (2005). Briefly, a paraformaldehyde fixation was performed, and the samples were dilacerated with pins prior to hybridization and observation. After observation, the dilacerated ascocarp pieces were collected again and dried at 60°C in order to determine their dry weight. The probe set applied to the samples was almost the same as the one described in Bertaux and colleagues (2005): it comprised probes specific to most of the bacteria (bacterial probe) and several bacterial divisions (α,β,γ-*Proteobacteria*, *Cytophaga–Flavobacteria*, *Firmicutes* and *Actinobacteria*). Moreover, to focus on α-*Proteobacteria*, probe RHI1247 specific to the *Rhizobium–Agrobacterium* group (Ludwig *et al*., 1998), GRb specific to the *Rhodobacter–Roseobacter* groups (Eilers *et al*., 2000) and the SPH120 specific to the *Sphingomonas* group (Brinkmeyer *et al*., 2003) were used. Where applicable, equimolar mixtures of probes (Daims *et al*., 1999; Meier *et al*., 1999) or competitor oligonucleotides (Manz *et al*., 1992) were used. For total bacterial counts, 4,6-diamidino-2-phenylindole DAPI was mixed with Citifluor AF1 antifading reagent (Citifluor, England) to obtain a final concentration of 2.5 μg ml^{-1} and this was used for mounting staining. The samples were

observed with a confocal laser scanning microscope (Bio-Rad Radiance 2100) built on a Nikon Eclipse TE2000-U microscope, at four excitation wavelengths (blue diod 405 nm, Argon laser 488 nm, Helium Neon laser 543 nm, red diod 633 nm), with an Apochromat 63x/1.2 water immersion lens. The images were recorded with the software Laser Sharp 2000 (Bio-Rad), and analysed with the software ImageJ version 1.3. Five independent countings (surface of one image: 0.033 mm^2) were performed at random on the whole surface of each 8-mm hybridization well. The mean value of these five countings was used to calculate the number of cells of each bacterial group *per* gram of dry-weight ascocarp or *per* total EUB probe-positive cells.

Statistical analysis

The mean value of the number of cfu *per* gram of dry-weight ascoma was calculated for all of the 18 sporocarps, log-transformed (Loper *et al*., 1984) and analysed by a one-factor ANOVA (maturation stage) ($P = 0.05$) and the Bonferroni–Dunn test. The mean value of the number of isolates and clones was calculated for the 18 sporocarps, then analysed with one-factor (identity of the bacteria) ANOVA ($P = 0.05$). The different identities of the clones (α,β,γ-*Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*) were compared using the Bonferroni–Dunn test.

The mean numbers of EUB-cells and DAPI-stained cells *per* gram of dry-weight ascoma were calculated for 11 sporocarps log-transformed and analysed by a one-factor ANOVA (maturation stage) ($P = 0.05$) and the Bonferroni–Dunn test. The mean numbers of cells of each specific bacterial group *per* EUB counts were analysed by a two-factor ANOVA (bacterial group and maturation stage) ($P = 0.05$) and the Bonferroni–Dunn test. The mean numbers of cells of each specific bacterial group *per* gram of dry-weight ascocarp were log-transformed before performing the same two-factor ANOVA.

DDBJ/EMBL/GenBank accession numbers

Sequences obtained in this study are available from DDBJ/EMBL/GenBank under accession numbers DQ303296–DQ303380 for the α-*Proteobacteria* and DQ279303–DQ279399 for the β-,γ-*Proteobacteria*, *Bacteroidetes*, *Firmicutes* and *Actinobacteria*.

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Supplementary materials

The following supplementary material is available for this article online:

Fig. S1. *Tuber magnatum* ascomata at different stages of maturation calculated on the basis of the presence of the asci containing mature spores: (a) immature ascocarp I; (b) intermediate ascocarp II; (c) mature ascocarp III. Scale bar: 100 μm.

Fig. S2. Fluorescence *in situ* hybridization of *T. magnatum* ascoma tissue (sample N8) with group-specific 16S rRNA oligonucleotide probes.

1. (A) no probe, (B) EUB338mix-FITC, specific to the eubacteria domain, (C) GRb-Cy3, specific for many members of the *Rhodobacter* group and (D) the superimposition of the (A), (B) and (C) images shows an exact colocalization of the signals obtained.
2. (A) no probe, (B) EUB338mix-FITC, specific to the eubacteria domain, (C) RHI1247-Cy3, specific to many members of the *Rhizobium* group and (D) the superimposition of the images (A), (B) and (C) shows an exact colocalization of the signals obtained.
3. (A) no probe, (B) EUB338mix-Cy3, specific to the eubacteria domain, (C) SPH120-FITC, specific to members of the *Sphingomonas* group and (D) the superimposition of the images (A), (B) and (C) shows an exact colocalization of the signals obtained. Scale bars: 5 μm.

Table S1. Affiliation of 16S rRNA gene clones and strains sequenced in this study.

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