

Compartmentalization Rather Than Host Tree Drives Truffle Microbiome

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Research

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Abstract

Background: Truffles are some of the among the most expensive edible fungi worldwide whose value in international markets is worth billions of US dollars annually. They form ectomycorrhiza which is a symbiotic relationship with host trees and produce hypogeous ascomata. Their whole life-cycle is closely related to their associated microbiome. However, whether truffle-associated compartments or host trees are drivers for truffle microbiome is unclear.

Methods: To identify and compare bacterial and fungal communities in four truffle-associated compartments (*Tuber indicum* bulk soil, adhering soil to peridium, peridium and gleba), associated to three host trees we sequenced their ITS (fungal) and 16S (bacterial) rDNA with Illumina MiSeq high throughput platform. We further applied the amplicon data to analyze the core microbiome and microbial ecological networks.

Results: *Tuber indicum* microbiome composition was strongly driven by their associated compartments rather than by their symbiotic host trees. Truffle microbiome was bacterial-dominated, and its bacterial community formed a substantially more complex interacting network compared with that of fungal community. Core fungal community changed from Basidiomycota-dominated (in bulk soil) to Rozellomycota-dominated (in interphase soil); while core bacterial community shifted from Bacteroidetes to Proteobacteria dominance from truffle peridium to gleba tissue. At the truffle and soil interphase, an interphase-sieving process was confirmed by i) a clear exclusion of four bacterial phyla (Rokubacteria, Nitrospirae, Chloroflexi and Planctomycetes) in gleba; ii) a significant decrease in alpha-diversity (as revealed by Chao 1, Shannon and Simpson indices); and iii) a substantial decrease in the complexity of the network from bulk soil, to soil-truffle interphase, to peridium and finally to gleba. The network analysis of microbiome showed a more complex and higher number of positive microbial interactions in truffle tissues than in both bulk soil and peridium-adhering soil. *Cupriavidus*, *Bradyrhizobium*, *Aminobacter* and *Mesorhizobium* were the keystone network hub genera associated to truffle gleba.

Conclusion: This study provides novel insights into the factors that drive the truffle microbiome dynamics and the recruitment and function of the microbiome components, showing that they are more complex than previously thought.

Background

As the most famous ectomycorrhizal fungi, truffles establish symbiotic relationships with a wide variety of host trees such as *Pinus* and *Quercus* [1], playing vital roles in maintaining healthy and sustainable forest ecosystems through their interactions with soil microbiome. Previous researches have explored the microbiome composition of some truffles and the physiology of associated trees by studying a single host species. For example, *Tuber indicum* can shape microbiome in ectomycorrhizosphere soil in a *Pinus armandii* forest while influence the host physiology in a *Quercus acutissima* stand [2] and metabolic profiles in a *Q. aliena* stand [3]. Microbiome's long-term stability is vital due to the fact that the uninterrupted presence of beneficial microbes and their associated functions guarantee host health and fitness.

A differentiation of the ascomata-associated microbiome might be developed between peridium and gleba due to the process of ascomata development. Colonization of the gleba tissue by soil microbiome may occur in advance to the differentiation of the truffle peridium when the primordium (a yellowish mycelial pellet) is in close contact with the soil [4]. As a consequence certain microbes are gradually caught by the ascomata tissue during the evanescence of primordium wrapped hyphae and thus protected from soil exchange by the outer part of peridium. During this process, a part of the microbiome can be 'sieved' and excluded from colonization as demonstrated by a significant reduction of ascomata's bacterial diversity compared to that of bulk soil [5]. Several studies have shown that truffle ascomata are strongly colonized by bacteria [6–8] and explored their roles in ascomata maturation [4], aroma formation [9, 10] and nutritional fixation [4, 11]. However, the co-existing fungi have received little attention, and whether the ascomata fungal communities are separated from those in the bulk soil is currently unknown. In order to comprehensively understand the truffle-

associated niche and the selection of a specific microbe, the microbiome at the interphase between truffle and soil should not be ignored.

After the primordium formation, the peridium is formed in soil during the whole ascomata developing process [4]. The peridium-adhering soil is a microhabitat hotspot for microbiome in terms of exchanging nutrients and signals [12–14]. Thus, identifying soil-truffle interphase microbiome could deepen our understanding on truffle ecology.

In a truffle-associated microhabitat, microbiomes are dynamic and highly diverse, making them challenging to understand [12, 14, 15]. The identification of core microbial taxa in soil, truffle and/or their interphase could provide insights into complex microbiomes for their interactive roles in establishing microbial consortiums and resistance to external environmental disturbances [16, 17]. Molecular ecological network analysis (MENA) has been widely utilized as a powerful way to reveal the complicated community assembling and microbial taxa under a diverse range of soil and plant microbiome interactions [18–20]. As positive cooperation or negative competition, such interactions can be described by network models [20–22], in which individual nodes within a network represents microbial taxa (OTUs) and the edge bridging two nodes refers to their inter-relationships (positive/negative) [20–22]. The MENA is also applied to identify which keystone taxa are relevant in the preservation of the community's structure and function [23–25]. In addition, spatial heterogeneity is able to influence the keystone taxa's distribution in any environment. For example, different keystone taxa may function individually, while multiple taxa with similar functioning (e.g., nitrogen fixation) are able to establish keystone guilds altering in this way the structure and dynamics of their ecosystems [25]. In the soil microbiome, keystone taxa have been computationally inferred/identified using network scores [25–27]. It has been demonstrated that in many cases, keystone taxa identified using statistical tools have a strong influence on the composition and performance of microbiome [18, 25, 27, 28]. Microbial keystone taxa have been widely identified in agroecosystems [29–31], but not in forest ecosystems, especially in those having ectomycorrhizal woody trees, despite their great ecological and functional importance. A compartmentalization of biogeochemical cycles has been found in truffle ascocarp peridium and gleba [4, 32]. As a result, knowledge of how keystone taxa could respond to ectomycorrhizal fungi or their fungal sporome compartments could increase our better understanding of their ecological values.

The central idea of this work was to better understand the effects of host tree and truffle-associated compartments (bulk soil, soil adhere to truffle peridium, peridium to gleba) on the variation in truffle microbiome, including the truffle ascomata-soil interphase that links the outer and inner parts of the truffle. Specific objectives were to explore: i) the dominance, composition and keystone species of bacteria and fungi in truffle tissues and its inhabiting soils; ii) microbial alpha taxonomic and their phylogenetic diversity; and iii) the relative importance of host tree and truffle-associated compartments in microbiome composition, using bacterial and fungal network analyses. In doing so, we used 16S and 18S rRNA gene sequencing to explore the microbiome's taxonomic and phylogenetic diversity. We employed a random matrix theory analysis (based on amplicon data) to investigate the core microbiome and microbial ecological networks. We hypothesized that the OTUs in the four truffle-associated compartments would be substantially higher in bacterial than in fungal microbiomes (H1); due to the existence of interphases (peridium and its adhering soil) between soil and truffle, there would be a clear selection of microbiome by soil and peridium leading to a significant decrease in microbial diversity (including evenness, richness and diversity) (H2); and, under such selecting influence, the truffle microbiome composition would tend to be compartment- rather than host-driven (H3). Moreover, with the consideration of microbiome diversity and composition changes, we finally hypothesized that the OTU numbers and their associations would be more complex in the bacterial than in the fungal networks, and such a network complexity would gradually decrease from the bulk soil, then to the soil-truffle interphase, and finally to the gleba tissue (H4).

Results

Differences in microbial richness and diversity indices

Microbial alpha diversity indices (Chao 1, Shannon and Simpson) did not differ among the three symbiotic host trees ($P > 0.05$; Fig. 1). When comparing the ascomata compartments, the three indices were all significantly higher in both the bulk soil and adhering soil to peridium than in the truffle's peridium and gleba ($P < 0.05$; Fig. 1A). The Shannon and Simpson indices in all the four different compartments (bulk soil, adhering soil to peridium, peridium and gleba) of *Tuber indicum* were significantly higher for bacteria than for fungi ($P < 0.05$; Fig. 1A).

Variations In Microbial Community Structures

Beta-diversity of microbial communities was analyzed by using non-metric multidimensional analyses (NMDS) for bacterial (Fig. 2A) and fungal (Fig. 2B) OTUs. For the bacterial community, NMDS distinguished four truffle-associated compartments (ANOSIM test, $R = 0.90$, $P = 0.001$), whereas the influence of host tree on bacterial community was not significant (ANOSIM test, $R = 0.18$, $P = 0.15$). The difference of bacterial community structure, as shown by diverging of the shape, enlarged from bulk soil, soil adhered to peridium, peridium to gleba (Fig. 2A). Contrastingly, the fungal communities showed no clear separations between the four compartments and three host trees (ANOSIM test, $R = 0.19-0.22$, $P = 0.89-0.99$, Fig. 2B). Both the bacterial and fungal communities exhibited a distinction between soil (BS and SP) and truffle tissues (P and G), with a stronger difference (stress = 0.053) in the bacterial than in the fungal community (stress = 0.103). The weighted UniFrac in all truffle-associated compartments was significantly different for both the bacterial and fungal communities (Table 1), indicating that the bacterial and fungal phylogenetic structure were also related to compartmentalization. In contrast, the identity of the host tree did not influence their microbial community structure in truffle-associated compartments (Table 1).

Table 1
UniFrac distance significant values when comparing the influences of host tree and truffle compartments

^a Group 1	Group 2	Bacteria		Fungi	
		t statistic	p-value	t statistic	p-value
All within Group	All between Group	(0.2929)	0.7697	(0.5835)	0.5598
Between Group	Pa vs. Py	0.6035	0.5464	2.1019	0.0360
	Pa vs. Qf	(0.8669)	0.3863	0.1775	0.8591
	Py vs. Qf	0.2534	0.8001	(2.2462)	0.0251
All within Group	All between Group	(13.8434)	0.0000	(8.6129)	0.0000
Between Group	P vs. SP	1.9867	0.0474	(2.7700)	0.0058
	P vs. BS	(5.6141)	0.0000	(3.9043)	0.0001
	P vs. G	7.7697	0.0000	14.9809	0.0000
	SP vs. BS	6.2622	0.0000	(1.0445)	0.2967
	SP vs. G	(2.0313)	0.0427	(2.8379)	0.0047
	BS vs. G	(8.5964)	0.0000	(3.9611)	0.0001

^aAlternative hypothesis: Group 1 mean = Group 2 mean. The tests of significance were performed using a two-sided Student's two-sample t-test ($n = 3$). In the group 2, the abbreviations for host trees are Pa = *Pinus armandii*, Py = *Pinus yunnanensis*; Qf = *Quercus franchetii* and for truffle compartments: BS = bulk soil; SP = soil adhere to peridium; P = peridium; G gleba.

Common Otus And Distributing Features Of Microbial Community

We built the phylogeny of common OTUs over the four truffle-associated compartments to assess their distributing and phylogenetic diversity. The number of common OTUs presenting in all compartments was 995 for bacteria and 157 for fungi (Additional file 1; Figure S1). For bacteria, all compartments were dominated by Proteobacteria (59.5% of all OTUs) and Actinobacteria (11.0%), followed by Bacteroidetes (7.2%), Acidobacteria (7.0%), Firmicutes (4.3%), Chloroflexi (3.3%), and Planctomycetes (2.0%) (Fig. 3A; Additional file 3; Table S1). Rokubacteria, Nitrospirae, Chloroflexi and Planctomycetes were only found in soils, but not in the peridium and gleba. Contrastingly, Bacteroidetes predominated in the peridium while Proteobacteria was mainly abundant in the gleba (Fig. 3A). *Sphingomonas*, *Afipia*, *Amycolatopsis*, *Acinetobacter*, *Bradyrhizobium*, *Cupriavidus* and *Pseudomonas* preferred to colonize the gleba (> 70% relative abundance). The dominating fungal phyla were Ascomycota (79.3%), followed by Basidiomycota (9.7%), Mucoromycota (3.7%), Mortierellomycota (1.1%), Chytridiomycota (0.6%) and Rozellomycota (0.4%) (Fig. 3B; Additional file 3; Table S1). Fungal species belonging to Basidiomycota mostly occupied bulk soil, while taxa assigned to Rozellomycota preferred to exist in the soil adhering to peridium (Fig. 3B). In the peridium, 6 species (5 from phyla Ascomycota: *Cosmospora_gigas*, *Clonostachys_intermedia*, *Exophiala_canceriae*, *Phaeoacremonium_hungaricum*, *Penicillium_chermesinum*, and 1 from Basidiomycota: *Anthracoidea_aspera*) had high relative abundance (averaged 52%). Only 3 taxa *Simplicillium_aogashimaense*, *unidentified_Rozellomycota* and *Cutaneotrichosporon_cyanovorans* (~ 65%) showed a very high abundance in the gleba.

Distinct Microbial Networks And Keystone Taxa

Eight networks were constructed using the bacterial and fungal OTUs from the four truffle-associated compartments (Figs. 4 and 5). The networks showed the positive or negative interactions among taxa and the nodes were distinguished by different colors at the phylum level. The similarity thresholds of the networks went from 0.83 to 0.94 (Table 2), which were higher than those of most reported networks by this method [33, 34]. Average connectivity, average clustering coefficient, average path length and modularity were all higher in the ecological network than in the random network (Table 2), showing that the constructed networks from the present study can be used for subsequent studies on the potential interactions among the bacterial and fungal communities based on their network characteristics such as modular and scale free [21, 35, 36].

Table 2

Topological parameters of the empirical molecular ecological networks (MENs) of microbial communities in truffle compartments and their associated random MENs

Compartments	Empirical networks ^a							Random networks		
	St	Node	Link	Modularity	GD	avgCC	avgK	GD ± SD	avg CC ± SD	Modularity ± SD
BS (Bacteria)	0.94	2782	4522	0.932	11.6	0.156	3.1	5.4 ± 0.02	0.003 ± 0.001	0.613 ± 0.003
SP (Bacteria)	0.94	1958	3098	0.938	11.4	0.148	3.2	5.3 ± 0.03	0.004 ± 0.001	0.625 ± 0.003
P (Bacteria)	0.91	960	1657	0.821	7.80	0.126	3.5	4.6 ± 0.04	0.01 ± 0.01	0.57 ± 0.01
G (Bacteria)	0.89	618	1550	0.673	6.29	0.208	5.0	2.5 ± 0.06	0.25 ± 0.02	0.22 ± 0.01
BS (Fungi)	0.83	138	338	0.615	4.46	0.254	4.7	3.2 ± 0.05	0.07 ± 0.01	0.41 ± 0.01
SP (Fungi)	0.85	101	201	0.639	4.50	0.200	3.7	3.5 ± 0.08	0.04 ± 0.01	0.48 ± 0.01
P (Fungi)	0.89	51	299	0.176	2.60	0.366	10.9	2.1 ± 0.04	0.39 ± 0.02	0.16 ± 0.01
G (Fungi)	0.87	61	241	0.281	3.17	0.287	7.8	2.4 ± 0.05	0.25 ± 0.02	0.22 ± 0.01

^a St, similarity threshold; network size, the number of nodes; avgK, average connectivity among nodes, GD, average geodesic distance between nodes; avg CC, average clustering coefficient of nodes. BS, bulk soil; SP, soil adhere to peridium; P, peridium; G, gleba.

The bacteria network was more complex than that of fungi, containing ~ 10–15 times more nodes and links, a comparative higher modularity and average geodesic distance between nodes, but a comparative lower average clustering coefficient of nodes and average connectivity between nodes (Table 2). When considering the aggregate/loose structure of network (as shown by the distance and connection between nodes), the average geodesic distance (GD) was lower, whereas the average clustering coefficient and connectivity were generally higher in truffle (P and G) as compared to soil (BS and SP) networks (Table 2; $P < 0.05$). A similar trend in all these above-tested parameters in the empirical networks was also observed between the two soil compartments of bulk soil (BS) and soil adhered to peridium (SP) and the two truffle compartments of peridium (P) and gleba (G) (Table 2).

In terms of the competitive/cooperative relationships, bacterial taxa exhibited a co-occurrence pattern, with positive correlations accounting for > 60% of potential interactions observed in the ecological networks of four compartments (Fig. 4). In addition, a stronger tendency of positive/co-occurrence associations was found within truffles (~ 72% positive links) than in soil (averaged 65% positive links). In contrast, for fungal taxa competitive relationships were identified in the

interface between soil and truffle, including 65% and 52% negative links in the soil adhering in peridium (SP) and peridium (P) networks (Fig. 5), whereas with 61% and 62% positive links in bulk soil and gleba networks, respectively (Fig. 5).

In order to depict the microbial variation of the interphase established from the bulk soil to the inner truffle, we visualized those changes at phylum/class level by alluvial diagrams (Fig. 6). The bacterial taxa re-allocation in dominating (six largest) modules occurred extensively at the interphase between soil and truffle, especially for the following three phyla: an obvious expansion for Proteobacteria and strong exclusion for Acidobacteria and Actinobacteria (Fig. 6A), and such a trend was also for the individual network modules (Additional file 2; Fig. S2). For fungi, the number of modules clearly decreased from the bulk soil to the truffle gleba (Additional file 2; Figure S2). The interphase that links the outer and inner parts of the truffle substantially excluded the Sordariomycetes while Pezizomycetes were highly persistent (Fig. 6B). Moreover, we determined the topological role of individual OTUs in the bacterial and fungal networks consisting of all truffle-associated samples via the random matrix theory-based network analyses (Figs. 7 and 8). No network hubs (super-generalists) were detected in the four bacterial networks. Most (> 90%) of the OTUs were peripherals with the majority of their links inside their modules. The number of bacterial keystone taxa (including both module hubs and connectors) at the phylum level decreased from soil (8), peridium (4) to gleba (2). Similarly, the number of keystone taxa decreased dramatically from soil to truffle: bulk soil (107) > soil adhered to peridium (61) > peridium (25) > gleba (9). The 107 bacterial keystone taxa in the bulk soil were dominated by Proteobacteria (36), Actinobacteria (30) and Acidobacteria (20), while the rare taxa belonged to Chloroflexi (7), Gemmatimonadetes (6), Bacteroidetes (4), Planctomycetes (3) and Rokubacteria (1). In contrast, the other three compartments were overwhelmingly occupied by Proteobacteria (31/66, 21/25 and 8/9) (Fig. 7B, C and D).

For fungi, the keystone species were *Oidiodendron*, *Tomentella* and *Sebacinales* in bulk soil (BS), while was *Lecanicillium* in the peridium-adhering soil (Fig. 7) but no keystone taxa were observed in the truffle peridium and gleba.

Discussion

Distinct microbiomes occupied in forest ecological niches such as phyllosphere [37, 38], roots [39], ectomycorrhize [34] and fruiting bodies [41, 42]. Constitutionally, the niches that microbes are inhabiting and substrates that microbes are consuming are the most important inherent factors to shape a unique host-related microbiome. Thus, we proposed that such a 'substrat-determining' theory could be proper in a narrow ecological niche that a typical ectomycorrhizal symbiosis fungus (truffle) associated microbiome. Four typical compartments of the bulk soil, interphases (soil adhered to peridium, and peridium) and gleba tissue were chosen for testing our hypotheses regarding to the effect of host and compartment on a truffle microbiome's diversity and ecology network changes.

In accordance with our first hypothesis (H1), the OTUs assigned to bacteria were substantially higher than those assigned to fungi in the four truffle-associated compartments (Fig. 1), and such dominances extended to truffle's ascomata (Fig. 1). Truffle biology studies have revealed bacteria-associated aroma formation [9, 10], maturation [4], and nutritional fixation [4, 11]. In our case, we found that there were 308 unique bacterial OTUs whereas 78 unique fungal OTUs in the truffle gleba (Additional file 1: Figure S1). Among truffle-associated compartments, soil would harbor more complex microbiome, compared to truffle that develops from soils (Figs. 4 and 5). Except for comparing the soil and truffle gleba, we included the two interphases that bridge these two distinctive compartments. We hypothesized that due to a clear microbiome exclusion existing in the interphase, microbiome diversity might be gradually decreasing from soil to inner truffle tissue. The results proved this hypothesis (H2), there was a clear selection of microbiome by soil and peridium, which leads to a significant decrease in the alpha-diversity (Fig. 1). Spatially, the microbial diversity, evenness and richness all decreased successively from the bulk soil, adhering soil to peridium, peridium to gleba (Fig. 1). Notably, these results were not only obtained from a single host tree, but from three typical truffle symbiont host trees (*P. armandii*, *P. yunnanensis* and *Q. franchetii*) (Fig. 1). However, there was not a clear influence of host on microbiome alpha and beta diversity (Figs. 1 and 2). Although certain ectomycorrhizal mycelia were sensitive to changes in host tree leaf litter composition [43] while such

changes were not transmitted to microbiome during the subsequent ascomata development (Table 1 and Fig. 2). In agreement with our third hypothesis (H3), the truffle microbiome composition was strongly driven by its-associated compartments rather than by their symbiotic host trees (Table 1 and Fig. 2). Among the compartments, the microbial structure variation was stronger in bacterial community than in fungal community. A higher microbial diversity (and richness) seems to be in line with strongly differentiated microbial community structure. For instance, there were significant higher bacterial richness, evenness and diversity in truffle-associated compartments than those of for fungi (Fig. 1). Studies showed that the change in bacterial assembling structure ought to be closely related to their roles in the sensitive biological processes of nutrients exchange among soil, truffle tissue and their interphases [44]. This aforementioned interphase microbiome exclusion proves that an environmental selection allows certain common microbes to 'pass' through soil to truffle peridium, and further colonize into inner gleba tissue (Fig. 6). In the present study, we initially identified the core microbiome number - the common OTUs number was around 6 times higher for bacteria than for fungi (995 vs. 157), which was in line with substantial differences between bacterial and fungal OTUs and agrees with the fourth hypothesis (H4). Such differences might be explained by the microbiome differences in the initial truffle ectomycorrhizal developing stage in ectomycorrhizospheric soil, because Li et al. (2017) reported that the number of species of bacteria were 10 times higher than that of fungal species in ectomycorrhiza (1514 vs. 100), and 6 times in ectomycorrhizospheric soil (1350 vs. 181) [1]. We further distinguished the variations on core microbiome among truffle-associated compartments. In gleba, the core bacterial OTUs were dominated by Proteobacteria (Fig. 3). Benucci et al. (2016) compared truffle microbial communities on the gleba tissue from eight truffle species [42] and recorded similar findings. Especially, the particularly abundant *Bradyrhizobium* and *Sphingobium* inside the gleba strongly reflected their roles in nitrogen fixation as well as glucose and fructose fermentation, respectively [32, 45, 46]. In contrast, with its functioning in carbon fixation [4] and ascospores release [47] Bacteroidetes predominated in the peridium. Notably, four bacterial phyla (Rokubacteria, Nitrospirae, Chloroflexi and Planctomycetes) exclusively colonized the truffle ascomata (Fig. 3), supporting the aforementioned interphase-excluding hypothesis. For fungi, our results demonstrated i) the Basidiomycota had their superiority of inhabiting in soil and the Rozellomycota tended to live in soil adhere to peridium, that is, a clear shift from Basidiomycota- to Rozellomycota-associated soils compared to truffle ascomata; ii) the number of fungal taxa with high relative abundances tended to increase from gleba (3) to peridium (6). *Phaeoacremonium* was one of the six most abundant genera in the truffle peridium, which could be a great potential contributor to the inchoate peridium formation since it enriched in the ectomycorrhizae of the same truffle species [1]. The potential interactions of individual taxa within truffle microbiome were then explored on how the whole microbiome could respond to external changes as a 'network' (Figs. 4 and 5). With the consideration of a higher bacterial richness and diversity that was evaluated via bacterial OTUs number and their associations would be more complex, we hypothesized that the bacterial network than that of fungi, and such a complexity of microbiome would be gradually decrease from bulk soil to soil-truffle interphase, and further to gleba tissue (H4). Results from the molecular ecological network analysis verified this final hypothesis (Table 2). Our data indirectly proved that the complexity of microbiome steadily weakened from bulk soil to soil-truffle interphase, and further to gleba tissue. Such phenomenon could be explained by i) the influence of substrate and its screening on residing microbes, as reflected by the distinctly varied microbial OTUs in compartments; ii) positive links between phylogenetic richness and the strength of their significant relationships - more significant correlations existing in a compartment harboring high microbial richness; and iii) modularity discrepancy among compartments - more functional modularity of microbiome performing nutrient cycling and substrate degradation etc. [48, 49] in soils than in truffles (Figs. 4 and 5). These network complexities could deepen our better understanding of internal characteristics or interactive dynamics of microbial communities. Based on the network variables (i.e., average clustering coefficient, connectivity etc.) for evaluating the aggregate/loose structure of network, the truffle microbiome networks, rather than soil microbial networks, did harbor closer and interconnected microbial OTUs (Table 2), indicating that truffle microbiome could be easily interfered. Furthermore, we estimated the taxa competitive/cooperative relationship between individual networks and found that bacterial OTUs exhibited a co-occurrence (in comparison to fungi; 56% negative links). The positive co-occurring trend was stronger in truffle (~ 72% positive links) than in soil (~ 65% positive links), which might be

related to the truffle's stronger interconnected/aggregated network structure for avoiding environmental perturbations (Figs. 4 and 5). Recently it has been demonstrated that microbial communities harbor keystone taxa (irrespective of their abundance), which drives microbiome composition and functioning [26, 50]. In line with the changes in a network complexity, the number of keystone taxa from the random matrix theory-based network analysis dramatically decreased from soil to truffle. The truffle inhabiting micro-niche heterogeneity can be put forward to explain such distribution existing between soil and truffle. In the present work, the bulk soil harbored the most complex microbiome network and highest number of keystone (107), and accordingly the highest keystone diversity (taxa assigned to 8 bacterial phyla), indicating that multiple keystone taxa, e.g. 107. in bulk soil and 61 in soil adhere to peridium (Fig. 7) might form a keystone guild and could influence a broadly microbial processes including organic matter decomposition and denitrification [25]. Whereas, in truffles, the keystone taxa were almost assigned to only one dominating proteobacterial phylum that could reflect certain N-fixing bacteria (such as *Bradyrhizobium* and *Cupriavidus*; Additional file 5; Table S2) or were function alone within truffle fruiting bodies. This indicates that truffle keystone taxa tended to have stronger effects on a relatively narrow process such as nitrogen and/or carbon fixation [4, 32]. For fungi, no keystone taxa were detected in truffle peridium and gleba, there were only three keystone taxa found in the bulk soil, and one in soil adhere to peridium (Fig. 8), showing their weak involvements as connectors and module hubs with a community. Even though network analysis is a useful tool in the computational identification of keystone taxa in microbial networks, it is of great relevance to connect these taxa to ecosystem processes. For the next step, the challenge is to complement theoretical with empirical evidence (RNA-stable isotope probing coupled with metaproteomics or metatranscriptomics) for identifying keystone taxa in microbial communities.

Conclusion

We conclude that the truffle-associated compartments, rather than the host trees, are a more important driver to influence the bacterial and fungal communities associated to *Tuber indicum* ascomata. This study highlights the multiple and complex potential interactions between bacterial and fungal associations in truffles, which are among the most expensive edible ectomycorrhizal fungi, with a market valued in billions of US dollars annually. A relevant future perspective would be to study the transcriptomic activities of the truffle's associated microbiome and to evaluate the functional profiles and metabolic pathways related to truffle cultivation at a global scale, in order to increase the productivity of these profitable highly prized fungi.

Methods

Sampling method and Storage

This research was conducted in the Puding Karst Ecological Experimental Station (Puding, Guizhou, 26°54'59.7"N, 105°42'35.5"E, 1325 m above the sea level), belonging to the Institute of Geochemistry, Chinese Academy of Sciences (CAS). *Tuber indicum* samples from three host trees (*Quercus franchetii*, *Pinus armandii* and *P. yunnanensis*) were collected during the truffle maturation season between September and December, 2018. A total of 10–20 truffles were harvested from the rhizosphere of an individual host tree, and five intact and healthy truffles were chosen for truffle-associated compartments tissue separation. Bulk soil (BS) was also collected 5.0 m away from the truffle production areas to avoid the influence of the truffle's sphere. Soils adhered to the peridium (SP) were those tightly adhering on the truffle surface (< 0.5 cm). SP was collected on a sterile petri dish with a sterilized soft-metal brush and then transferred into a 5.0 ml tube. After soil collection, all truffles were cleaned with sterilized milli-Q water and dried with sterilized absorbent paper. The ascomata were then cut by sterilized scalpels and the truffle peridium (P) and gleba tissues (G) were collected by sterilized forceps. The respective compartments of G and P from five truffles were mixed together and stored in alcohol sterilized self-sealing bags (60 mm × 85 mm) as a composite sample at – 20 °C for subsequent species

identification (ITS and SSU) and DNA extraction. The experimental design was a two-factor design (host × compartment) with $3 \times 4 = 12$ treatments. There were three replicates per treatment for a total of $12 \times 3 = 36$ biological replicates.

Dna Extraction And Pcr Amplification

Two extraction methods were used to isolate microbial DNA from truffle and soil samples. Soils (BS and SP) were extracted using the MoBioPower Soil DNA kit (12888), and truffle samples were processed using the DNeasy Plant Mini Kit (Qiagen SA). Polymerase chain reaction (PCR) amplifications were carried out following the previously described method [51]. Briefly, a 25 μ L reaction mixture was set up containing 5 μ L of 5 × Reaction buffer, 5 μ L of 5 × GC buffer, 2 μ L of dNTP (2.5 mM), 1 μ L of Reverse primer (10 μ M), 1 μ L of Forward primer (10 μ M), 8.75 μ L of ddH₂O, 2 μ L of DNA Template and 0.25 μ L of Q5 DNA Polymerase. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the primers (338F, 806R) as described by Mori et al. [52]. Internal transcribed spacer 1 (ITS 1) was used for fungal identification, using the primers ITS5F and ITS1R [53]. PCR thermal cycling conditions were set under the following conditions: 98 °C for 2 min (initial denaturation), 25 – 30 cycles of 15 s at 98 °C, 55 °C 30 s, 72 °C 30 s, and concluded with a final extension for 5 min at 72 °C. Amplicons were extracted from 2% agarose gels and purified with the Axygen Axy Prep DNA Gel Extraction kit (AP-GX-500) according to the manufacturer's guidelines and quantified by the Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen (P7589) on a Microplate reader (BioTek, FLx800).

Illumina Miseq Sequencing And Bioinformatics

Purified amplicons were pair-end sequenced 2×300 on the Illumina MiSeq platform, Miseq-PE250 (Personalbio, Shanghai, China) using the MiSeq Reagent Kit v2 (600-cycles-PE, MS-102-3003). Sequences were processed and quality-filtered using the QIIME (Quantitative Insights into Microbial Ecology) pipeline. The 300-bp reads ends were truncated from the first site with low quality (average quality value < 20 over a 10-bp sliding window, the undesirable truncated reads (length < 150 bp) were filtered. Then, the overlapping sequences (≥ 10 bp and passed through quality screening) were assembled using the FLASH software (v1.2.7) [54]. After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% similarity cutoff. The obtained OTU taxonomic clusters were compared with BLAST against the GenBank database [55], OTUs with an abundance < 0.001% of the total sequences across all samples were removed [56] from the final analysis. OTUs assigned to same phylum, class, genus and species level were grouped together based on their taxonomic affiliations.

Data Processing And Statistical Analysis

For each bacterial and fungal community inhabiting in various truffle-associated compartments from different host trees, Chao 1 richness index was calculated from the total OTUs, and so did for the two diversity indices, i.e., Shannon index (which considers both the numbers of individual taxa and of OTUs; Fig. 1) and Simpson evenness index (a measure of the relative abundance of the different individual taxa making up the richness of an area/sample).

Microbial alpha-diversity was estimated by richness (Chao 1) and diversity (Shannon and Simpson) indexes. One-way analysis of variance (ANOVA) followed by Tukey HSD (at $P < 0.05$) was used to compare significant differences in diversity indices.

Beta-diversity of the overall microbial communities between paired samples were determined using the UniFrac metric (Lozupone and Knight, 2005) in the MOTHUR program (<http://www.mothur.org>) and the NMDS analysis was performed by the vegan package of R software based on the weighted and unweighted unifrac distance matrix, and the obtained points were plotted using an Origin 2018.

We constructed the phylogeny of the OTUs (with a threshold of relative abundance at the top 100, in order to provide a relatively equal comparison and to reduce complexity of a tree) commonly shared to the four compartments to assess i) the phylogenetic selection of these OTUs and ii) the domains of a compartment in individual species. For the bacterial and fungal dataset, two phylogenetic trees were constructed in QIIME using maximum likelihood method and visualized using the online Interactive Tree of Life.

Network And Keystone Taxa Analyses

Individual networks were constructed for various compartments/host trees based on 16S rRNA or ITS1 gene sequence data. The molecular ecological network analysis (MENA) pipeline (<http://ieg4.rccc.ou.edu/mena/help.cgi>) was used to analyze the networks. Summarily, there were four main steps for network constructions: i) original data collection (OTU table); ii) data standardization (with 'lg' transformation); iii) pair-wise correlation/similarity estimation, and iv) adjacent matrix formatting based on a random matrix theory method [21, 36]. Indices to evaluate the features of the nodes were: i) Degree, a node with higher degree means that it is highly connected with other nodes (that is, high degree = strong relationship with others); ii) betweenness centrality (BC) = among-module connectivity (Pi), the parameter was used to indicate nodes connecting modules (connectors), $P_i > 0.62$; iii) closeness centrality (CC) = within-module connectivity (Zi), referring to highly connected nodes within modules (module hubs), $Z_i > 2.5$; iv) important nodes to both the network and its own module coherence = network hub ($Z_i > 2.5$, $P_i > 0.62$); v) peripherals - for the nodes within module but few outside connection, $Z_i < 2.5$ and $P_i < 0.62$). The nodes with either a high value of Zi or Pi were defined as keystone taxa, including network hubs, module hubs and connectors [57]. To reveal stories in the large networks, we used alluvial diagrams to highlight and map the significantly structural changes in the network data [58]. The microbial module data of each truffle compartment were obtained from the online platform MENA, and were re-arranged in an excel file. As the bacterial network contained a high number of modules (> 100), we set two criteria to capture major information in the complex functional modules: i) selecting top 6 modules that had the highest number of highly interconnected OTUs; and then ii) filtering and keeping the same OTUs numbers in individual modules. In this way, we could map the trend of changes in bacterial phyla participating in these major modules. For fungi, no selective criteria were needed considering their limited number of modules. Detailed changes in bacterial and fungal diagrams were constructed and visualized with the ggplots2 package in R, based on the demonstrating link (<https://cran.r-project.org/web/packages/ggalluvial/vignettes/ggalluvial.html>).

Abbreviations

16S rRNA gene

16S subunit of the ribosomal RNA gene; ANCOVA:Analysis of covariance; ANOSIM:Analysis of similarity; ANOVA:Analysis of variance; BC:Bray-Curtis; cDNA:Complementary deoxyribonucleic acid; FDR:False discovery rate; ITS:Internal transcribed spacers; NMDS:Non-metric multidimensional scaling; PCR:Polymerase chain reaction; PD:Phylogenetic distance; PERMANOVA:Permutational multivariate analysis of variance; QIIME:Quantitative Insights into Microbial Ecology; OTU:Operational taxonomic unit; rRNA:Ribosomal RNA; SD:Standard deviation

Declarations

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Availability of data and materials

The sequences of 16S rRNA and ITS genes have been deposited in the NCBI Sequence Read Archive under the accession number SRP126991.

Authors' contributions

FQY developed the concept. JPM designed all the experiments, performed the lab experiments. DL performed the statistical analyses, constructed the figures and interpreted data. DL, JPM and XH wrote the manuscript. RGO and XH critically reviewed the manuscript. All authors discussed the results, critically reviewed the manuscript and approved its publication.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

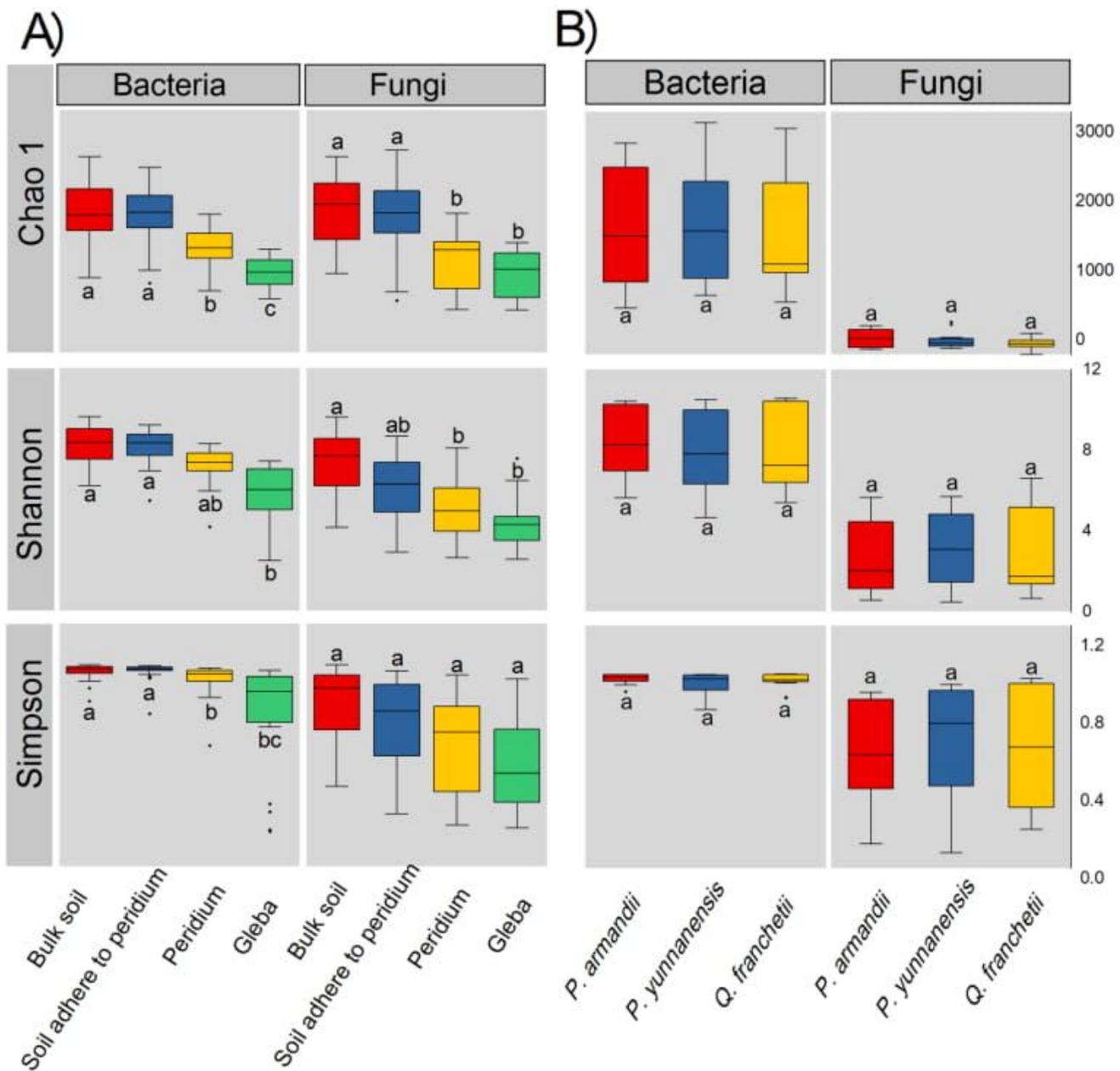


Figure 1

Bacterial and fungal diversity indices in four different compartments (A) of *Tuber indicum* ascomata associated with three host trees (B). Alpha diversity indices were based on microbial richness (Chao 1 index), diversity (Shannon index) and evenness (Simpson index). For individual index boxes, significant differences for compartments or host trees and post-hoc grouping are indicated with letters (Tukey HSD, $P < 0.05$).

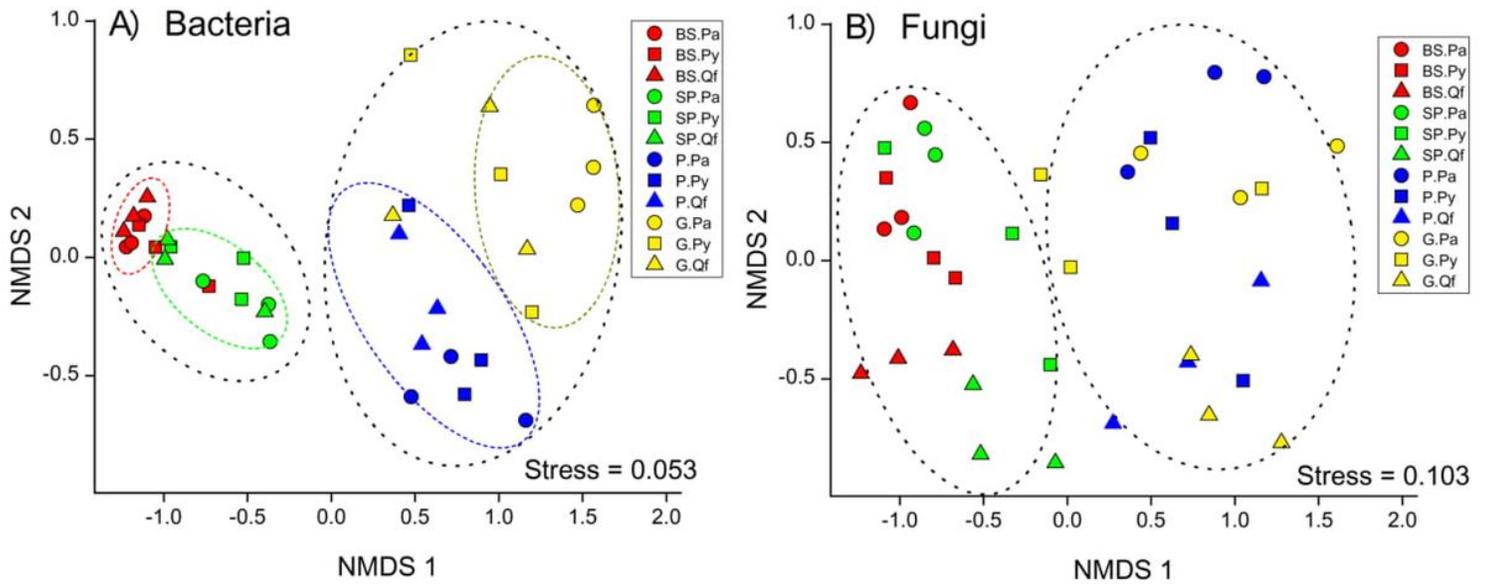


Figure 2

Bacterial (A) and fungal (B) community compositions as indicated by unweighted no-metric multi-dimensional scaling plots (NMDS) of pairwise UniFrac community distance across the *Tuber indicum* recorded in four different ascomata compartments and three different host trees. The abbreviations for truffle compartments are: BS = bulk soil; SP = soil adhere to peridium; P = peridium; and G gleba; and for host trees are: Pa = *Pinus armandii*; Py = *Pinus yunnanensis*; and Qf = *Quercus franchetii*. Interpretation of the abbreviations is described in Table 1.

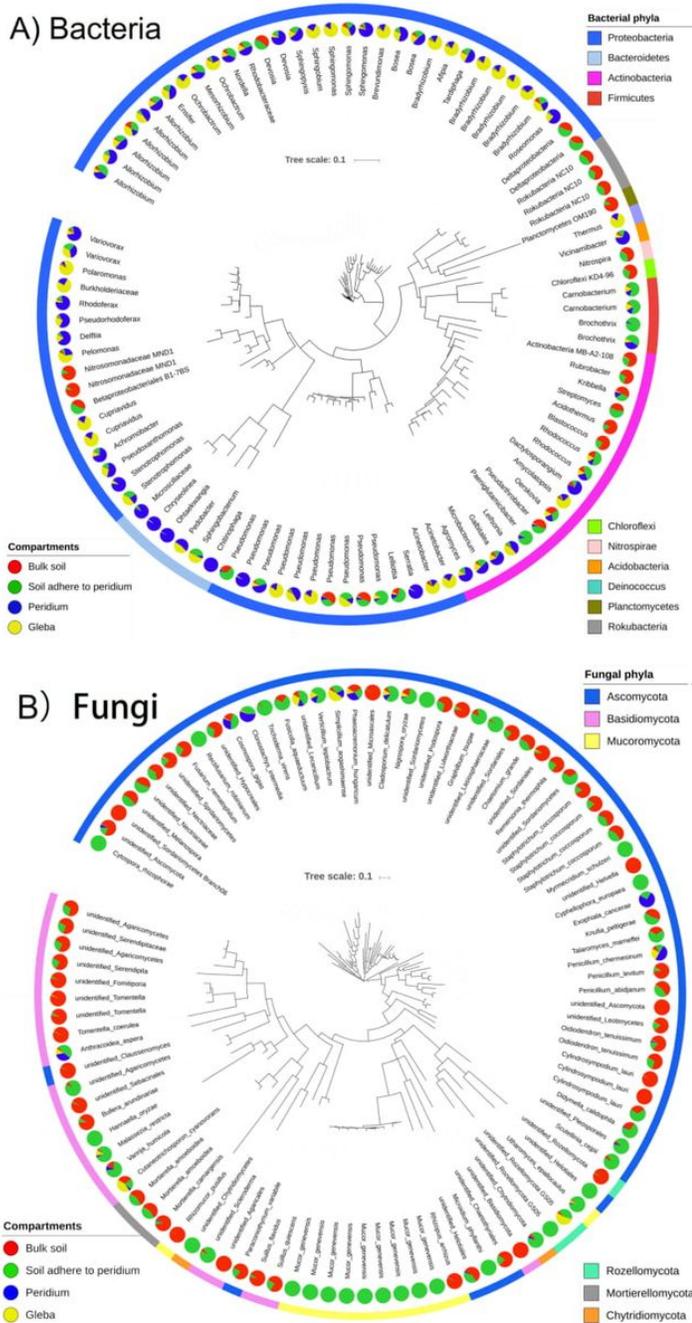
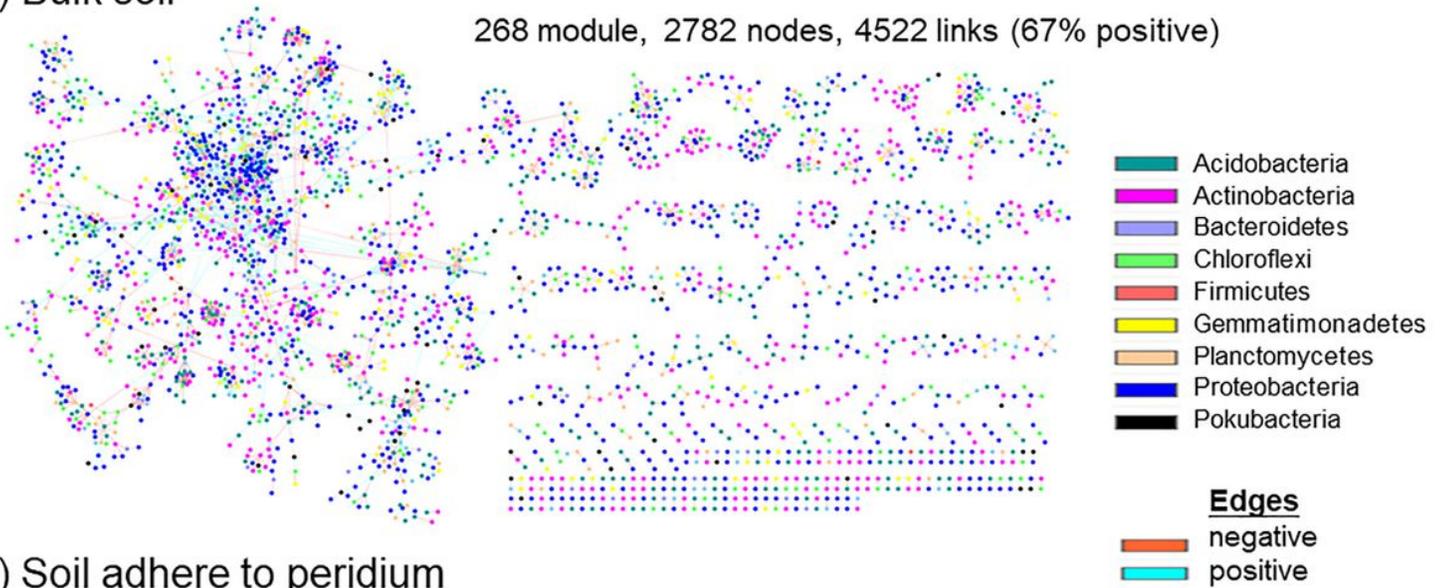


Figure 3

Phylogenetic trees of common OTUs common to the four truffle-associated compartments based on analysis of 16S rRNA genes (a) and fungal ITS (b). Colours of stripes indicate different major phyla. The pie charts represent the relative abundance of each OTU, and the pie slice colours the distribution across compartments (bulk soil in red, soil adhere to peridium in green, peridium in blue and gleba in blue).

A) Bulk soil

268 module, 2782 nodes, 4522 links (67% positive)



B) Soil adhere to peridium

183 modules, 1958 nodes, 3098 links (62% positive)

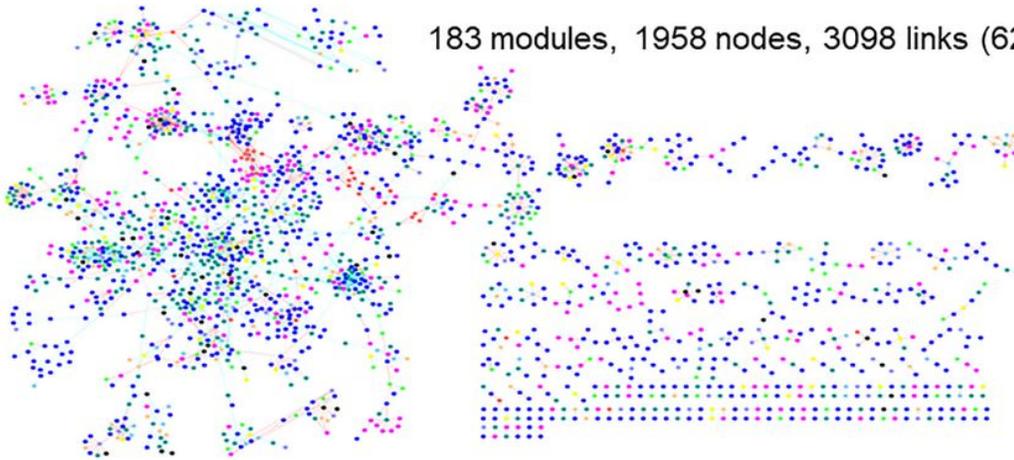
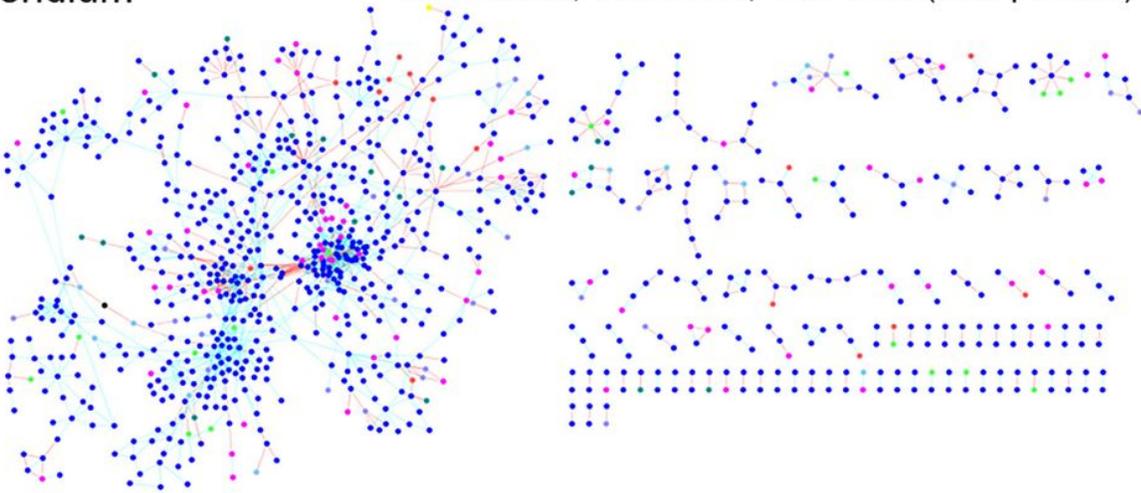


Figure 4

Potential interaction Network interactions of bacteria in truffle-associated compartments. Points represent operational taxonomic units (OTUs) and the colour of points indicates different major phyla. Solid lines represent relationships among nodes. A module is a cluster of highly interconnected nodes. Interpretation of the abbreviations is described in Table 1.

C) Peridium

108 modules, 960 nodes, 1657 links (70% positive)



D) Gleba

72 modules, 618 nodes, 1550 links (74% positive)

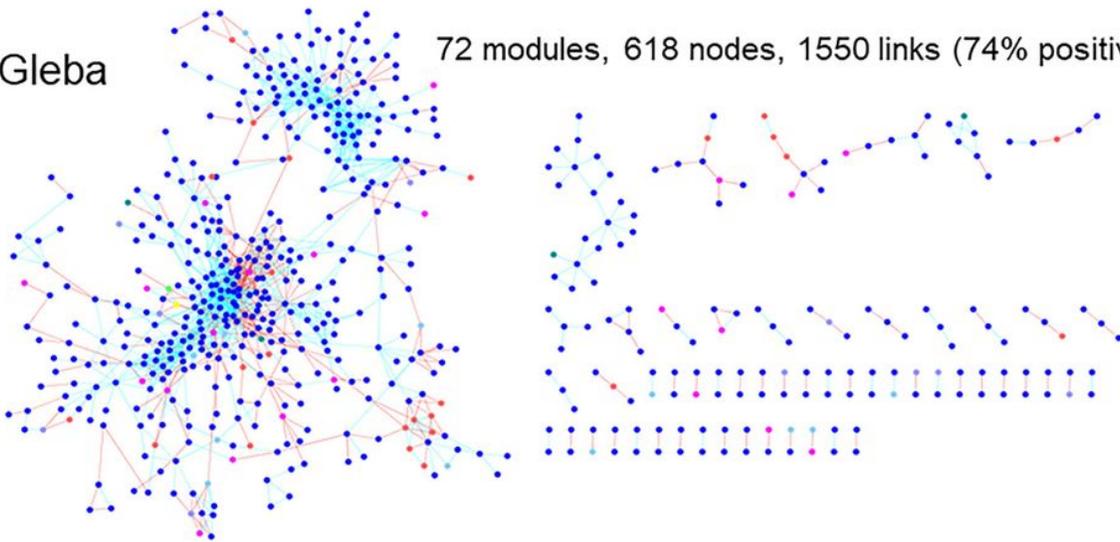
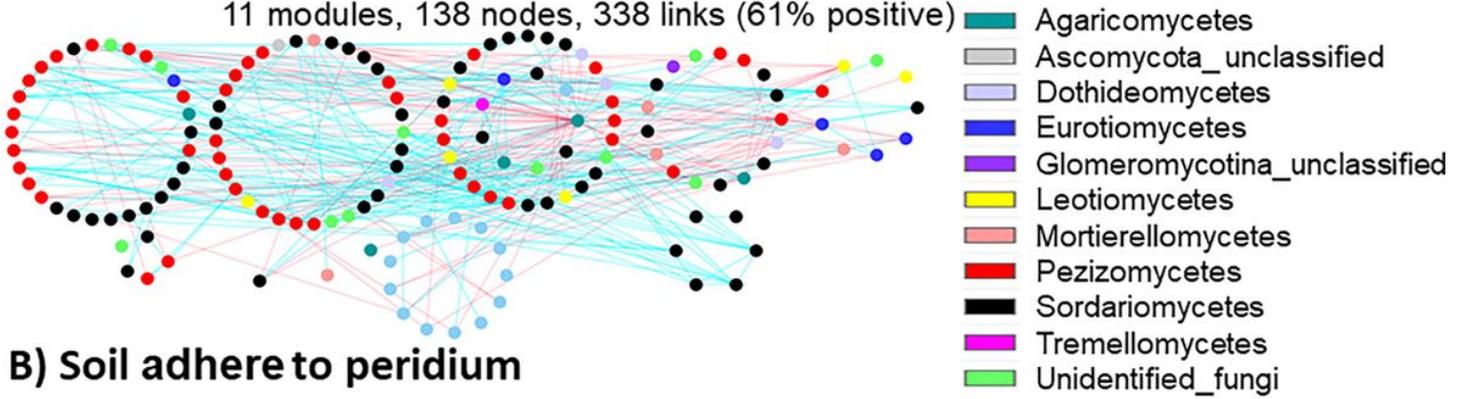


Figure 5

Potential interaction Network interactions of bacteria in truffle-associated compartments. Points represent operational taxonomic units (OTUs) and the colour of points indicates different major phyla. Solid lines represent relationships among nodes. A module is a cluster of highly interconnected nodes. Interpretation of the abbreviations is described in Table 1.

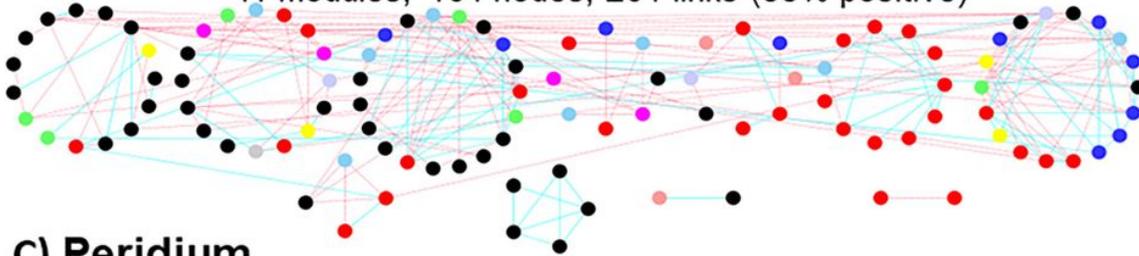
A) Bulk soil

11 modules, 138 nodes, 338 links (61% positive)



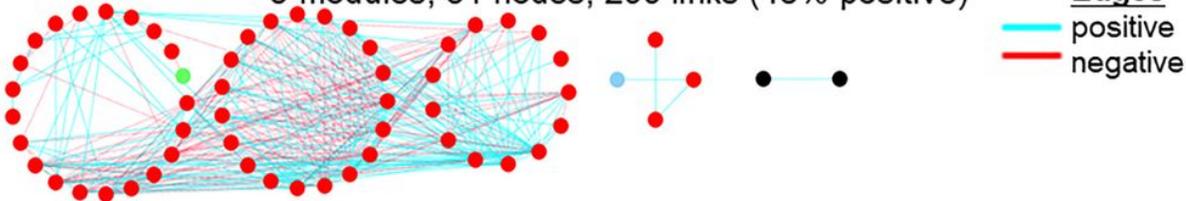
B) Soil adhere to peridium

11 modules, 101 nodes, 201 links (35% positive)



C) Peridium

5 modules, 51 nodes, 299 links (48% positive)



D) Gleba

7 modules, 61 nodes, 241 links (64% positive)

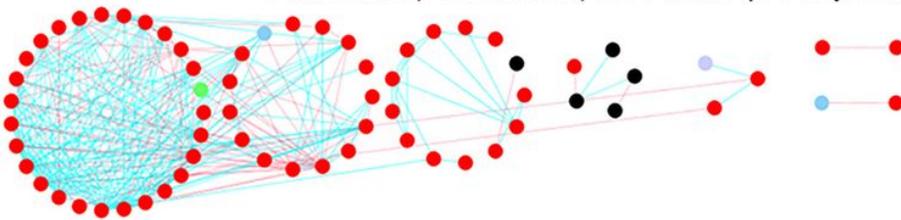


Figure 6

Network interactions of fungi in truffle-associated compartments. Nodes represent operational taxonomic units (OTUs) and the colour of nodes indicates different major classes. Solid lines represent relationships among nodes. A module is a cluster of highly interconnected nodes. Interpretation of the abbreviations is described in Table 1.

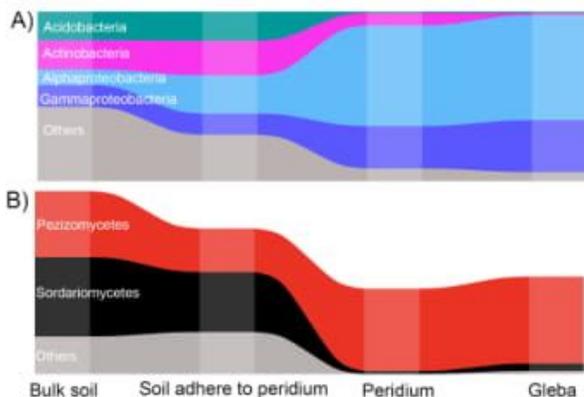


Figure 7

Focused alluvial diagram of A) bacteria and B) fungi in truffle-associated compartments. Each column represents a compartment. The flows among compartments represent the re-distribution of clusters of OTUs that involved in network functional modules. To avoid colour saturation, only the dominant microbial taxa were coloured.

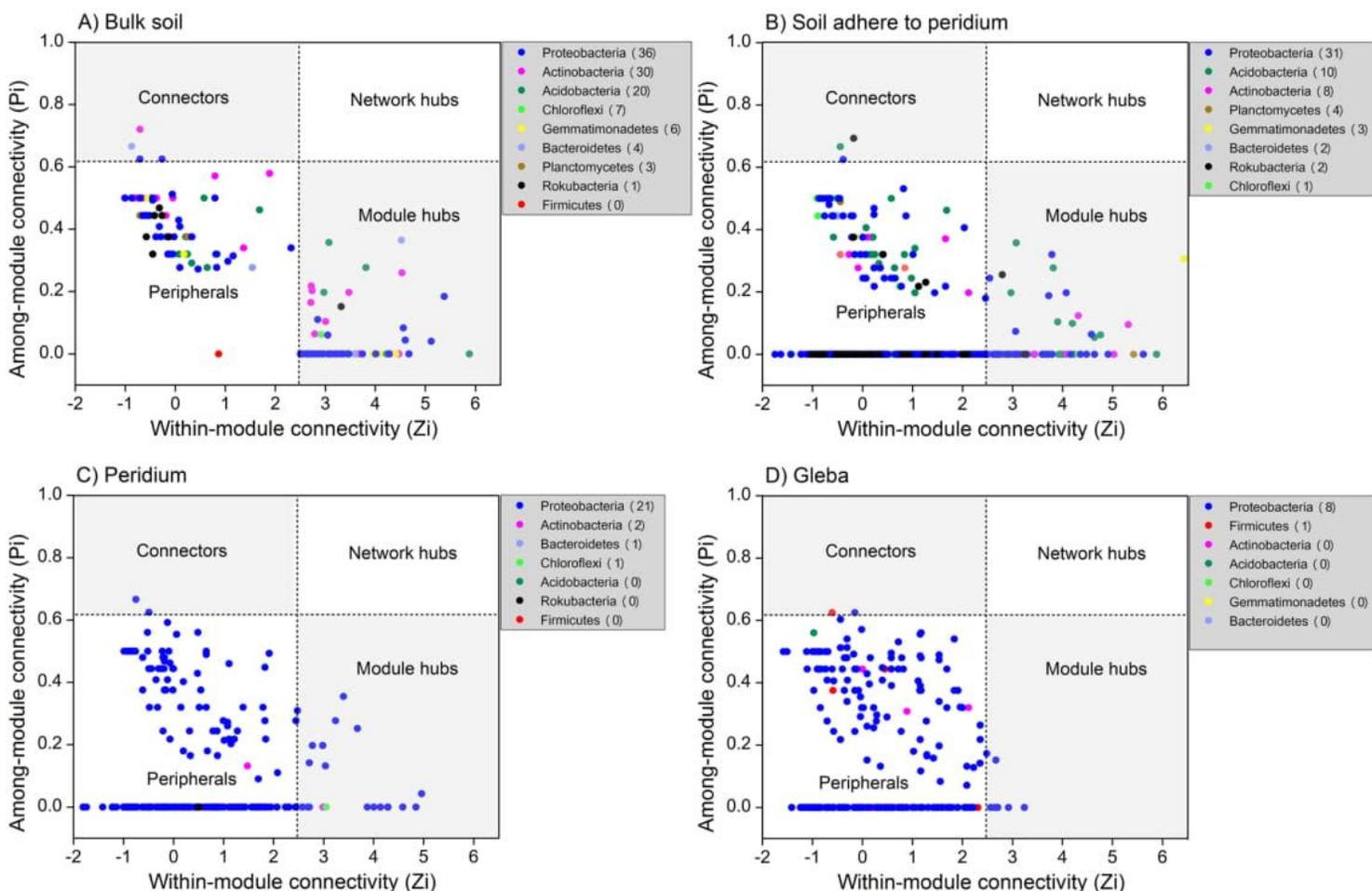


Figure 8

Z-P plots showing the classification of nodes to identify potential bacterial keystone species in four truffle-associated compartments. Each point represents an OTU. The topological roles of individual OTUs were determined based on the scatter plot of within-module (Z_i) and among-module (P_i) connectivity. The module hubs ($Z_i > 2.5$) and connectors ($P_i >$

0.62) are highlighted by grey-shady area. For figure caption, colour represents OTUs assigned to bacterial phyla and the value in bracket indicate the total number of keystone species (including module hubs and connectors).

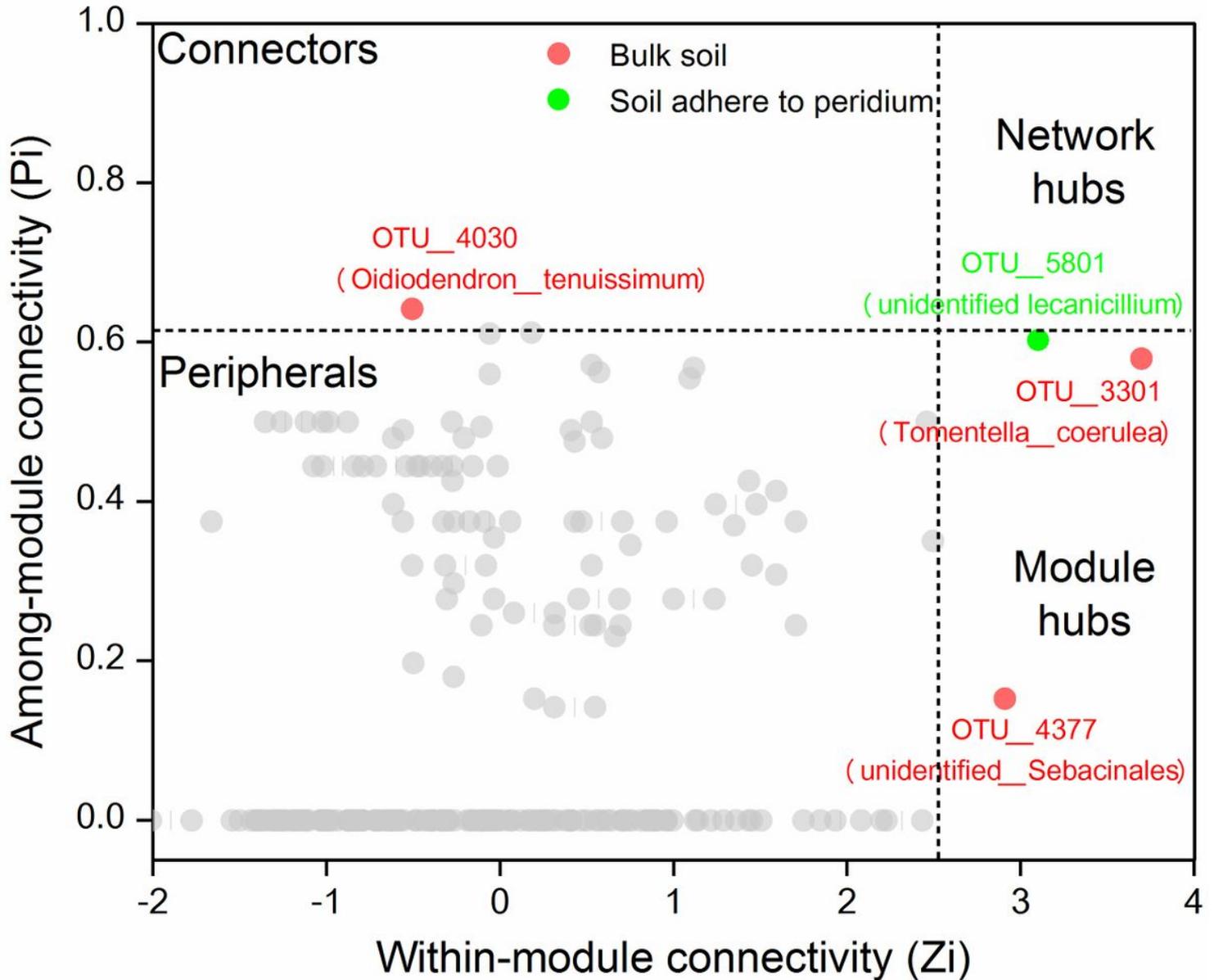


Figure 9

Z-P plots showing the classification of nodes to identify potential fungal keystone species in four truffle-associated compartments. Each point represents an OTU. The topological roles of individual OTUs were determined based on the scatter plot of within-module (Z_i) and among-module (P_i) connectivity. The module hubs ($Z_i > 2.5$) and connectors ($P_i > 0.62$) are highlighted by color point. No keystone taxa were detected in truffle peridium and gleba, whereas there were three keystone taxa found in bulk soil (BS) and one in soil adhere to peridium (SP) which are labeled with OTU numbers and species name.

Supplementary Files

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