Functional Response of Ectomycorrhizal Fungal Community to Nitrogen Deposition on Slash Pine (*Pinus elliottii*) Plantation in South-central China

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Chen Ning

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Committee members:

Printed name	Signature	Department/Affiliation
Gregory Mueller, Major advisor		Plant Biology + Conservation
Louise Egerton-Warburton		CBG / PBC
Andrew Wilson		CBG / PBC

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Signature of major advisor confirming final approval — Date: 29 July 2014

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Plant Biology and Conservation Program Director Signature — Date: 6 August 2014

Abstract

- Nitrogen (N) deposition can alter belowground microbial communities, especially ectomycorrhizal (ECM) fungi, resulting in a reduced ability of associated trees to access organic nutrients. This study tested whether N addition decreases ECM species richness and shifts ECM species composition across spatial scales in a subtropical slash pine (*Pinus elliottii*) plantation in Hunan China (113°02 '-03' E, 28°06 '-07' N).
- Combined molecular (sequencing of fungal ribosomal DNA) and morphological approaches were used to measure ECM fungal operational taxon unit (OTU) richness, community structure and composition at three spatial scales; soil core, plot and forest at a study site where three different nitrogen addition treatments (control, low, and high) had been applied over a three year period.
- High N deposition reduced taxonomic richness of the ECM fungal community on roots. N addition reduce the relative abundance of observed mycorrhizae formed by Thelephoraceae and *Cenococcum* sp., whereas the observed frequency of Atheliaceae sp and *Russula* sp. was increased in N addition plots.
- At the level of ECM morphotype group,, the activity of three enzymes (acid phosphatase, AP; polyphenol oxidase, PO; and protease, PRO) showed different patterns along the N gradient: AP activities of ECM root tips were repressed by N addition, whereas potential PO was stimulated by high N input. PRO activities did not significantly vary among the three different treatments even though there was a change in ECM community diversity and composition, suggesting ecologically functional across the pollution gradient.
- Slash pine (*Pinus elliottii*) as an exotic pine species has been frequently planted in deforested areas in south-central China. Local, potentially introduced, and undescribed ECM species were recovered during the study.
- Additional study, with increased sampling intensity, both spatially and temporally, is needed to better differentiate the impacts of abiotic from biotic factors.

Key words: ectomycorrhizal (ECM) fungal community, nitrogen (N) deposition, extracellular enzyme activities, *Pinus elliottii*, Chinese pine plantation

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Introduction

Nitrogen (N) deposition is considered to have great influence on the biota (Dupra *et al.*, 2010) and functioning of terrestrial ecosystems (Vitousek *et al.*, 1997). Elevated N can alter the soil carbon (C) /N, litter decomposition, and fine root biomass, thus causing a change in mycorrhizal symbioses (Pregitzer *et al.*, 2000; Ramirez *et al.*, 2010). N availability through deposition or fertilization also has been shown to influence ectomycorrhizal (ECM) fungal communities and their biodiversity in a variety of systems (Kårén & Nylund, 1997; Fransson *et al.*, 2000; Jonsson *et al.*, 2000; Lilleskov *et al.*, 2001, 2002a; Peter *et al.*, 2001; Frey *et al.*, 2004; Berch *et al*. 2006; Carfrae *et al.*, 2006; Avis *et al.*, 2003, 2008; Sun *et al.*, 2010). Changes in enzyme activity have also been documented under elevated nitrogen (reviewed by Lilleskov *et al*., 2011; Jones *et al.*, 2012). However, few studies have investigated the correlation between changes in ECM community composition and enzymatic activities under increased N deposition. This study investigates these relationships in an exotic pine plantation (*Pinus elliottii*) in subtropical China.

ECM fungi play a critical role in the diversity, function and renewal of terrestrial ecosystems. They form symbiotic relationships with host plants and receive a large, direct share of net primary productivity in return they facilitate nutrient acquisition of the host plant, protect their root system from microbial pathogens, and enhance their drought tolerance (Dahlberg, 2001). Many trees such as those in Betulaceae, Pinaceae, Fagaceae, Myrtaceae, and Dipterocaraceae are important and obligate ECM plants, forming vast boreal, temperate and subtropical forests (Smith & Read, 2008). ECM fungal species have a high diversity, which has been estimated to be as high as 20,000-25,000 species around the world (Mueller *et al*., 2007; Rinaldi, 2009). This estimate may be low as many morphological species concepts are proving to be species complexes, many species in North America and Asia have been given European names but in fact are unique

species, many areas of the tropics and the Asian continent have not been thoroughly surveyed, and some species fruit rarely or cryptically (Lilleskov *et al.*, 2011). In China, 40 families, 80 genera, and c. 500 species of ECM fungi have been reported (Gao & Guo, 2013). Because of inherent fungal traits, such as the sporadic occurrence of the sexual fruit bodies (sporocarps), the cryptic nature and the lack of morphological characters of the vegetative state, and the difficulty in culturing most species for lab work, identifying species of concern has been challenging (Lilleskov *et al.*, 2011). Though molecular methods have greatly improved the ability to identify and quantify ECM fungi from environmental samples and mycorrhizal root tips (Horton & Bruns, 2001), we currently cannot use this information by itself to name new species in the absence of sporocarp data. However, due to the greater understanding of the biology and taxonomy of the fungal partner, research into ECM ecology has progressed further with respect to a mechanistic or theoretic understanding of what drives community assembly and structure (Bruns & Kennedy, 2009). Even so, further work is needed to explore how the reciprocal influence between plant and ECM fungal communities may play a fundamental role in determining the species composition (biodiversity of both plant and fungal communities) and their ecosystem functions.

Two general conclusions can be addressed from previous N - ECM fungal community studies: First, the effect on species richness and composition of ECM fungi in forest ecosystems varies depending on the level and duration of N deposition. These studies have generally found that short-term belowground responses to increased N availability are more subtle than sporocarp responses, typically resulting in small changes in relative frequency or abundance rather than major community shifts (e.g. Kårén & Nylund, 1997; Jonsson *et al.*, 2000; Peter *et al.*, 2001). By contrast, response to long-term elevated N input or annually high load N input can be quite dramatic, leading to a major shift in dominant fungi and reduced belowground diversity (e.g. Frans-

son *et al.*, 2000; Peter *et al.*, 2001; Lilleskov *et al.*, 2002a; Frey *et al.*, 2004). In addition, the effects of experimental N addition vs. ambient N deposition levels can vary dramatically among different studies. For example, Lilleskov *et al.* (2002a) measured a 70% decline in ECM fungal richness belowground and a dramatic shift in the species composition in high-N input sites (ten fold increase in the N deposition plots over ambient deposition levels) of Alaskan spruce forest which was impacted for c.30 yrs by high levels of atmospheric N deposition from an industrial ammonia production facility. By contrast, Jonsson *et al.* (2000) and Avis *et al.* (2008) reported that ECM fungal species richness was c. 20% lower in treatment plots receiving threefold greater experimental N deposition than in ambient control plots in conifer forests and temperate deciduous ecosystems, respectively.

Second, though the structure and diversity of ECM communities have been extensively studied across a wide range of forest types, as well as their response to environmental disturbance, the structure-function relations (e.g. ecological preferences, resilience, functional redundancy, and functional complementarity) in these communities have been incompletely addressed (Fransson *et al.*, 2000; Elmpvist *et al*., 2003; Peay *et al*., 2008; Courty *et al*., 2010). Studies of fungal communities have evolved from observations of sporocarps above ground (e.g. Wallenda & Kottke, 1998; Lilleskov & Bruns, 2001) to analyzing belowground composition. Previous studies revealed that sporocarp inventories of ECM fungi correlate poorly with community structure on mycorrhizal root tips due to episodic fruiting and that some ECM species either do not produce sporocarps or form inconspicuous reproductive structures (Gardes & Bruns, 1996; Dahlberg *et al.*, 1997; Kårén & Nylund, 1997; Jonsson *et al.*, 2000; Lilleskov *et al*., 2002a).

Understanding the connection between these two variables is a major goal in ecological studies of both micro- and macro-organisms (Robinson *et al.*, 2010; Thompson *et al.*, 2012) and will be

essential to understanding how both plant and their microbial symbionts respond to environmental change (Deslippe *et al*., 2011; Pickles *et al*., 2012). Many ECM fungi can break down soil organic matter through excretion of hydrolytic and oxidative enzymes, and patterns of extracellular enzyme production across ECM species suggests that ECM diversity and assemblages may affect soil C and N cycling rates (Eaton & Ayres, 2002; Lilleskov *et al.*, 2002b; Courty *et al.*, 2005, 2006; Talbot *et al.*, 2008, 2013; Rineau & Garbaye, 2009; Pritsch & Garbaye, 2011; Brzostek & Finzi, 2011; Burke *et al.*, 2011). Thus, extracellular enzyme activities can be considered functional traits to study functional diversity and resilience of ECM communities (Cullings & Courty, 2009).

The high diversity of soil biota, including ECM fungi, has been interpreted as evidence that a high degree of functional redundancy may exist, but interpreting shifts in ECM community structure is complicated by the fact that the degree of intraspecific variation in physiology may be high (Wagner *et al.*, 1988; Gay *et al*., 1993). Cairney (1999) points out that it is not yet possible to draw general conclusion about links between community structure and function because relatively few investigations of ECM fungal physiology have been conducted with more than five isolates of the same species. Changes in the relative abundance of different species might lead to changes in function if accompanied by large changes in morphology and metabolic activities: relevant factors to consider are the amount and distribution of extrametrical mycelium, the capacity to utilize more or less complex organic substrates, and C-use efficiency (Fransson *et al*., 2000).

Changed nutrient availability may alter species composition as well as result in changed activity within a species (Taylor *et al*., 2000). Bending & Read (1995) found that the differing abilities of *Suillus bovinus* and *Thelephora terrestris* to obtain nutrients from litter was consistent with their

differing patterns of mycelial growth and enzymatic capabilities. In a more recent study, Jones *et al*. (2012) points out that the identity of fungal symbionts is a more dominant factor influencing activities of mycorrhizal exoenzymes than fertilization. For example, their study shows that although all the *Cenococcum* and *Suillus* samples were taken from different biogeoclimatic zones, edaphic conditions, and genetically distinct population of host pine species, these two species showed non-overlapping ecological functions (potential enzyme activities) and were distinct from each other.

Agerer (2001) developed the concept of exploration types to describe and organize the diversity of anatomical features seen in fungal mycelium of ECM fungi. This system provides a framework for discussing anatomical features that influence fungal exploration of the soil and the organic N use. Some taxa (e.g. species of *Tricholoma*, *Cortinarius*, *Piloderma*), have medium distance fringe category of exploration types (Agerer 2001, 2006; http://www.deemy.de), and dramatically decline in abundance in response to N deposition condition. Recent development of methods for determining the potential enzymatic activity profiles of individual ECM makes it possible to investigate the roles of ECM fungi in soil processes (Courty *et al*., 2005; Pritsch *et al*., 2011). Using these approaches, some studies have reported that the activity profile of ECMs formed by the same species may change significantly according to ever-changing ecological conditions. (e.g. Courty *et al*. 2005; Rineau & Garbaye 2009; Jones *et al*. 2010, 2012; Walker *et al.*, 2014) Since more than 95% of the N and sulfur (S) and 20-90% of the phosphorous (P) in surface soils is present in soil organic matter (Guggenberger & Haider, 2002), N addition may affect cycling of N, P, and S through its effects on ECM fungi. For instance, Jones *et al*. (2012) found that cellobiohydrolase, β-glucosidase, xylosidase, and laccase associated with *Piloderma* and *Cenococcum* mycorrhizae increased under annual N fertilization treatment; β-N-

acetylglucosaminidase, sulfatase and phosphatase activities did not vary with fertilization; and enzyme activities of *Cenococcum* mycorrhizae were positively correlated with total soil N, whereas those of *Piloderma* mycorrhiza were negatively correlated with soil pH. Based on these studies, it is reasonable to suggest that if different assemblages contain species that are functionally redundant with respect to these traits (e.g. phosphatase activity), rates of C and N cycling may be consistent across fungal communities regardless of differences in species composition (Courty *et al.*, 2005, 2006).

In China, the emission of reactive N increased from 1.4×10^{7} $t\cdot yr^{-1}$ in 1961 to 6.8×10^{7} $t\cdot yr^{-1}$ in 2000 and is likely to be 1.05×10^{8} $t\cdot yr^{-1}$ by 2030 (Zheng *et al.*, 2002). The emission of reactive N leads to deposition of 8-40 kg $N\cdot ha^{-1}\cdot yr^{-1}$ in some forest of southern China (Chen & Mulder, 2007; Du *et al.*, 2008; Fang *et al.*, 2011). The possible impact of elevated N input on vegetation, N cycling, acidification, and microbial activity in tropical and subtropical Chinese forests have only been addressed in few studies (Liu *et al*., 2004; Mo *et al.*, 2004; Song *et al.*, 2011, Yuan *et al.*, 2013). In addition, slash pine (*Pinus elliottii*), an introduced pine species, has been frequently planted in deforested areas in south-central China because of its tolerance to drought and arid condition (Bracho *et al.*, 2012). Land use change from native forest to exotic pine plantation can affect soil organic matter and result in quantitative and qualitative changes to soil carbon and nitrogen pools along with soil biological properties (Chen *et al.*, 2004, 2005). Little is known regarding the likely consequences of these conversion on soil fungal communities, but conversion from mixed native forest to plantation monoculture is likely to exert an influence on ECM fungal diversity (Chapela *et al.*, 2001; Bastias *et al.*, 2007). A better understanding of ECM community and functioning in slash pine plantations is also needed to develop an effective reforestation strategy in south central areas of China.

To address these questions, I tested the hypotheses: (I) Chronic nitrogen enrichment will result in decreased ECM fungal species richness and shifts the ECM community structure and composition. (II) Higher level of N enrichment will affect ECM communities to a greater extent than lower N treatment (III) N enrichment will suppress enzyme activities in mycorrhizospheres, where high N has greater negative effects on enzyme activity than low N (IV) Slash pine (*Pinus elliottii*) as an exotic pine species frequently planted in deforested area in south-central China, will be inhabited by a low diversity of, largely introduced ECM fungal taxa.

In testing these hypotheses, I used a spatially explicit sampling design to capture fungal community composition represented in soil cores from slash pine stands of south-central China. These plots under-went a 3-yr nitrogen deposition experiment. They were assayed for the extracellular activities of three enzymes involved in depolymerization or release of soluble nutrients from soil organic matter.

Materials and Methods

Study sites

The study site is located in Hunan Forest Botanic Garden (113°02 '-03' E, 28°06 '-07' N), south-central China. The climate is typical subtropical humid monsoon, with an annual average temperature of c.17.2 ℃. The hottest moth is July, with an average temperature of 29.4 ℃, with a extreme maximum temperature of 40.6 ℃; the coldest is January, with an average temperature of 4.7 ℃ and extreme minimum temperature of -11.3 ℃. Annual average sunshine hours is 1677.1 h. Rainfall is abundant, and the average annual rainfall is about 1400 mm. Annual frost-free period is 270-310 d. The study site is at an altitude of 50-100 m and the soil formation is mainly of quaternary Pleistocene patterned ground, belonging to typical red soil hilly land. Tree composi-

tion is dominated by slash pine (*P. elliottii*) and camphor tree (*Camphora officinarum*) with a forest age of around 30 years. The average ambient annual input of dissolved inorganic N (including both NO_3^- and NH_4^+) is c. 25 kg $N \cdot ha^{-1} \cdot yr^{-1}$ (Fang *et al.*, 2011).

Experimental fertilization

Nine plots (10×10m that enclose at least three pine trees) were established in June 2010 in the study site. These encompass three N fertilization levels: control (CK, 0 kg $N \cdot ha^{-1} \cdot yr^{-1}$), low nitrogen (LN, 50 kg $N \cdot ha^{-1} \cdot yr^{-1}$), and high nitrogen (HN, 300 kg $N \cdot ha^{-1} \cdot yr^{-1}$). Each treatment was replicated three times and randomly located in the study site. A buffer zone of >3m was set between each plot. The appropriate concentration of NH_4NO_3 was dissolved in 5 L water and uniformly sprayed in the targeted treatment plot. The same amount of water was sprayed on the control treatment plots to reduce the influence of extra water on forest biological geochemistry cycles. N fertilization was applied twice each year (January and June) for 3 years.

Enzyme assays

Three mature slash pine trees were randomly selected in each plot as 'focal trees. ' Four soil cores (10 cm diameter and 15 cm deep, at the four cardinal directions) were collected 45 cm from the base of each focal tree. Soil cores were placed in a cooler and stored at 4 ℃ for a maximum of 7 days at National Engineering Laboratory for Applied Technology of Forestry and Ecology in South China until analyzed further.

Root tip preparation followed Pritsch *et al.* (2004). After being washed free of soil, roots from individual trees were pooled. Each ECM tip was placed into a morphological category (i.e. 'morphotyped' using a dissecting microscope) based on ECM mantle features such as color, texture and surface ornamentation (Agerer 1987-2002; Agerer 2001, 2006; http://www.deemy.de).

Seven active root tips with the same diameter from each morphotype were chosen from each pooled sample and cut into equal lengths (c. 4 mm) to minimize the error of assaying mycorrhizae in different physiological conditions. Then each root tip was placed in a tube of a sieve strip that consisted of a row of eight polymerase chain reaction (PCR) tubes (8×200 µl Multi Ultra Strips®, Roth, Karlsruhe, Germany), the eighth tube was left as a control. After that, the strips were used in all functional assays to transfer seven tips simultaneously from one row of wells to another in a 96-well plate (Sarstedt, Inc., Newton, NC, USA), allowing the same tips to incubate, be rinsed for 3 min, reincubated and rinsed again until the end of an experimental series. After all the enzyme assays had been completed, the root tip was rinsed, dried, weighed and then frozen at -80 °C for molecular analysis.

Enzyme assays were derived from (Colpaert & van Laere, 1996; Pritsch *et al.*, 2004) Three enzymes were tested: acid phosphatase (AP), polyphenol oxidase (PO), and protease (PRO). The substrates for AP and PO were 5 mM p-NP (p-nitrophenyl phosphate) and 25 mM L-DOPA (L-3, 4- dihydroxyphenylalanine), respectively, prepared in 50mM sodium acetate-acetic acid buffer (pH=5.0, Sigma Chemical, Co., St. Louis, MO, USA). 100 µl substrate was added to each well and root tips were incubated in the substrate for 1 hour at room temperature (c.30 ˚C). After that, 20 µl of 1M NaOH and 80µl sterile water were added to finish AP assays and 100 µl sterile water was added for PO. Measurements were carried out with a Tecan Infinite M1000 (Tecan, Mannedorf, Switzerland) spectrophotometer. The absorbance of p-NP was measured at 410 nm and 460 nm for L-DOPA. PRO activity was measured with a general proteolytic substrate (Azocoll®, <50 mesh supplied by Calbiochem-Behring Corp., La Jolla, CA, USA) that released a colored soluble degradation-product. To 200 mg of Azocoll®, 10ml 50mM sodium acetate-acetic acid buffer was added. Once the substrate had been hydrated (fully for 15 min), 100 µl of Azo-

coll® solution was added to each well. The plate was then covered with a lid and incubated at 37 ˚C for 2 hours; agitated gently every 30 min so that fresh solution came in contact with the root tip. After that, 100 µl sterile water was added to each well and absorbance was measured at 520 nm. For each root tip and enzyme assay, a net index of activity was calculated as follows:

$$\text{Net Activity} = \text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{control}}$$

After which, Net Activity was adjusted for incubation time and root tip weight as:

Activity per g root tip = Net Activity × Time × (1/ weight (g)) × (final well volume/ incubation volume)

Molecular analysis of ECM fungi from colonized root tips

DNA from three preserved root tips randomly chosen from each mophotype from each pooled sample was extracted using DNeasy Plant Mini Kit (Qiagen SA, Coutaboeuf, France) with the following modifications. Ethanol was removed after the wash step by incubation for 5 min at 65 ˚C. 50 µl (total 100 µl) buffer AE was added to dilute the extracted DNA. The internal transcribed spacer (ITS) region of ribosomal DNA was amplified using the primer combination of ITS-1F and ITS-4 (White *et al.*, 1990; Gardes & Bruns, 1996). Thermocycling conditions for polymerase chain reaction (PCR) were: 96 ˚C for 3 min followed by 35 cycles of 96˚C for 30 s, 54 ˚C for 30 s, and 72 ˚C for 1 min, followed by a final 10 min at 72 ˚C. PCR products were visualized by gel electrophoresis and strong, single-band PCR products were sequenced directly using reverse primers ITS-4 on an Applied Biosystems 3130xl Genetic Analyzer. For sequence verification, some samples were also run with forward primer ITS-1F. Weak PCR products and products showing multiple bands were cloned, using TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Successfully cloned colonies were PCR amplified using primer pair M13F/M13R and then screened using gel electrophoresis. Multiple colony amplicons of the appropriate sized

PCR product were selected for sequencing (detailed in Data Analysis below).

Soil nutrients analysis

One hundred gram soil subsamples were taken from each soil core after fine roots selection and sieved using a 2 mm screen in which gravel and coarse organic matter were discarded. A portion of each soil sample was air-dried and then manually milled and sieved through a 0.5 mm mesh to measure available N ($NH_4 + NO_3$) and P concentration. Available N was determined using sodium chloride extraction and Zinc (Zn) - ferrous sulfide reduction method (Institute of Soil Science, Chinese Academy of Science 1978) and P was measured using an acid-extracted molybdenum colorimetric method with a digestion of HCl-NH_4F. To determine the organic matter and C, total N and P, and available potassium (K), another portion of the soil sample was oven-dried at 105 ˚C, milled and sieved through 0.25 mm mesh. Organic C and organic matter were determined by applying the wet combustion method (the Walkley-Black procedure; Institute of Soil Science, Chinese Academy of Science 1978) using oxidization of potassium bi-chromate. The converting factor of soil organic C to organic matter was 1.724. Following ammonium acetate extraction, available K was determined by FP6410 flame photometer (Jingke limited Co., Shanghai, China). Total N was measured using Semimicro-Kjedahl digestion (Institute of Soil Science, Chinese Academy of Science 1978) with a mixture of H_2SO_4, K_2SO_4, $CuSO_4$ and Se. Following digestion with a mixture of HNO_3 and HCl (1:3), total P was determined using a molybdenum colorimetric method and K, calcium (Ca), magnesium (Mg), and minor elements: iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), lead (Pb), cobalt (Co), nickel (Ni), and cadmium (Cd) within the harvest residue were measured using atomic absorption spectrophotometer mode AA-7000 (Shimadzu Corp., Nakagyo-ku, Kyoto, Japan)

Data Analysis

To determine patterns of resources and nutrients in soil, analysis of variance (ANOVA) (aov in R 3.0.2; R Development Core Team, 2013) with site, treatment, and plot (nested with treatment) as independent variables and soil chemistry parameters as the depend variables was performed. Significance was assessed using Tukey Honest Significant Difference test (TukeyHSD in R 3.0.2; R Development Core Team, 2013) at 5% significance level. In all cases, the three plots per treatment were used as replicate experimental units.

Forward and reverse sequences were aligned and quality-trimmed in CodonCode Aligner 4.2.4 (CodonCode, Co., Centerville, MA, USA). Sequence identity was determined using the Basic Local Alignment Search Tool algorithm (BLAST; Altschul *et al*., 1990) in databases associated with the National Center for Biological Information (NCBI) and User-friendly Nordic ITS Ectomycorrhiza (UNITE; Abarenkov *et al*., 2010). Fungi were identified to species if their ITS sequences match a named sporocarp or voucher specimen with at least 97% sequence similarity over at least 450 base pairs with an 80% query coverage.

Molecular data were also assembled to identify unknown operational taxonomic unites (OTUs). Sequences were first assembled using Codoncode Aligner 4.2.4 (Codoncode, Co., Centerville, MA, USA). Original sequences, plus sequences downloaded from GenBank, were aligned using MUSCLE (Edgar, 2004) with additional manual adjustment to the alignment performed in Mesquite 2.75 (Maddison & Maddison, 2011). Maximun likelihood and bootstrapping analyses were performed on each dataset using RAxML (Stamatakis, 2006) using the default parameters as implemented on the CIPRES web portal (http://www.phylo.org/portal2) (Miller *et al.*, 2009), with bootstrap statistics calculated from 1000 bootstrap replicates. This phylogenetic analysis was conservative and necessary since not all ECM root tips sampled produce reliable molecular iden-

tifications because of the many unknown species and lack of BLAST match in GeneBank.

Chao2, jackknife2, Abundance-based Coverage Estimator (ACE), and Michealis-Menten (MM Means) estimators of total expected OTU richness as well as Shannon-Wiener (*H'*) and Simpson's diversity (1/*D*) indices were calculated for ECM OTU diversity in each treatment using Estimate S (Colwell, 2009; version 9.1.0). Differences in ECM fungal OTU assemblages on pine roots under different fertilization regimes were visualized using non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities. Shepard plots were constructed to test for goodness of fit of the NMDS and then compared using non-parametric MANOVA (functions in the package vegan 1.17-8 in R 3.0.2). Spearman's correlation test (cor.test in R 3.0.2; R Development Core Team, 2013) was used to test for significant correlations between axis scores and each soil nutrients. Significantly correlative elements were combined and then presented in NMDS plot as environmental variables.

To assess variation in enzyme activities among treatments, I tested each variable with a fixed-factor (Treatment × morphotype) two-way ANOVA, followed by Tukey's HSD at 5% significance level to determine if there were significant differences between mean activity among treatments. To meet normality assumptions, the raw root tip activity data of all enzymes were log transferred. Boxplot was applied to show differences of enzyme activities among treatments per morphotype. The potential enzyme activity of individual root tips was also used to calculate the proportional contribution to community enzyme activity when abundance and enzymatic activity were considered together (as in Walker *et al.* 2014). This value was derived by dividing the total activities of root tips per treatment by the total community's potential enzyme activity.

Results

Soil nutrients

The results of soil analyses are summarized in Table 1. Though the differences in total N and available N were not significant among plots, the high N deposition plots showed higher levels of total and available N in the soil. Organic matter decreased in low N plots but increased under high N deposition, thus low N plots and high N plots show a significant difference ($P \leq 0.05$). There was significant higher total K and available P in low N plots than control and high N plots, but total P did not vary significantly among all the plots. Surprisingly, available K in high N plots was significantly lower than in control and low N plots. In all cases, other soil element concentrations vary among different treatments. Mg, Fe, Mn, Zn, and Co was higher in N deposition plots, while Ca, Cu, Cd, Pb, and Ni showed insignificant variation among plots.

ECM fungal community structure

From the 742 root tips collected, 318 root tips were randomly selected depending on morphotype per focal tree. One hundred and forty-one of these yielded a PCR product of sufficient quality for sequencing. Fifty-nine of the weak PCR products were successfully cloned and yielded 222 successfully M13F one-side sequences. Two hundred and fifty-seven of the sequences representing 21 OTUs were identified as ECM fungal species (Table 2 and Fig S1, S2), with 14 species present on more than one tree. Three of the four unidentified Helotiales species were resolved in clades with samples collected from Hunan and Jiangxi province during other studies (e.g. Huang *et al*., 2012), with samples of Helotiales sp.2 resolved as a unique clade (Fig S1). *Russula* sp.1, *Russula* sp.2 and *Lactarius* sp. are resolved as unique clades and are not clustered with any known species or environmental samples from GenBank (Fig S2). Many species, such as Atheli-

Table 1 Rhizosphere soil characteristics in different N treatments.

Soil properties	Treatments[1]		
	Control (n=36)	Low N (n=36)	High N (n=36)
Organic matter[2] (g kg^{-1})	26.19 ± 1.83 ab	20.89 ± 1.75 a	28.27 ± 1.78 b
Total N (mg kg^{-1})	1337.23 ± 71.44	1259.11 ± 63.56	1478.82 ± 113.63
Total P (mg kg^{-1})	109.54 ± 3.63	122.29 ± 4.53	115.30 ± 4.38
Total K (mg kg^{-1})	2309.87 ± 130.09 a	3359.07 ± 156.87 b	2664.21 ± 116.96 a
Available N (mg kg^{-1})	25.67 ± 1.96	24.86 ± 1.29	26.6 ± 1.73
Available P (mg kg^{-1})	3.49 ± 0.13 a	4.62 ± 0.16 b	3.04 ± 0.13 a
Available K (mg kg^{-1})	69.53 ± 6.75 a	64.25 ± 2.71 a	41.31 ± 1.79 b
Total metals (mg kg^{-1})			
Ca	0.19 ± 0.02	0.26 ± 0.05	0.21 ± 0.02
Mg	0.90 ± 0.02 a	1.05 ± 0.02 b	0.98 ± 0.02 b
Cu	84.92 ± 6.90	79.97 ± 18.60	68.41 ± 7.67
Fe	15986.24 ± 266.67 a	16272.82 ± 140.03 a	17034.31 ± 169.30 b
Mn	79.52 ± 7.00 a	100.82 ± 6.04 b	113.91 ± 5.76 b
Zn	52.32 ± 1.20 a	59.96 ± 1.84 b	56.00 ± 1.38 ab
Cd	1.67 ± 0.09	1.57 ± 0.12	1.48 ± 0.07
Pb	35.11 ± 1.35	41.56 ± 1.86	37.34 ± 1.82
Ni	42.30 ± 2.72	40.82 ± 5.50	51.70 ± 6.08
Co	5.00 ± 0.53 a	5.04 ± 0.55 a	7.31 ± 0.68 b

[1]Soil samples were collected from each soil core per tree per plot per treatments (n=36, *N*=108)

[2]Mean values ± SE are presented for all variables, Lower case letters denote a significant difference at $P \leq 0.05$ derived from Tukey's honestly significant difference (HSD) tests of the interaction terms.

Table 2 Identification of ectomycorrhizal fungal OTUs associated with *P. elliottii* growing at the study site in Hunan botanic garden, China.

OTUs	Closest Blast match accession in Genbank[1]	Morphotype[2]	Query/aligned portion length (bp) (similarity, %)[3]	Frequency in each treatment[4]		
				Control	Low N	High N
Atheliaceae sp.1	KF007260 Uncultured *Tylospora*	1,2,5,6,10,15	636/640 (99)	2	1	3
Atheliaceae sp.2	AB769886 Uncultured Atheliaceae	2	475/512 (93)	0	1	0
Cenococcum geophilum	JQ347051 Uncultured *Cenococcum*	1,5,10,14	491/495 (99)	1	2	0
Cenococcum sp.2	FJ440882 Uncultured Dothideomycetes	1,2,5	466/497 (94)	3	0	0
Helotiales sp.1	KF007259 Uncultured ectomycorrhizal fungus	1,3,5,6,8,10,11,13	537/550 (99)	4	5	6
Helotiales sp.2	AB571492 Uncultured ectomycorrhizal fungus	1,2,5,6,13	581/582 (99)	2	1	1
Helotiales sp.3	AB769894 Uncultured Helotiales	1,3,5,9,10	549/551 (99)	2	1	2
Helotiales sp.4	HM208727 Fungal sp. Shylum141	13	563/564 (99)	1	0	0
Lactarius sp.	JF975641 *Lactarius* sp. XHW-2011	1,2,13	571/574 (99)	1	1	1
Pezizomycotina sp.	JX860472 Uncultured Pezizomycotina	5,6	462/490 (94)	1	0	1
Phialocephala fortinii	KF313098 *Phialocephala* sp. YJM2013	1,5	562/564 (99)	1	0	1
Russula sp.1	AB597671 Fungal sp. JK-02M	8,13	582/584 (99)	0	1	1
Russula sp.2	AB597671 Fungal sp. JK-02M	13	500/542 (92)	0	1	0
Russula sp.3	EU819437 *Russula virescens*	13	687/745 (92)	0	1	0
Scleroderma citrinum	HM237176 *Scleroderma citrinum*	13	600/600 (100)	1	0	1
Scleroderma sp.2	AB769913 Uncultured *Scleroderma*	2	561/569 (99)	0	0	1
Sebacina sp.	GU328531 Uncultured Basidiomycota	10	628/657 (96)	0	0	1
Thelephora sp.1	GQ240910 Uncultured *Thelephora*	6,11	619/624 (99)	1	1	0

Table 2 *Continued*

OTUs	Closest Blast match accession in Genbank	Morphotype	Query/aligned portion length (bp) (similarity, %)	Frequency in each treatment		
				Control	Low N	High N
Thelephoraceae sp.1	AB769927 Uncultured Thelephoraceae	5,9,12	663/665 (99)	2	1	0
Tomentella sp.1	AB769926 Uncultured Thelephoraceae	2,4,5,6,9,10	662/664 (99)	5	1	1

[1]Closest matched BLAST results with informative species and genera were used.

[2]Detailed morphotype description refers to Table 4.

[3]Similarity values were computed from the percent match between the portion of the query aligned and its reference sequence.

[4]Frequency refers to present/absent in each focal tree per treatment, totally 9 trees in each treatment.

aceae spp and Thelephoraceae spp. are resolved in clades that include samples collected from Chinese native tree (masson pine) forests. The dominant ECM fungal species included Helotiales sp.1, Atheliaceae sp.1, *Cenococcum* spp., and Thelephoraceae spp. (Fig 1). The N deposition gradient increased the relative frequency of Atheliaceae spp. and Helotiales sp.1; in contrast, Thelephoraceae spp. dramatically decreased from 11.27% in the control to 1.49% in high N plots. Some species, such as *Cenococcum* spp. only appeared in control and low N plots, while *Russula* spp. only were recovered in N addition plots.

N deposition did not significantly reduce ECM fungal richness (Table 3). The control and low N treatments had equal richness of ECM species (n=14), which is slightly higher than the high N plots (n=12). Though MM means was highest in control plots, the other three estimators of total OTU richness detected the highest richness in low N plots. Diversity of OTUs, as expressed by both Shannon's *H'* and Simpson's 1*/D*, which is influenced primarily by species evenness and dominant species respectively, was lowest in high N plots. N deposition effects were not large enough to be detected at individual plots. Compared to Huang *et al.*, 2012 study of masson pine (*Pinus massoniana* Lamb.) forest ECM community, the documented and estimated ECM fungal OTU richness of slash pine in my study was lower than that of the native tree species, except for one study site in Taoling Pb-Zn mine site (TLT). Even though many more trees were sampled in the TLT study (40 vs. 9 trees per treatment in my study), the ECM richness of native masson pine is much lower in the mine site. However, the reported Shannon's *H'* and Simpson's 1*/D*

Table 3 Estimators of OTU richness and diversity of ECM fungi in different treatments and the comparison of Huang *et al*., 2012, Masson pine (*Pinus massoniana* Lamb.) forest in Hunan province, China.

Sites	Number of trees	Observed ECM richness	Estimators of expected total species richness				Diversity indices	
			Michaelis-Menten	Chao2 ± SD	Jackknife2 ± SD	ACE ± SD	Shannon's *H'*	Simpson's 1/D
Control	9	14	20.89	14.95 ± 4.27	14.76 ± 6.15	14.73 ± 5.47	2.25	8.46
Low N	9	14	15.63	18.17 ± 9.09	16.62 ± 6.55	19.72 ± 13.54	2.1	7
High N	9	12	15.4	13.11 ± 5.01	13.47 ± 4.34	13.88 ± 5.41	2.02	6.4
HY1[1]	10	17	-	41.0 ± 22.7	35.3	-	1.8	4.5
HY2	10	13	-	37.5 ± 16.6	24.2	-	1.7	3.7
TLT	40	8	-	9.0 ± 8.1	11.9	-	1.5	3.8
TLC	24	23	-	29.3 ± 24.5	34.7	-	2.6	10.2

[1]Study sites was described in Huang *et al.,* 2012, the two sites (fragmented forest patches) located in excavated Huayuan (HY) Pb-Zn mine land, one in Taolin Pb-Zn mine tailing (TLT), and one non-polluted forest (TLT) in Linxiang City. All study sites are in Hunan province, China.

index were lower in HY1, HY2 and TLT of masson pine forest than my study of slash pine.

Non-metric multidimensional scaling of the frequency of all observed fungal OTUs (including all the ECM and non-ECMs) revealed that the fungal communities in N addition plots were more similar to each other than to the community recorded from the control plot (Fig 2, p=0.8 for non-parametric MANOVA among three treatments, performed by each plot). The environmental variables examined are included in Fig 3. Only environmental variables showing significant differences among treatments were considered; total K was considered significantly influencing the fungal community structure ($P \leq 0.05$).

Potential enzyme activity of fungal root tips

Seven hundred and forty-two root tips representing 15 morphotypes were assayed (Table 4). N treatment, morphotype and their interaction were shown to significantly influence AP and PO activity, but N treatment did not significantly influence PRO activity (Table 5). N deposition significantly suppressed AP activity, while PO activity increased significantly in high N treatment. The average PRO activity increased, but not significantly, in high N treatment (Fig 4). Morphotype 1 (relative abundance 23.8-25%) and 5 (relative abundance 19-28.1%) were the dominant morphotypes among the treatments; the relative abundance of morphotype 1, 6, 10 and 13 did not significantly vary among the treatments, while morphotype 5 and 14 were higher in low N treatment.

Other morphotypes (e.g. morphotype 3, 4, 7, 8, 12, and 15) only appeared in one or two treatments (Fig 5a). The AP activity contribution of morphotype 1 was highest in the control treatment (24.2%), while morphotypes 9 and 13 contribute 13.3% and 17.8%, respectively, even though they had low relative abundance (both 7% in control treatment); the relative abundance of morphotype 1 and 5 were close in low N plots, but the relative contribution of AP from morphotype 5 (43.6%) was higher than morphotype 1 or other morphotypes. Other morphotypes showed significant variation among different treatments: for example, morphotype 2 had its highest relative abundance in the control treatment, but its AP activity was highest in the high N treatment; morphotype 14 had high relative abundance in the low N treatment, but contribute little to AP activity in this treatment. The PO activity showed a similar trend to that seen for AP activity by most morphotypes. However, the contribution of morphotype 1 (20.4-30.3%) was close to morphotype 5 (11.7-27.8%), which differed in AP activity (14.0-24.2% vs. 15.4-43.6%). For the PRO activity, morphotypes 5 and 6 contributed more in low N treatment; even though the relative abundance of morphotype 6 did not significantly vary among treatments, the relative contributions of all the enzyme activities from morphotype 6 were high in low nitrogen treatments. To our surprise, the PRO activity contributions of morphotypes 9 and 10 dramatically increased in high N treatment, but showed lower activity in the other two enzyme activities compared to other morphotypes (Fig 5b-d). N deposition generally increased activities of enzyme associated with the breakdown of plant cell components. PO activity of morphotypes 1, 2, 11, 12, and 14 significantly increased in high N level, contributing 52.9% for the whole fungal commu-

Table 4 Morphotype descriptions of collected fungal root tips associated with *P. elliottii* growing at the study sites in Hunan botanic garden, China.

No.	Color	Surface texture	Matntle coverage[1]	Branch pattern	Exploration type[2]	Hydrophobicity[3]
1	Dark brown	Matte	Med-partial	Alternate	Cont/short/med	hi
2	Dark brown	Fuzzy	Thin-full	Alternate	Cont/short	hi
3	Black	Matte-cystidia	Thick-full	Alternate	Cont	hi
4	Light brown	Shiney	Thin-partial	Alternate	Cont	hi
5	Black	Matte	Thin-full	Branched	Cont/short	hi
6	Dark yellow	Fuzzy/glittery	Thin-full	Branched	Med-fringe	ho
7	Light brown	Matte/shiney	Thin-full	Alternate	Cont	hi
8	Yellowish	Fuzzy	Thick-full	Branched	Cont/short	hi
9	Brown	Shinny	Thin-partial	Branched	Cont	hi
10	Dark brown	Glittery	Thick-full	Alternate	Cont	hi
11	Dark brown	Glittery	Thick-full	Alternate	Med-smooth	hi
12	Brownish	Shiney	Thick-full	Branched	Cont	hi
13	White	Glittery	Thin-full	Branched	Cont	hi
14	Black	Matte	Thin-full	Alternate	Cont/short	hi
15	Brownish	Shiney	Thin-full	Alternate	Short	hi

[1]Mantle thickness: Med – medium thickness; coverage area: full – 100% coverage of fungal hyphae, partial – 50-75% coverage of fungal hyphae.

[2,3]Exporation types and hydrophobicity (ho) or hydrophilicity (hi) references and exploration types are from Agerer 2001 or 2006; for exploration types, cont – contact, short – short-distance, med – medium-distance; for sub-type, smooth – smooth subtype, fringe – fringe subtype.

Table 5 One- and two-way ANOVA analyses of dependent variables effects on enzyme activities.

Factors	Acid phosphatase		Polyphenol oxi-dase		Protease	
	F	*P-value*	*F*	*P-value*	*F*	*P-value*
N treatment	10.80	P<0.001	18.30	P<0.001	2.68	0.07, ns[1]
Morphotype	5.64	P<0.001	4.53	P<0.001	6.60	P<0.001
Treatment × morphotype	11.36	P<0.001	5.00	P<0.001	5.48	P<0.001

[1]ns, not significant.

nity enzyme activity; only morphotype 10 was suppressed by N addition (Fig 6b). AP activities of morphotype 1, 6, 9, and 13 were significantly suppressed by N addition, while morphotypes 2 and 14 significantly increased in high N treatment. The activities of morphotypes 5 and 10 did not vary among the treatments (Fig 6a). High N level significantly suppressed the PRO activities in morphotypes 2, 11, and 14, but stimulated the activities of morphotypes 9, 10 and 12; in addition, the most abundant morphotypes, 1 and 5, showed the same trend in that PRO activity did not significantly vary among treatments (Fig 6c).

Discussion

Soil nutrients

Increased N deposition resulted in significant variation in organic matter, total K, and available P and K to a depth of at least 10 cm in the mineral soil, but not in total or available soil N (Table 1). While soil in the high N plots contained more N than in the low N addition and control plots, we had expected to see larger differences in the total N and available N along the gradient of N treatments, similar to those noted in previous studies of N gradients (Lilleskov *et al.*, 2001, 2002a; Jones *et al*., 2012). As Fang *et al*. (2005) reported, high background N in the soil before N fertilization occurred might cause the soil to reach N saturation and become acidified. This possible explanation was supported by the large increase in metals such as Zn, Co, Mn and Fe in my study (Table 1). Some taxa may be sensitive to acidification effects. For example,

Amphinema byssoides and dark-mantled *Tomentella* species were reported to respond positively to liming but decline with increasing N inputs (Antibus & Linkens, 1992; Taylor & Brand, 1992; Veerkamp *et al*., 1997). Furthermore, *A. byssoides* is often found colonizing nursery seedlings (Danielson & Visser, 1990; Grogan *et al.*, 1994). This suggests that the intolerance of certain ECM to soil acidification or nutrient imbalance, rather than nitrogen availability *per se*, may have led to some of the patterns observed in the present study. The dramatic decline of *Tomentel-la* sp.1 observed in this studies N input plots match trends reported in previous studies (citation?).

Soil organic matter showed a similar trend to that reported by Frey *et al*. (2004) and Jones *et al*. (2012). Plants growing under increased nitrogen conditions will likely display a large increase in leaf production (relative to root growth) or understory plant abundance. This provides more leaf litter available for decomposition. If the leaf litter is also higher in N after N fertilization, then the decomposition rates will be faster, which would result in higher bacterial/fungal activity, and an increase in organic matter. Similar trends were reported in Lilleskov *et al.* (2001, 2002a) where high N deposition was associated with a significant increase of tree growth rates and un-derstory grass relative abundance.

My results show documented a significant increase in available P in low N (4.64 mg kg^{-1}) but a slight decrease in high N plots (3.04 mg kg^{-1}) (Table 1). This trend was also reported by Jones *et al.* (2012) whereby persistent increased N deposition resulted in an increase in available P in both fermentation-humic and mineral layer soils. The observed decrease in soil K levels in my

study, especially in high N plots (41.31 mg kg^{-1} in high vs. 69.53 mg kg^{-1} in control), is similar to the results reported by Carfrae *et al.* (2006). These authors reported that available K slightly decreased in both N addition and N-sulfur (S) addition treatments. Few studies have focused on changes in concentration of minerals such as Ca, Mg, Cu, Fe Mn, Zn, Cd, and Pb over N deposition gradients. However, some metals, such as Mn availability have been shown to increase as soils become acidified, and then decrease as prolonged acidification leaches Mn from the soil (Ulrich, 1995). In my study, I found that Mg, Mn and Zn were significantly higher with increasing N availability, but that other minerals showed no significant variation (Table 1). It is possible that the increase in soil metal concentration with increasing N fertilization may have influenced the ECM community composition and enzyme activity. Some studies investigating the effect of heavy metals on ECM fungi have shown strong negative effects of metals on ECM communities (Staudenrausch *et al*., 2005; Ruotsalainen *et al.*, 2009), but not always. For example Hui *et al.*, (2011) and Huang *et al*. (2012) indicated that elevated heavy metal concentrations in the soil may not be determining factors in the structure of ECM communities, but that soil maturity (e.g. available nutrients level, humidity and aggregate) could be much more pivotal. However, soil metal concentrations were not significant vectors explaining the dispersal of ECM communities in NMDS space (Fig 3) suggesting that the effect of metal concentration may be less important than other soil factors, such as N and K in this study. Compared to Huang *et al*., 2012, total K (10,590.3~13,649.5 mg kg-1) and total P (302.1~784.1 mg kg^{-1}) were much higher in HY and TLC than in my study (total K: 2,309.9~3359.1 mg kg^{-1}; total P: 109.5~122.3 mg kg^{-1}). In the

TLT mine site, the total soil K (8216.3 mg kg^{-1}) and total P (114.0 mg kg^{-1}) were close to my study, ant that site had lower ECM richness than the non-mine HY and TLC sites (Table 1).

In summary of this section, my results mostly agreed with results in other studies to suggest that N deposition can have persistent effects on soil solution chemistry at depth, with potential negative effects on colonization by, and species composition of, ECM fungi.

Effects of N deposition on ECM fungal communities

Twenty-one OTUs were identified as ECM species in my study, of which 14 species were reported from control and low N plots vs. 12 species in high N plots (Table 2, 3). The results support previous reports that ECM fungal communities are less species-rich and differ in structure and composition under elevated N deposition. Data from my low N plots documented changes in relative frequency rather than major community shifts similar to some previous studies (e.g. Kårén & Nylund, 1997; Jonsson *et al.*, 2000; Peter *et al.*, 2001). For high N plots, the effects were consistent with other studies (e.g. Fransson *et al.*, 2000; Peter *et al.*, 2001; Lilleskov *et al.*, 2002a; Frey *et al.*, 2004) in that high N input reduced ECM fungal richness and diversity.

The mycorrhizal community in my study was dominated by species that do not form conspicuous sporocarps. Helotiales sp.1 colonized 23% of the root tips sampled from plots while Thelephoraceae spp. colonized almost 20% of the root tips. N deposition increased the relative frequency of Helotiales sp. 1. In contrast, the relative frequency of Thelephoraceae spp. was highly sup-

pressed by N addition (11.27% relative frequency in control vs. 1.5% in high N plots) (Fig 1). This result was consistent with Lilleskov *et al.* (2002a), which reported that rough-mantled Thelephoroid (probably all *Tomentella*) species declined or disappeared with increasing N, especially *Tomentella subliacina*, which peaked at moderate N levels (80 mg N·kg soil^{-1}·28d^{-1}) and dramatically decreased at high N level (160 mg N·kg soil^{-1}·28d^{-1}). By contrast, Peter *et al.* (2001) found that the frequency of Thelephoraceae and Corticiaceae increased in their N-plots from 46.0% ± 1.6% in 1997 to 57.2% ± 3.5% in 1999 compared to 36.5-38.0% in their control-plot. *Cenococcum* was the most frequent and abundant ECM taxon in many studies and apparently has a high fitness or competitive ability relative to other ECM fungal species (Fransson *et al*., 2000; Jonsson *et al.*, 2000; Lilleskov *et al*., 2011). However, in my study *Cenococcum* spp. was only recorded from control and low N plots, and was absent in high N plots, similar to the pattern reported by Lilleskov *et al.* (2002a) and Berch *et al*. (2006). Since *C. geophilum* is known to be highly diverse genetically at small scales (e.g. LoBuglio & Taylor, 2002; Douhan & Rizzo, 2005), and even on a single tree (Bahram *et al.*, 2011), it is unclear how genetic differences versus edaphic effects explain the differences in reported *C. geophilum* responses to elevated N. *Russula* spp. were considered nitrophobic and were reported to significantly decrease under N input by Jonsson *et al*., 2000; Peter *et al*., 2001; Lilleskov *et al*. 2002a), but other *Russula* spp. seem to prefer elevated N conditions in other studies (e.g. Carfrae *et al*., 2006; Avis *et al*., 2008). For instance, Avis *et al*. (2008) found that *Russula amoenolens* and *Russula mariae* (terminal restriction fragment length polymorphism match type) are more frequent in N deposition

plots.Thus, species of *Russula* exhibit a clearly mixed response to N deposition, and taxon respsonses may be linked to fundamental nutritional or physiological shifts under different soil conditions (Lilleskov *et al*., 2011).

Increasing N effects C allocation in both partners of the symbiosis: on the one hand, C supply from the plant to the fungus may be reduced since the provision of C skeletons for N assimilation is enhanced by higher N levels in both roots and leaves (Peter *et al.*, 2001); on the other hand, a shortage of carbohydrates provided by plants for fungal growth under high N levels can negatively impact fungal growth. The ability to utilize other C sources might explain the better adaptation of some species, and saprotrophic capability of certain ECM fungi has been demonstrated (Durall *et al*., 1994; Perez-Moreno & Read, 2000), e.g., *Tylospora frillosa* was shown to have decomposing and proteolytic abilities (Cairney & Burke, 1994). Sporocarps of resupinate ECM including most of the Thelephoraceae and Corticiaceae, commonly form on litter and soil debris and on well-decayed wood, perhaps suggesting saprotrophic abilities (Erland & Taylor, 1999). In addition, given that ECM competitive outcomes appear to be environmentally context-dependent and that abiotic factors such as temperature (Erland & Finlay, 1992) and PH (Mahaood, 2003) vary considerably spatially and temporally in soil, maintenance of high richness of ECM communities can be explained by a shifting mosaic of competitive dominance.

To summarize, the variation in response of the same ECM species to N addition in different systems may be attributed to different abiotic factors in subtropical areas of China than that of Eu-

ropean and American temperate areas; alternatively, since these ecosystems differ in tree species, biotic interactions between the species *per se* and their hosts, or competitive interactions between the ECM species that differ in their response to N fertilization may be important in determining the response of species to elevated N conditions. Additional studies examining host interactions, biogeographic distribution patterns and ecological mechanism(s) responsible for ECM community shifts in response to N addition are needed.

Effects of N deposition on enzyme activities in the mycorrhizosphere

The results of the effect of elevated N on activity of the three tested enzyme systems were mixed in relation to my original hypotheses (Fig 4) and varied from expectations based on some previous studies. Increased levels of N deposition generally resulted in the suppression of AP activities. This result does not fit the findings of Jones *et al.* (2012) who reported that AP activities of three observed ECM fungal genus (*Piloderma*, *Cenococcum*, and *Suillus*) were unchanged among different N treatments. PO activity, an indicator of lignin-degrading potential, has been reported to be significantly reduced in elevated N treatment (Carreiro *et al.*, 2000; Sinsabaugh *et al*., 2002; Frey *et al*., 2004). However, my study documented a significant increase in PO activity in high N plots. Chalot *et al*. (1995) reported that high concentrations of ammonium represses PRO production and alters the ability of the fungal communities to utilize organic forms of nitrogen, but my study found no significant variation in PRO activity among different plots.

Following Agerer (2001), I grouped my observed morphotypes by exploration type (Table 4).

Taxa such as *Tricholoma*, *Cortinarius*, *Piloderma* that are classified in the medium-distance fringe category of exploration types have been shown to undergo a dramatic decline in response to N deposition. This type of ECM has a hydrophobic exploration subtype that typically involves a dense proliferation of hyphae forming loose, relatively undifferentiated rhizomorphs that ramify with high hyphae density around patches of organic matter, often in organic horizon. On the other hand, most genera with mixed or positive responses to N deposition (e.g. *Russula*, *Lactarius*, *Laccaria*, *Tylospora*, *Thelephora*, *Tomentella*) have hydrophilic exploration types that include contact, short-distance, and medium-distance smooth types, involving a lower investment in transport mycelium, and hence would be generally expected to be of lower C cost to their hosts than the more intensely rhizomorphic taxa. These patterns suggest two main ECM strategies for growth and N acquisition, one focusing on insoluble, complex organic resources, and one focusing on uptake of labile N forms such as amino acids, ammonium, and nitrate (Lilleskov *et al*., 2011).

From the enzyme activity results (Fig 5), morphotype 6, which is a hydrophobic, medium-distance mat-forming subtype, showed a highly repressed PRO activity in high N plots. This result fits the hypothesis put forward by Lilleskov *et al.* (2011) that an extensive capacity for organic N exists in this taxonomically unrelated but morphologically similar morphotype group. In contrast, the predominant morphotypes among the plots, morphotypes 1 and 5, are contact/short distance exploration types. The proteolytic ability in the soil of these two contact/short distance

exploration types was less impacted by N deposition, and appeared to drive the enzymatic activity of the entire fungal community. In addition, some morphotypes, such as morphotype 2, showed enhanced PO activity and helped moderate the total activity in the control plots, while morphotypes 9 and 10 showed steeply increased PRO activity and contributed almost 45.1% of the total PRO activity in high N plots. There are a number of possible explanations for this. First, it is possible that these that taxa included under ECM morphotype 9 and 10 specialize on distinct organic resources (e.g. organic N for the N-sensitive taxa and organic P for the N-tolerant taxa). Second, although most long-distance exploration types appear to explore for organic N, a sub-set of taxa may be adapted to high efficiency mining of P resources, suggesting that these taxa would have dramatically different enzymatic capabilities and effects on host N vs. P uptake (Lilleskov. *et al*., 2011). Third, fluctuations and temporal partitioning of enzymatic activities may result from the combination of constant changes of both the species structure of the community (Koide *et al*., 2007; Courty *et al*., 2008) and the specific activity level in each population. Such fluctuations may be driven at least in part by environmental factors such as season (Voříšková *et al*., 2014), litter quality (Aponte *et al.*, 2010; Cullings *et al*., 2010) or tree phenology (Barbaroux & Breda, 2002; Courty *et al*., 2007; Dickie *et al*., 2010). This high versatility across species may be a key characteristic for rapid adaptation of ECM communities to changing environmental conditions. This result also suggests that ECMs not only channel facilitating exchanges, but also contribute to organic nutrient cycling (as occasional saprotrophic fungi) in association with true decomposing fungi (Wu *et al*., 2005; Lindahl *et al*., 2007; Karst *et al*., 2008;

Koide *et a*l., 2008).

Although assaying enzymatic activity based on morphotype rather than identified species resulted in large variation in enzyme activities, the signal of enzyme activity was strong enough to allow us to draw conclusions on functional diversity of the ECM taxa and community. First, such enzymatic assays actually measure the total secreted potential activity of the ECM, which is a mixed organ composed of plant and fungal tissues and associated bacteria. All these components potentially contributed to the measured enzymatic activities, but the enzyme activity of the ECM is expected to comprise most of the total activity based on the nutrient foraging capacity of ECM relative to plant roots and soil bacteria (Courty *et al*., 2005). Second, some species, such as *C. geophilum* are frequently found to colonize and coexist with other ECM species (Zak & Marx, 1964). Thus it is likely that the heterogeneity of activity associated with the *Cenococcum* morphotype is due in part to superficial morphotyping and the coexistence of several symbionts in the same mycorrhiza (or sample). Third, my study found a pattern that many different species that were included in the same morphotype (black-matte, hydrophilic and contact/short exploration type) (e.g. *Tomentella*, Helotiales sp.1, *Phialocephala fortinii*, Thelephoraceae sp.1, *Cenococcum* sp.2, *C. geophilum*) had similar AP activities in control plots. Thus, the patterns elucidated in this study based on morphotypes may reasonablely be considered to correspond to the functional diversity of ECM fungi and their component species.

Although each ECM type appeared to be more or less specialized in one or more activities, activ-

ity profiles are also likely influenced by the location and horizon from which the samples were taken (Courty *et al*., 2005). Moreover, my method measured potential activities on ECM root tips only and do not take into account extraradical mycelium, which can be important for some long-distance exploration type ECM species (Agerer 2001). Talbot *et al*. (2013) also found that enzymatic activity on ECM root tips taken from the same soil cores used for bulk enzyme analysis did not correlate with the activity of any enzyme measured in the bulk soil. Further studies incorporating mycelial ingrowth bags and examining the impact on soil and ECM-associated bacterial communities and bacterial enzyme function are needed to further elucidate the impact of N deposition on enzymatic activity.

Conclusion

In the present study, I have shown that the main effect of high-level nitrogen deposition was a decrease in the standing richness and community composition of ECM fungi, whereas N addition produced variable effects on the tested enzymatic activities. The apparent functional redundancy in enzyme activities, notably protease, appeared to buffer the ECM community from negative effects of N on enzyme activity, but more work is needed to further document these findings.. Studies combining both morphotyping and species level identification plus data on the contribution of extramatricular mycelium and bacteria are needed (e.g. Griffiths *et al*., 1991; Agerer 2001; reviewed by Lilleskov *et al*., 2011).

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Zheng XH, Fu CB, Xu XK, Yan XD, Huang Y, Han SH, Hu F, Chen GX. 2002. The Asian nitrogen cycle case study. *Ambio* **31**: 79-87.

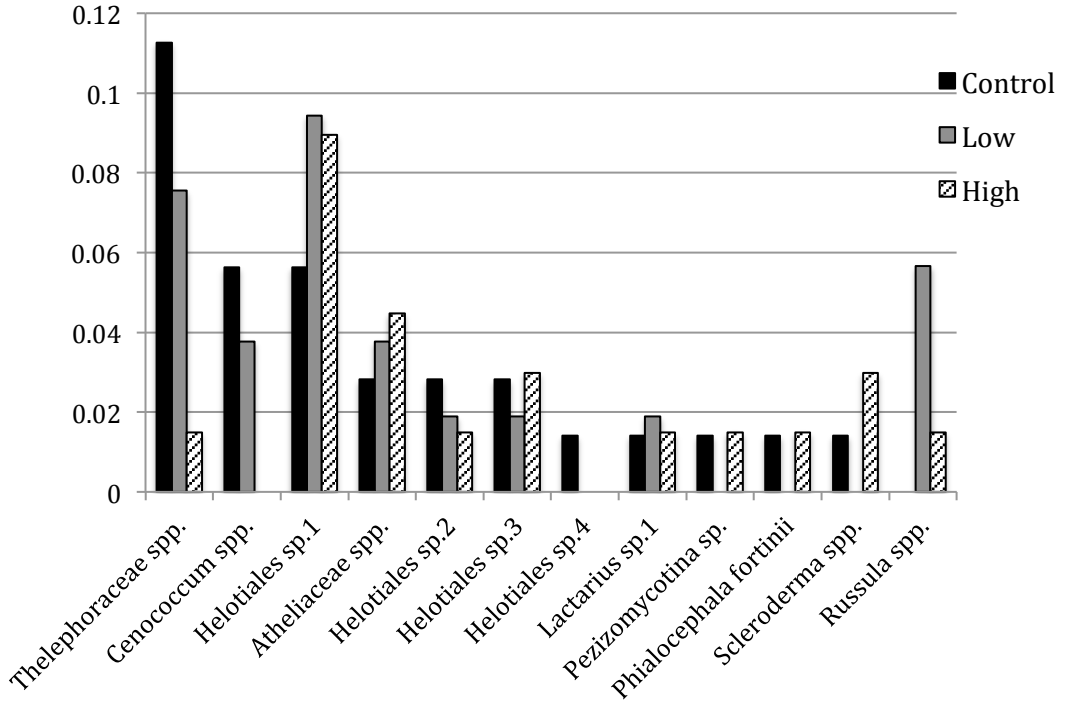


Fig.1 Relative frequency (%) of the genera and families of ectomycorrhizal fungi in different treatments.

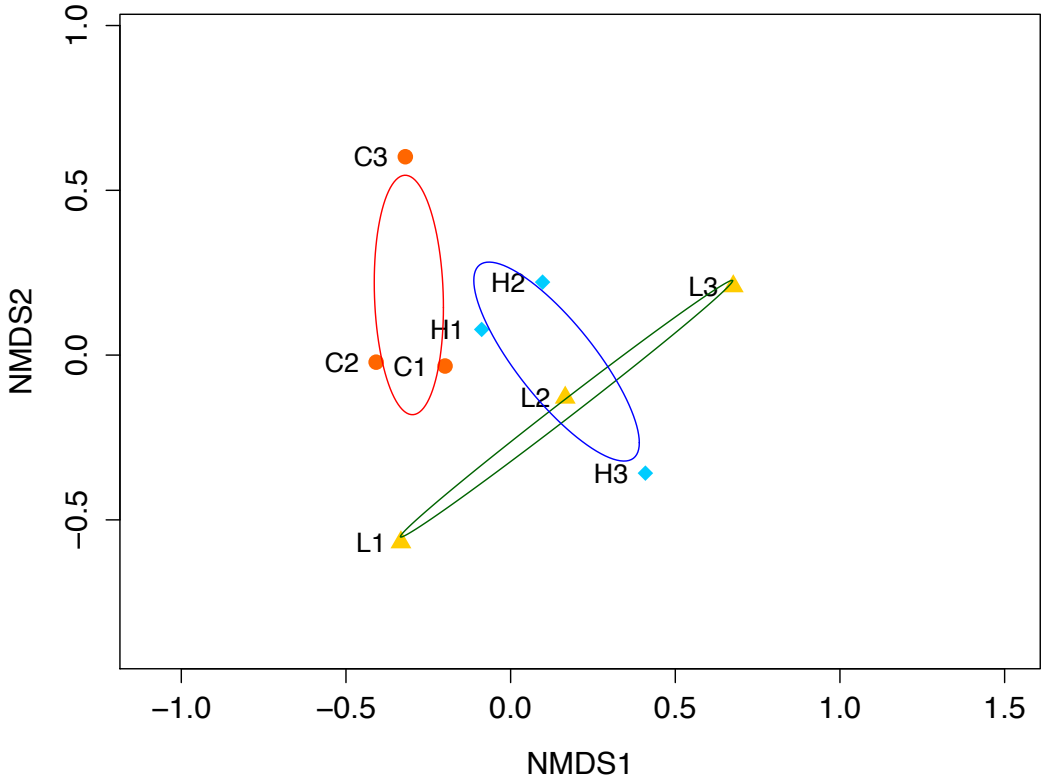


Fig.2 Non-metric multidimensional scaling ordination of fungal communities. Each point represents the fungal community composition in each plot. Circle-control; triangle-low N; diamond-high N. The circle surround data points from the same treatment. A Shepard plot found a non-metric fit with an R^2 of 0.985 and linear fit with R^2 of 0.901.

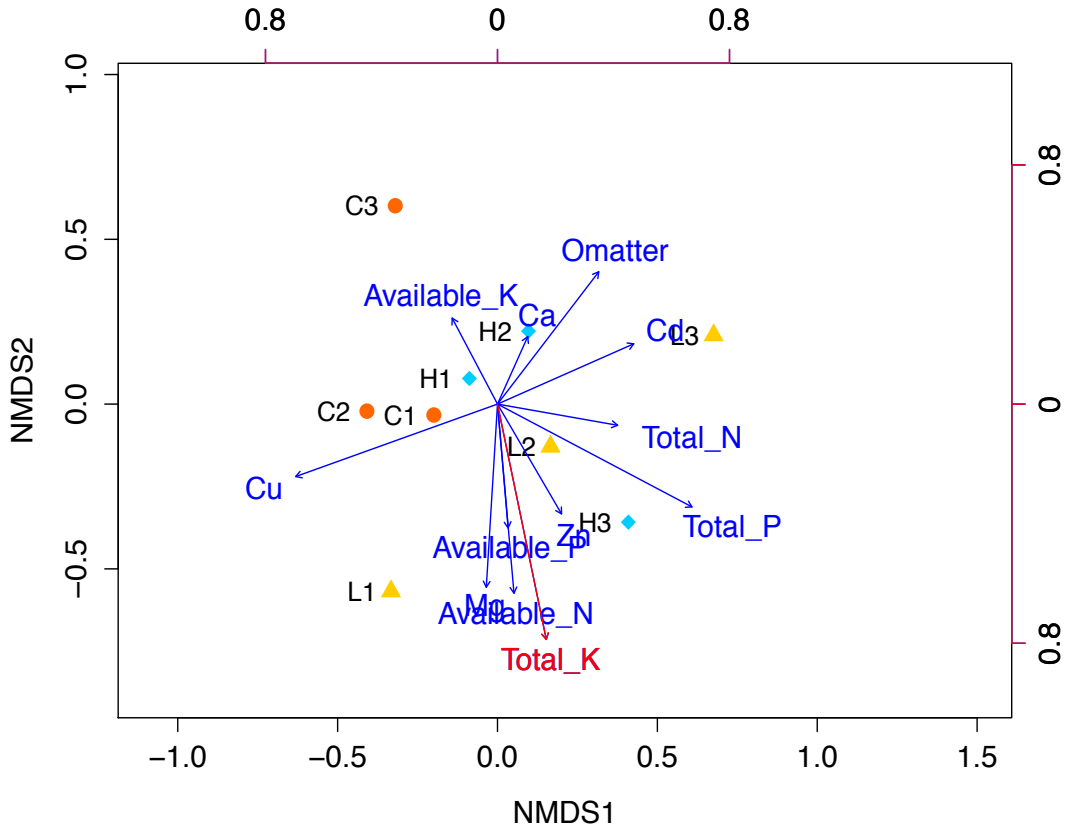
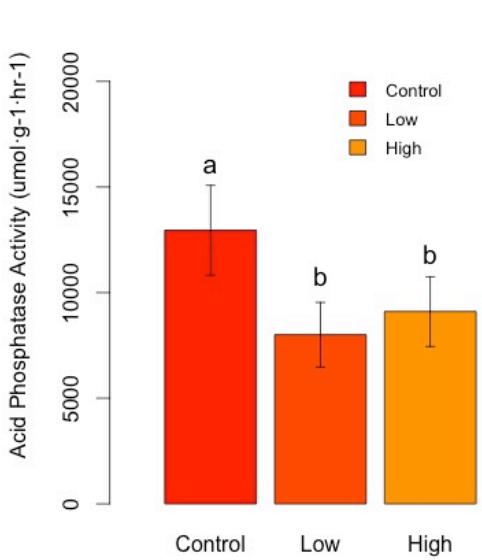
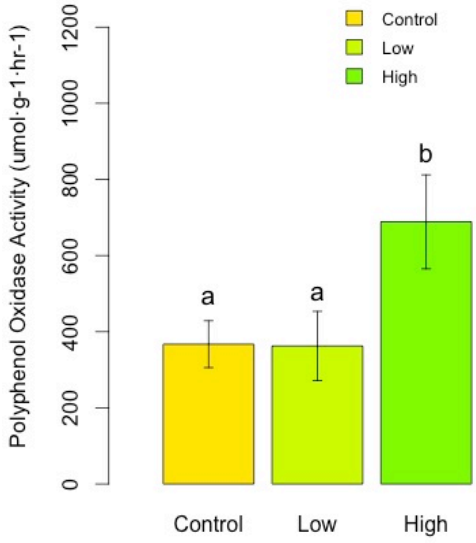


Fig.3 Non-metric multidimensional scaling ordination of fungal communities with environmental variables. Each point represents the fungal community composition in each plot. Circle-control; triangle-low N; diamond-high N. The circle surround data points from the same treatment. Only environmental variables showing significant differences among treatment were considered. Omatter-organic matter; N-nitrogen; P-phosphorus; K-potassium; Ca-calcium; Mg-magnesium; Cu-copper; Zn-zinc; Cd-cadmium. Environmental variables with 95% significant influence was targeted in red.





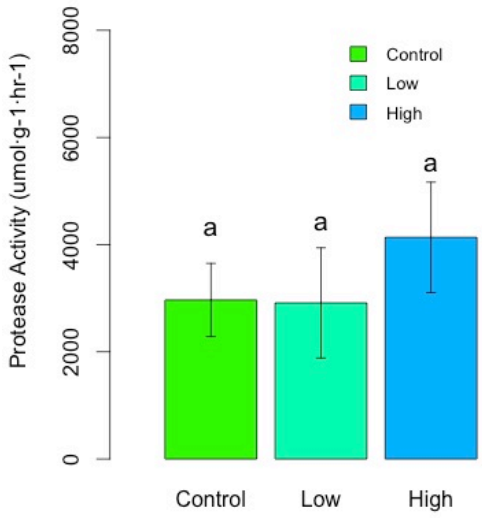


Fig.4 Potential acid phosphatase (a), polyphenol oxidase (b), and protease (c) activities of fungal communities of different treatments. Lower case letter designate significant differences at P≤0.05 based on Tukey's honestly significant difference (HSD) test. Bars represent SE.

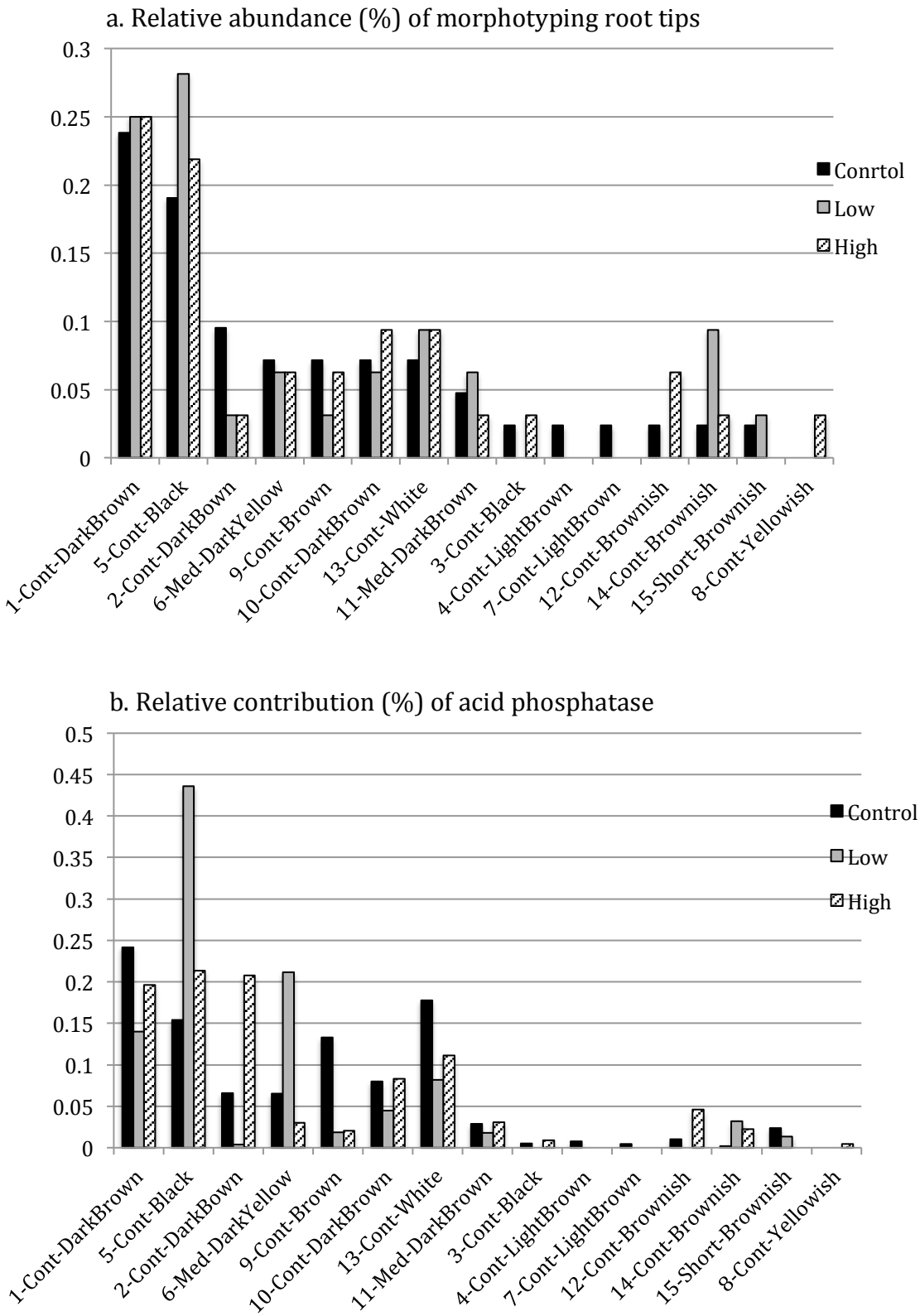
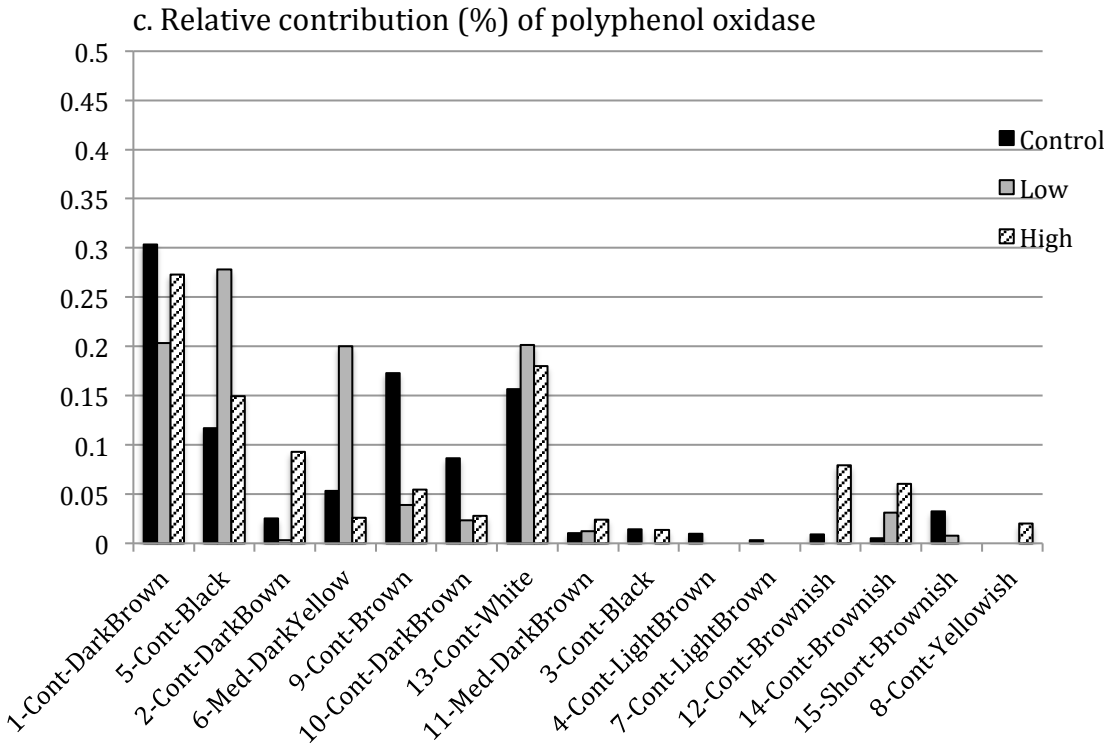


Fig.5 Relative root tip abundance (a) and the relative enzymatic contribution of three enzymes: (b) acid phosphatase (c) ployphenol oxidase (d) protease. See the Materials and Methods section for details on how each metric was calculated.



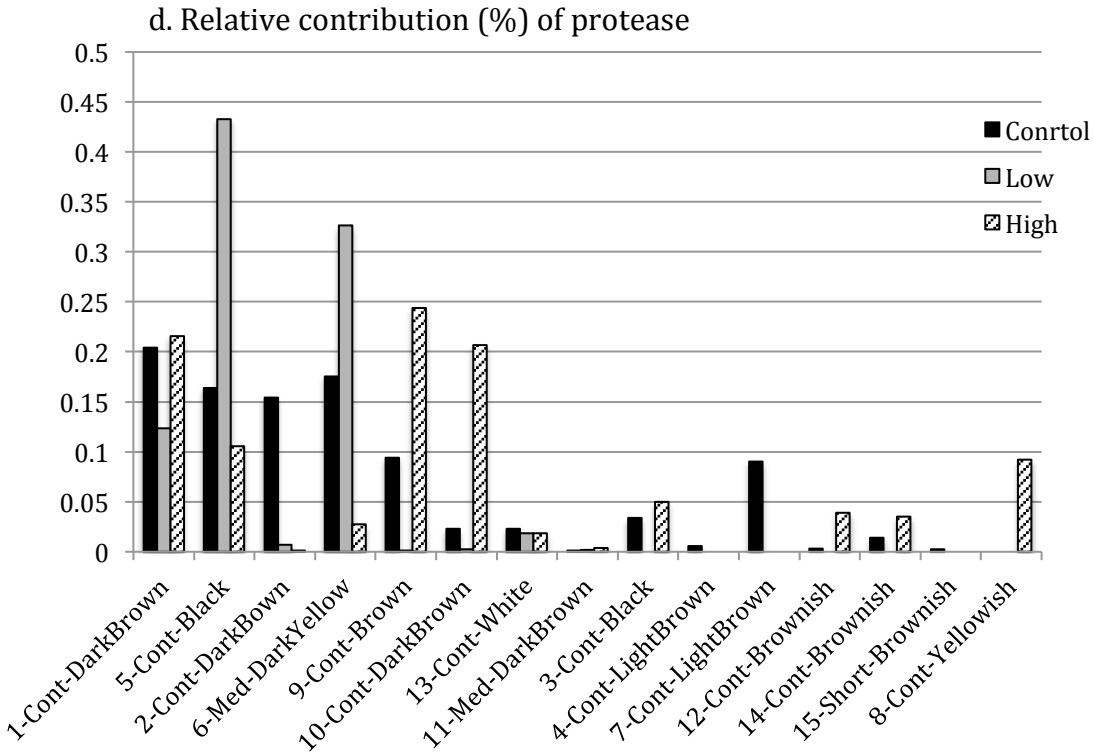
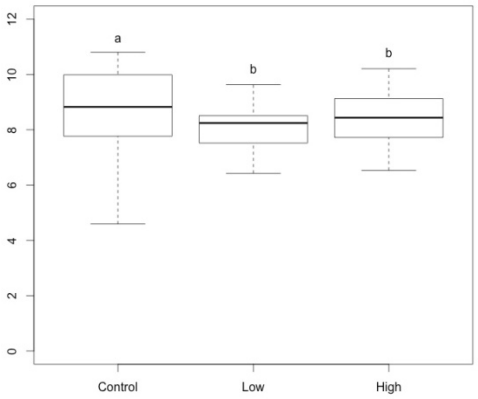


Fig 5 *Continued*

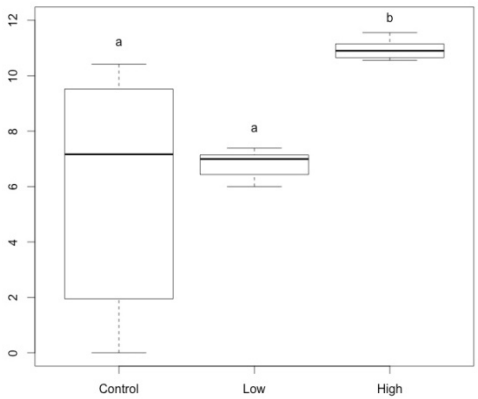
Fig.6 Potential activities of three enzymes: (a) acid phosphatase (b) polyphenol oxidase (c) protease for the morphotyping root tips. Raw data are presented in the figure, but all data were log transferred to meet assumptions of normally in statistical analyses. Lower case letter designate significant differences at $P \leq 0.05$ based on Tukey's honestly significant difference (HSD) test. Boxes surrounding median values represent the first and third quartiles, and whiskers show the smaller (and larger) of either the maximum (and minimum) values or 1.5 × the interquartile range (c. ±2SD). Morphotype 4, 7, and 8 were not shown since they just presented in only one plot.

a

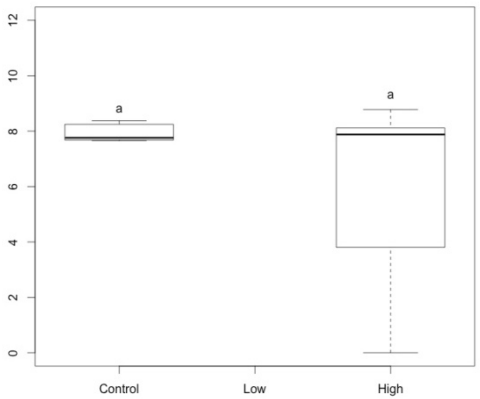
Phosphatase Morphotype 1



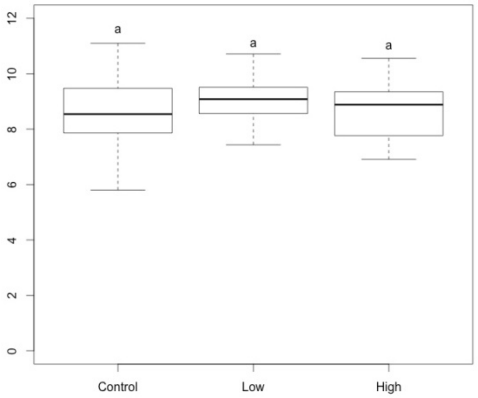
Phosphatase Morphotype 2



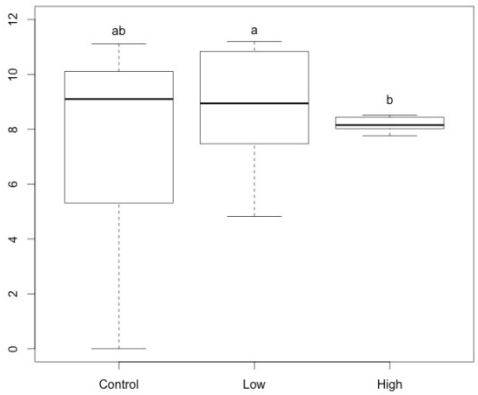
Phosphatase Morphotype 3



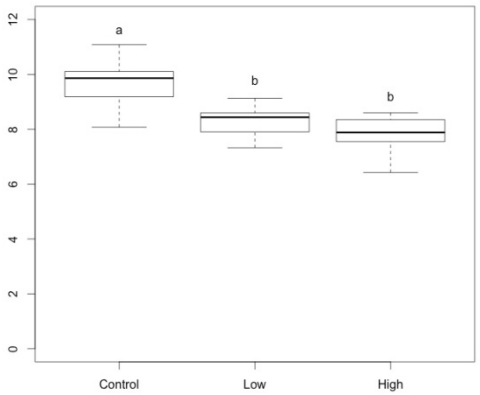
Phosphatase Morphotype 5



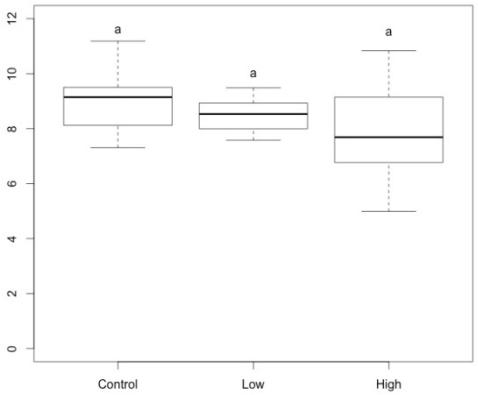
Phosphatase Morphotype 6



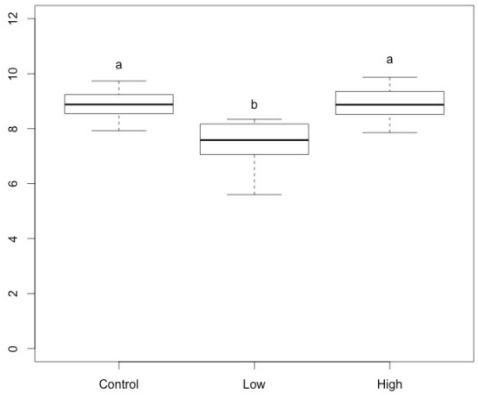
Phosphatase Morphotype 9



Phosphatase Morphotype 10



Phosphatase Morphotype 11



Phosphatase Morphotype 12

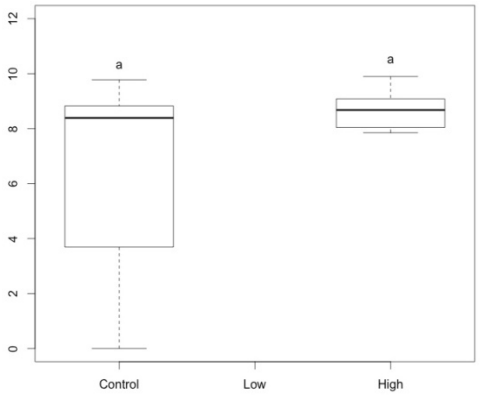
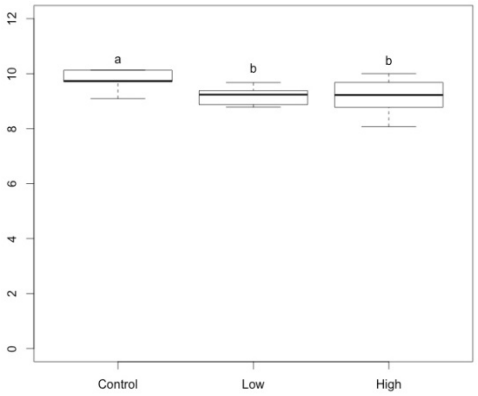
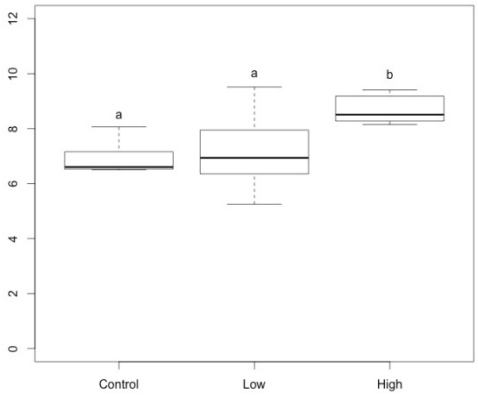


Fig.6 *Continued*

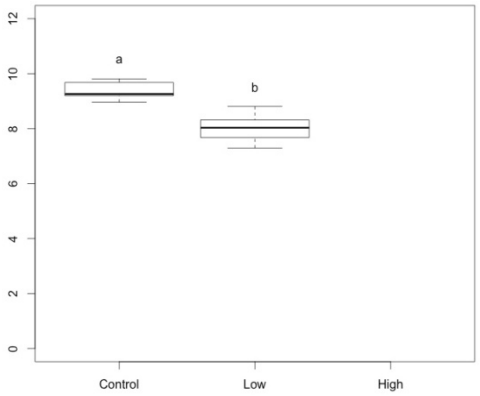
Phosphatase Morphotype 13



Phosphatase Morphotype 14



Phosphatase Morphotype 15



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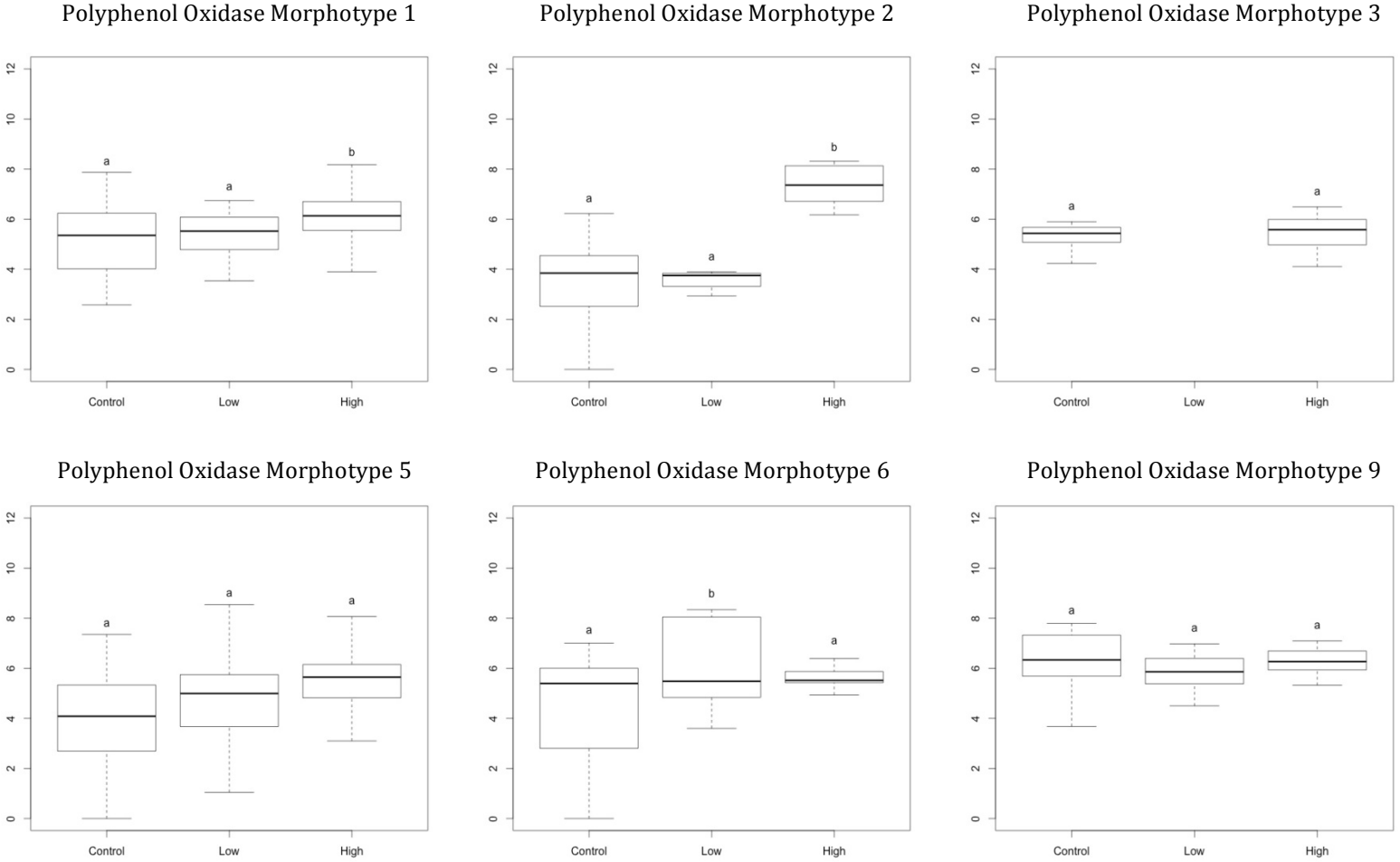
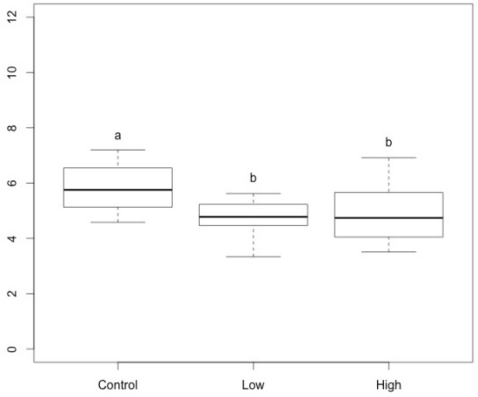


Fig.6 *Continued*

Polyphenol Oxidase Morphotype 10



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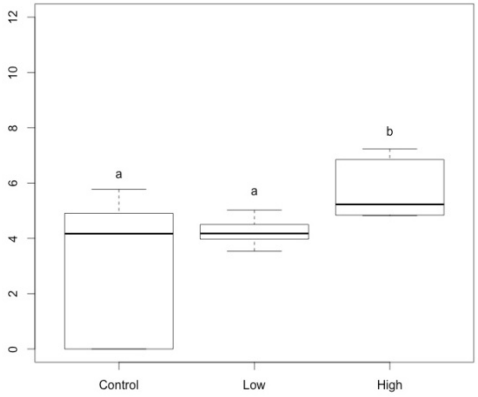

Polyphenol Oxidase Morphotype 12

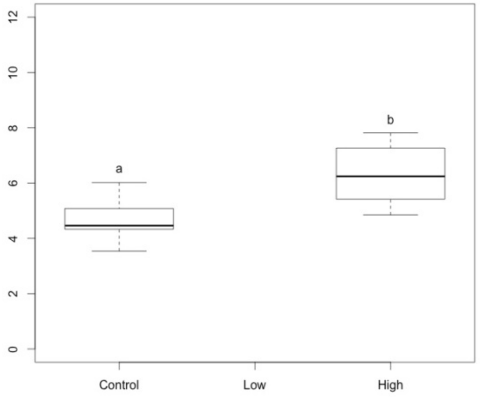

Fig.6 *Continued*

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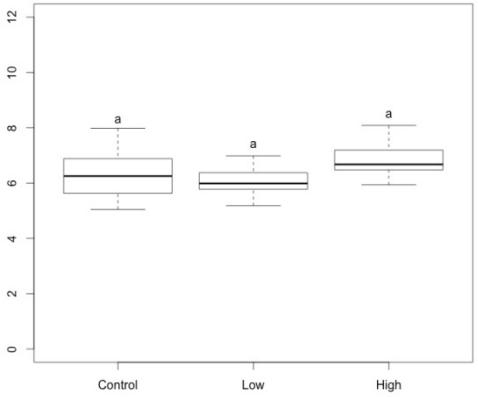

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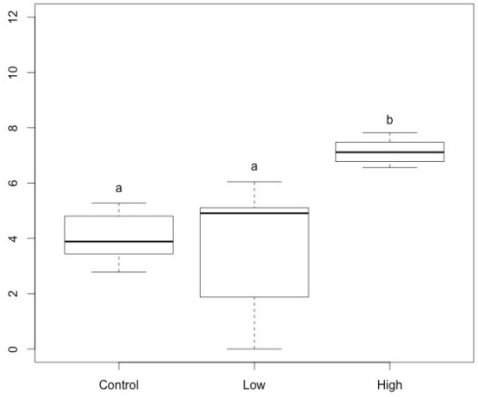

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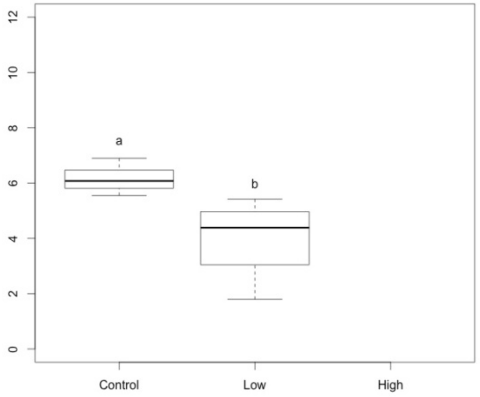

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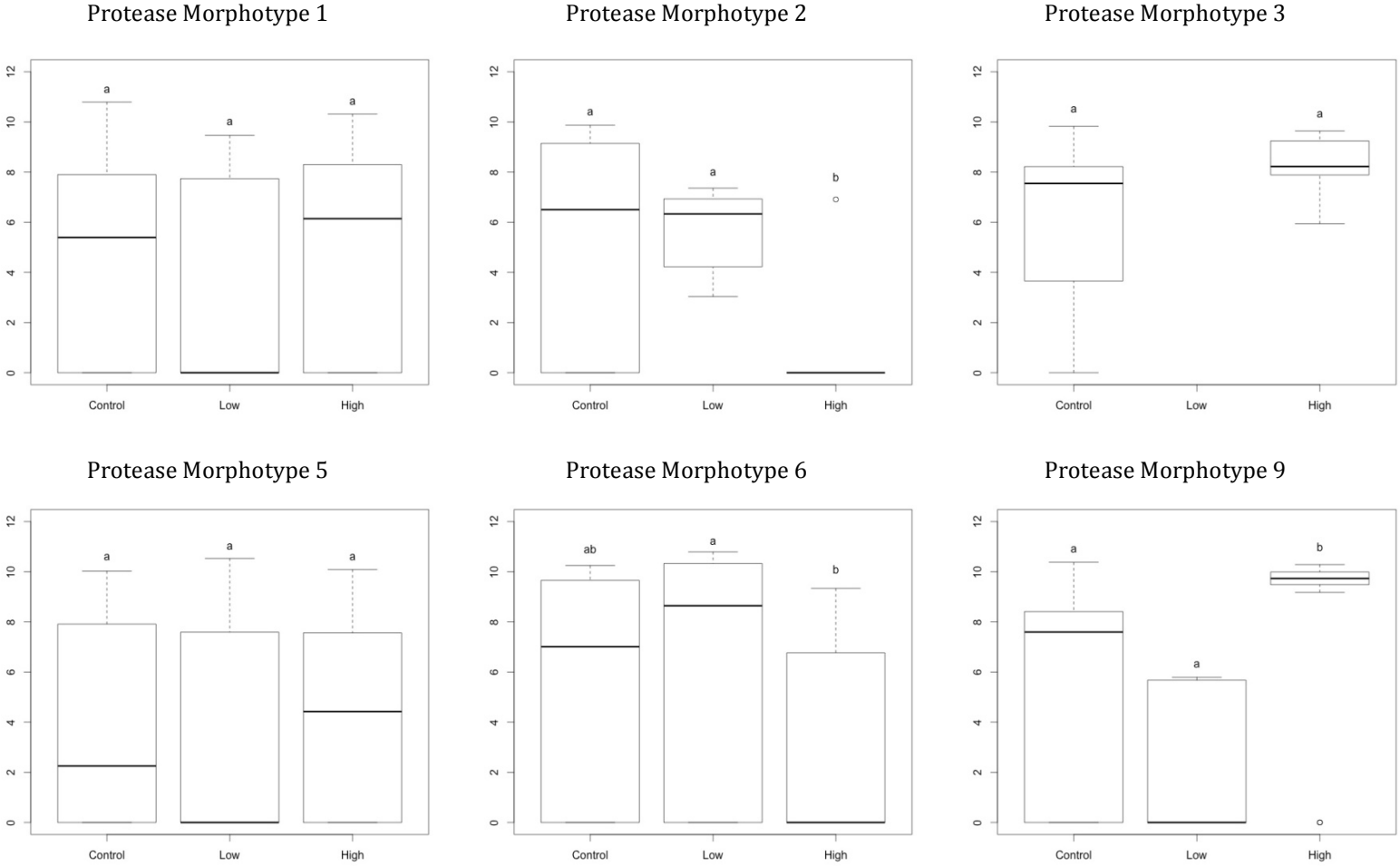

Fig.6 *Continued*

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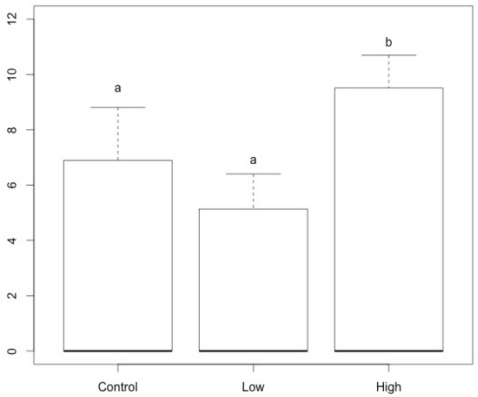

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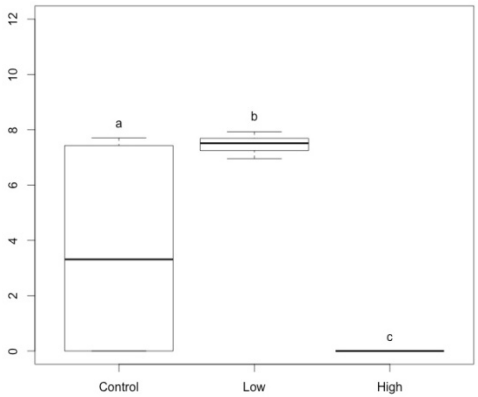

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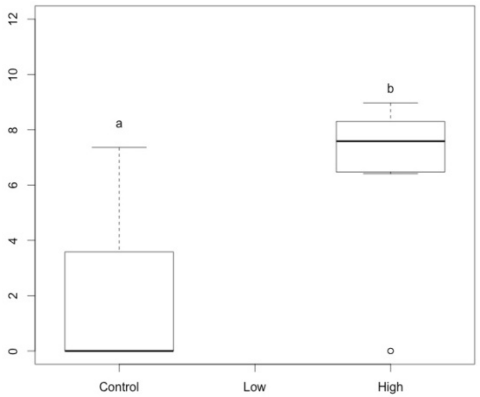

Fig.6 *Continued*

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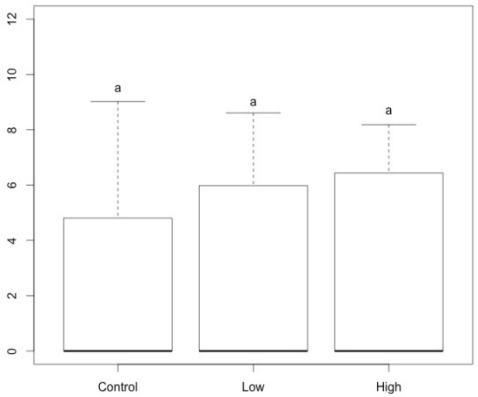

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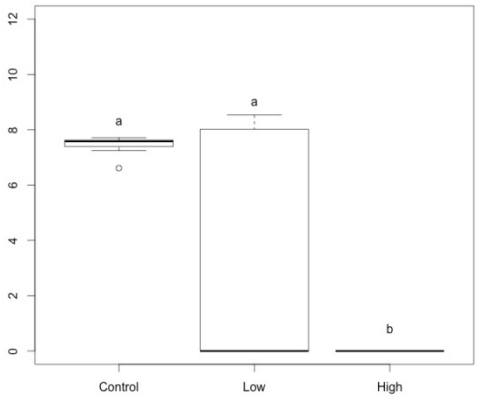

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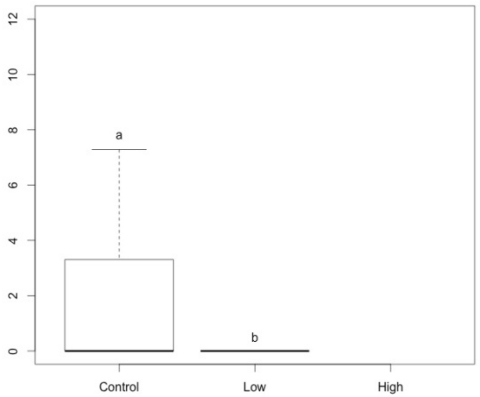

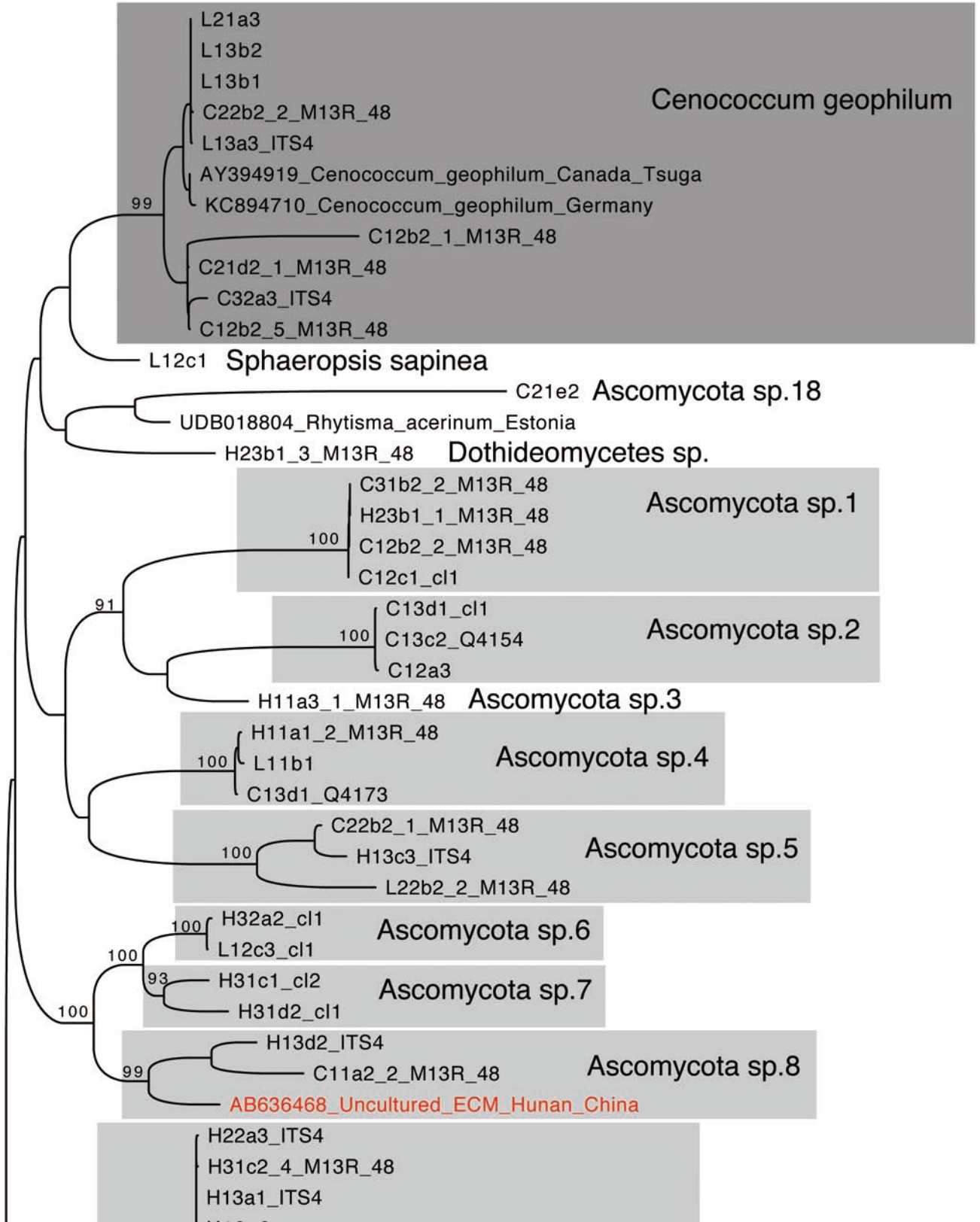
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L13b1
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Cenococcum geophilum
99
L12c1
Sphaeropsis sapinea
C21e2
Ascomycota sp.18
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Dothideomycetes sp.
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C12a3
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Ascomycota sp.2
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Ascomycota sp.4
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Ascomycota sp.5
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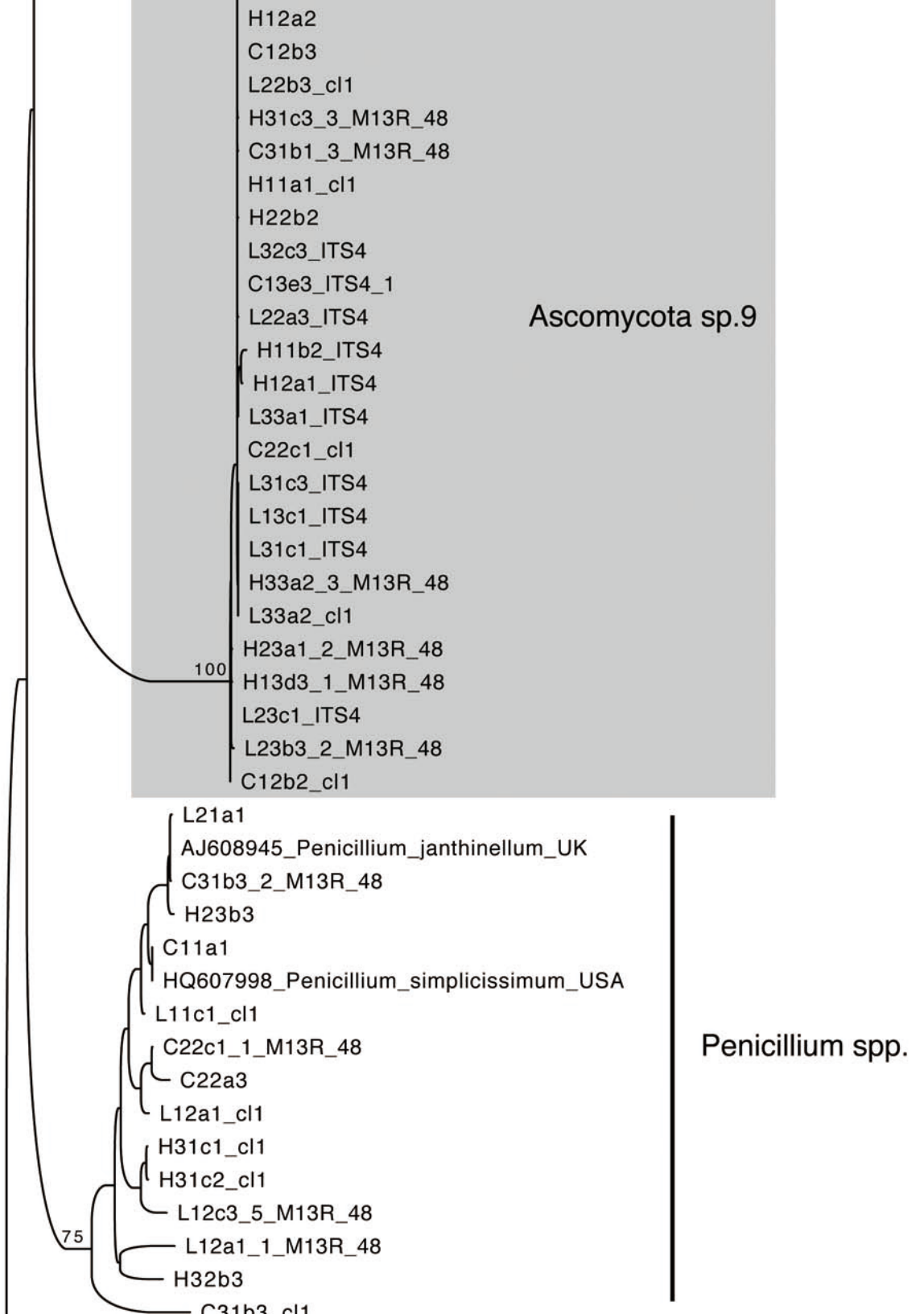
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C13e3_ITS4_1
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H11b2_ITS4
H12a1_ITS4
L33a1_ITS4
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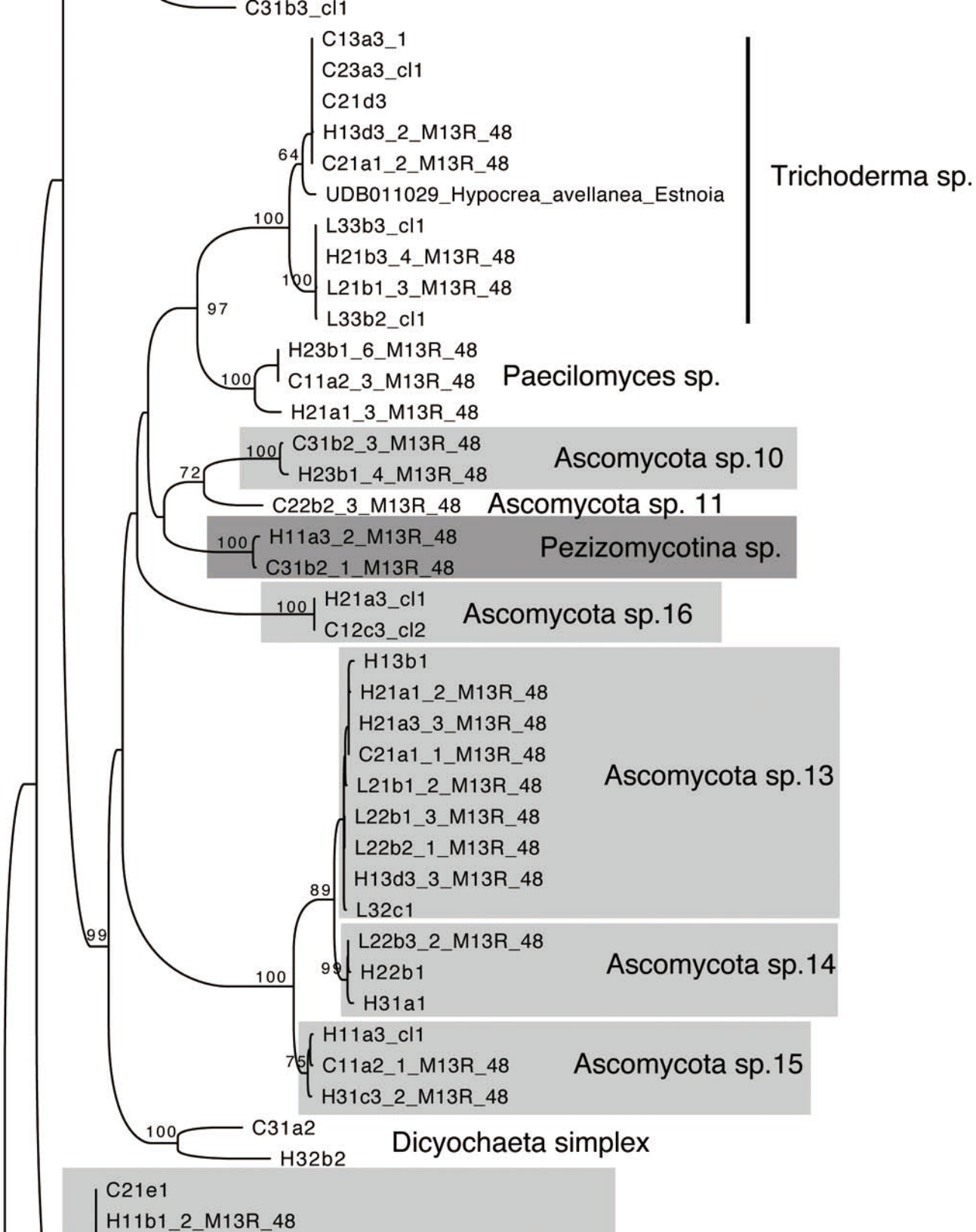
Penicillium spp.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Paecilomyces sp.
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H22b1
H31a1
Ascomycota sp.14
H11a3_cl1
C11a2_1_M13R_48
H31c3_2_M13R_48
Ascomycota sp.15
C31a2
H32b2
Dicyochaeta simplex
C21e1
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72
100
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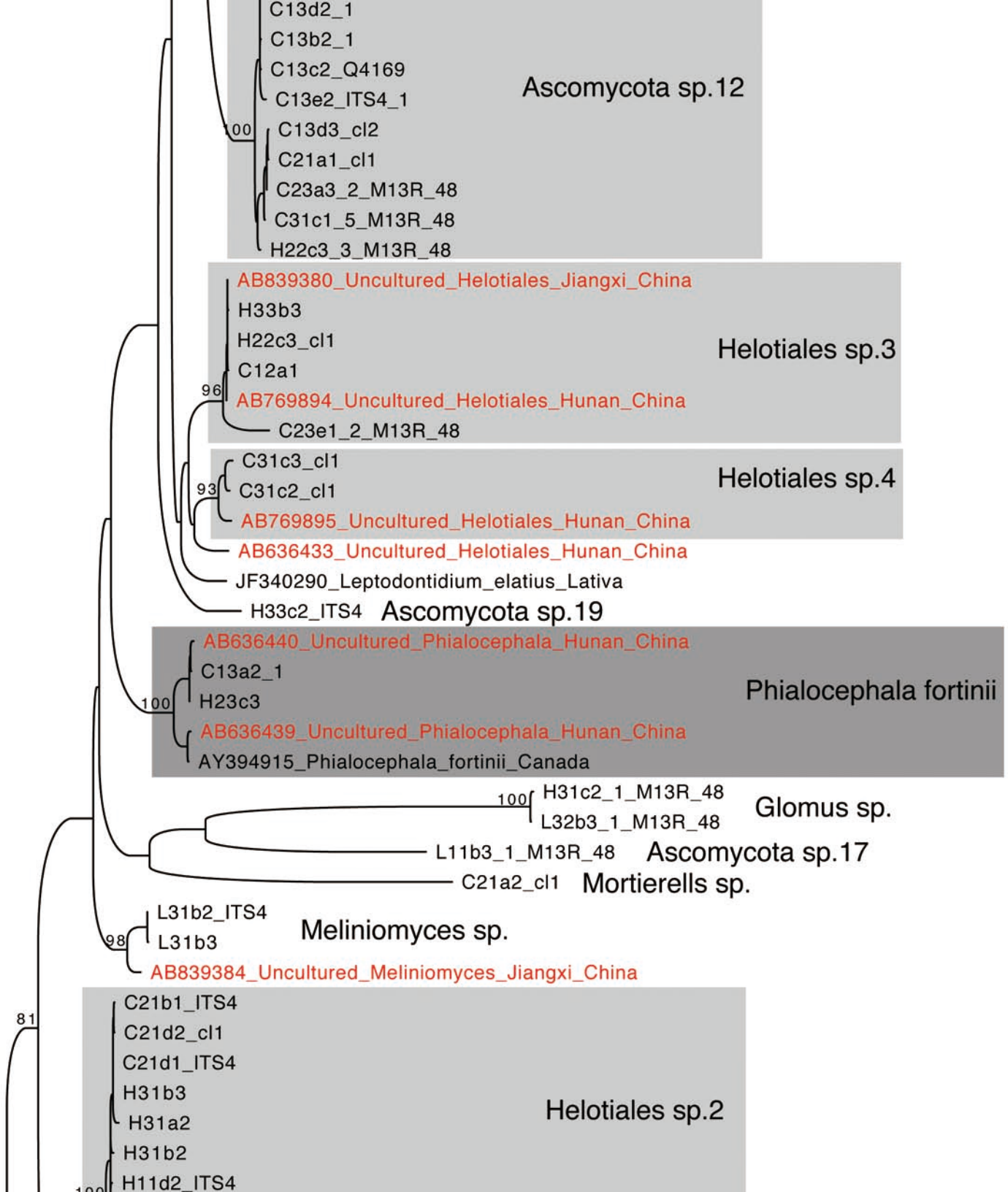
99
75
100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H22c3_3_M13R_48
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96
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H23c3
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L32b3_1_M13R_48
Glomus sp.
L11b3_1_M13R_48
Ascomycota sp.17
C21a2_cl1
Mortierells sp.
L31b2_ITS4
98
L31b3
Meliniomyces sp.
AB839384_Uncultured_Meliniomyces_Jiangxi_China
81
C21b1_ITS4
C21d2_cl1
C21d1_ITS4
H31b3
H31a2
H31b2
H11d2_ITS4
Helotiales sp.2

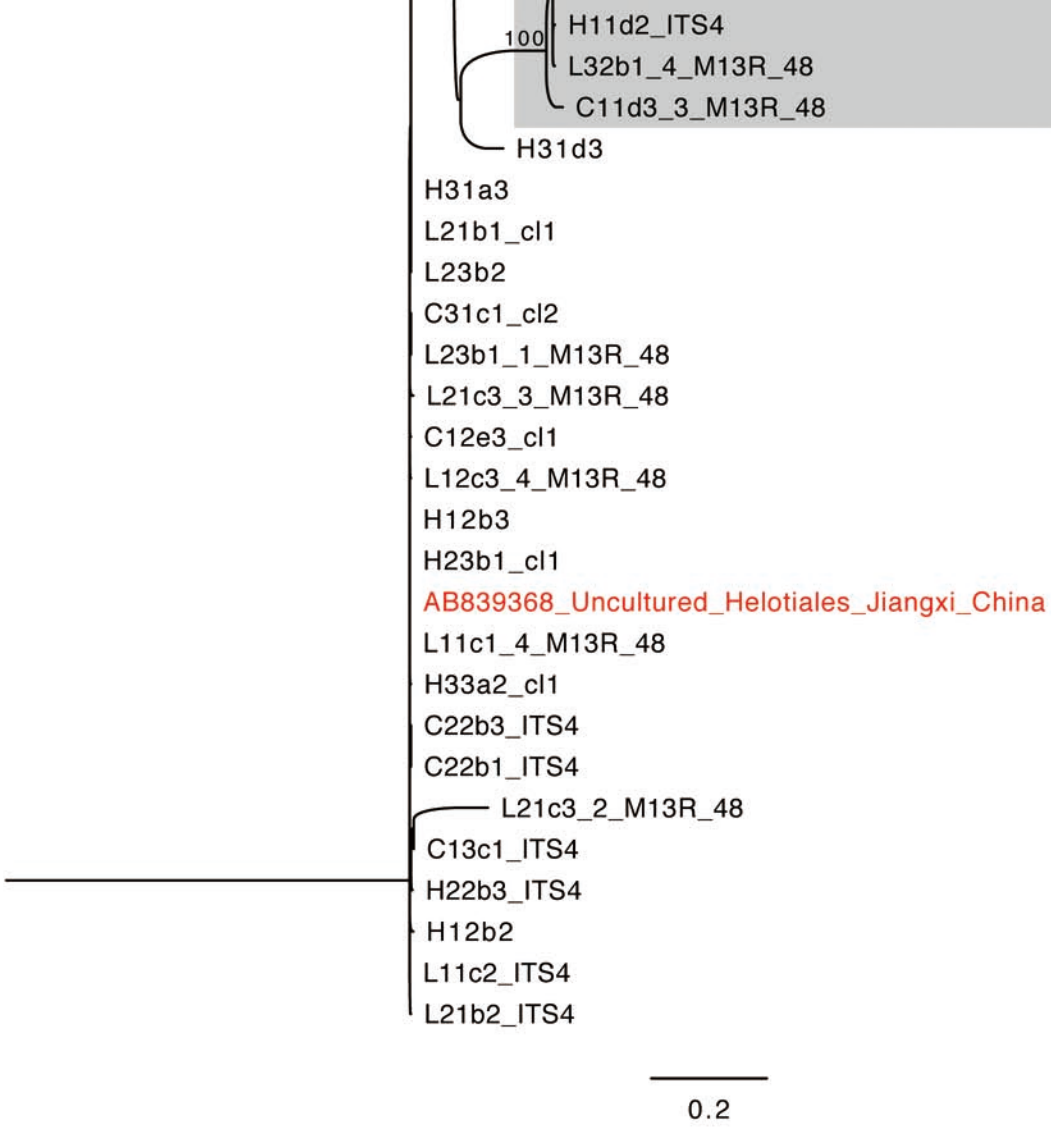
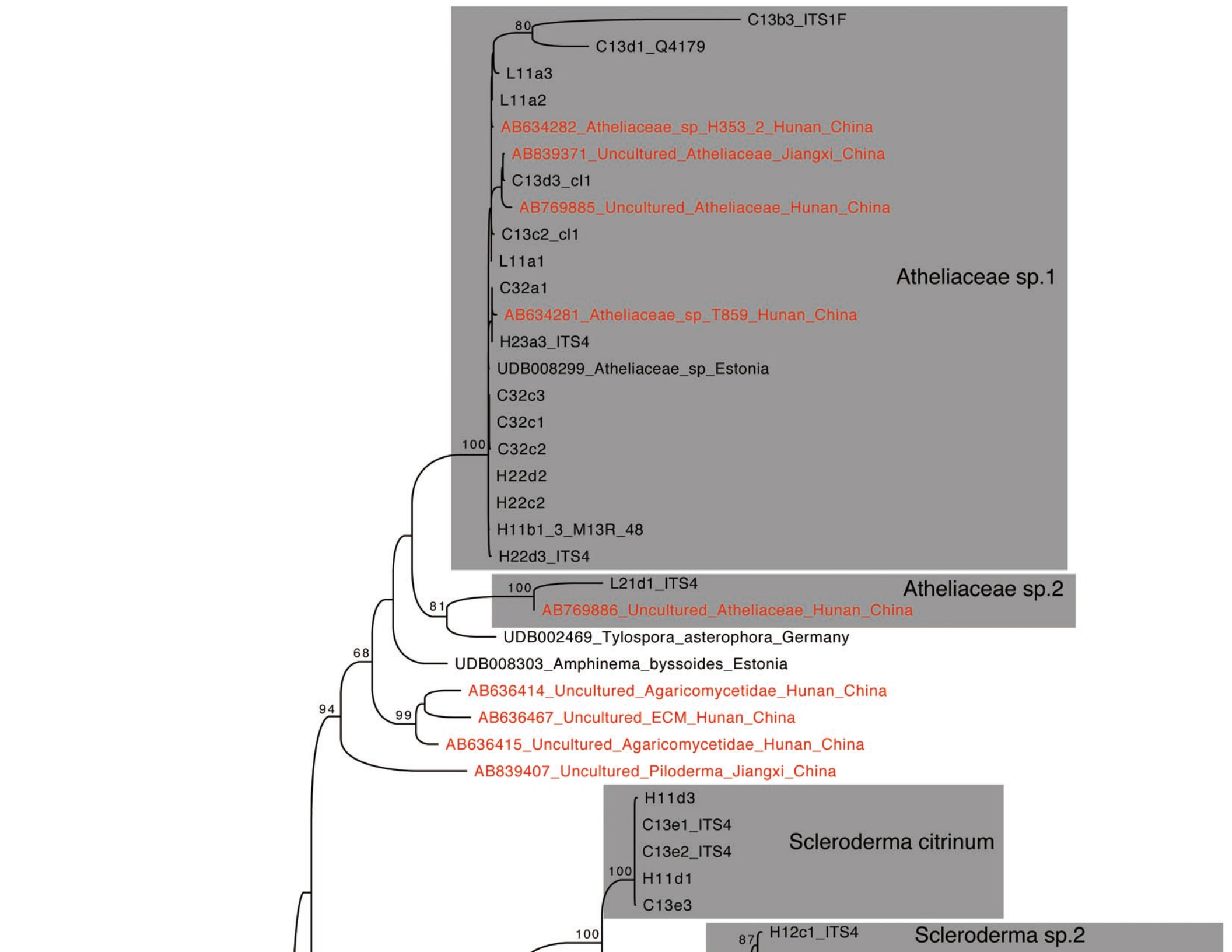
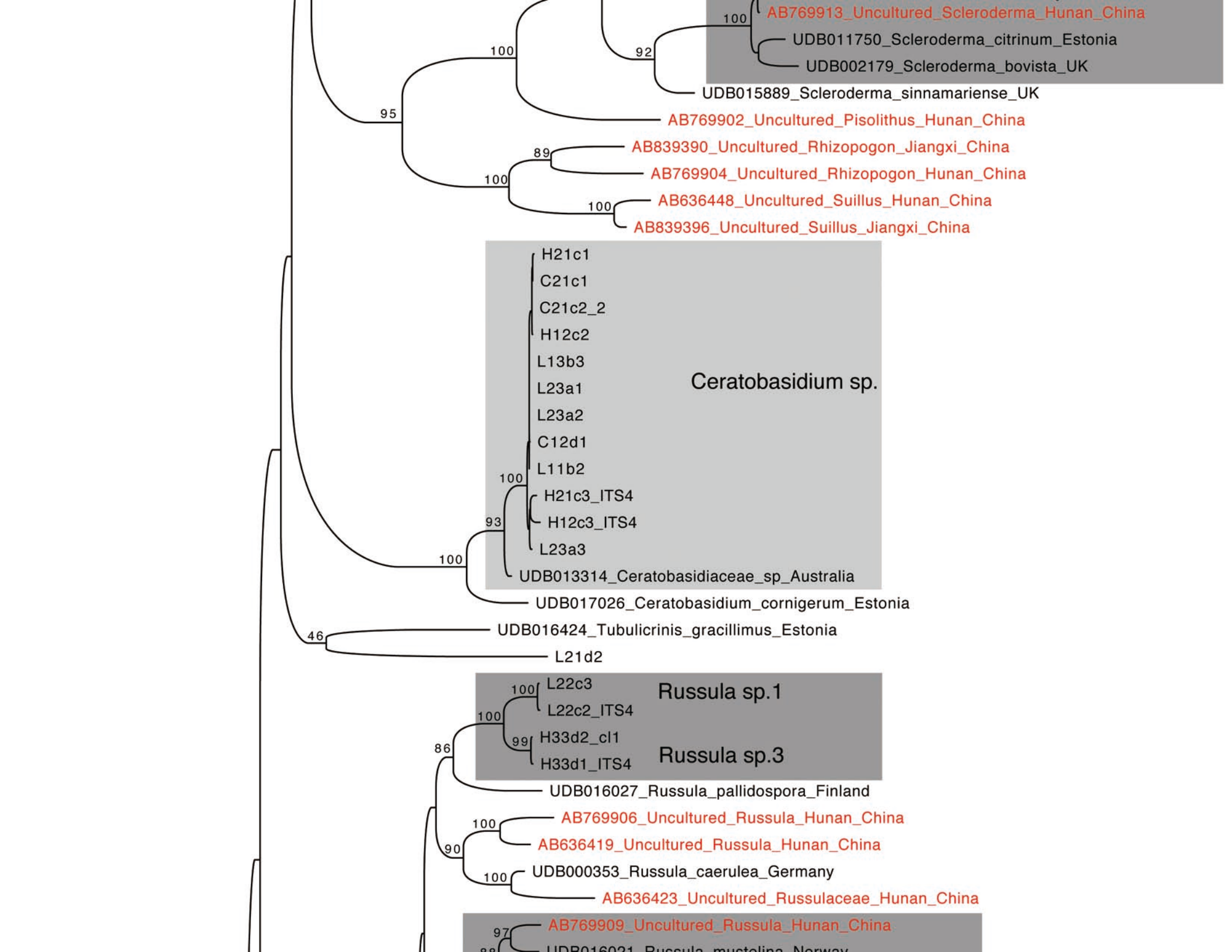
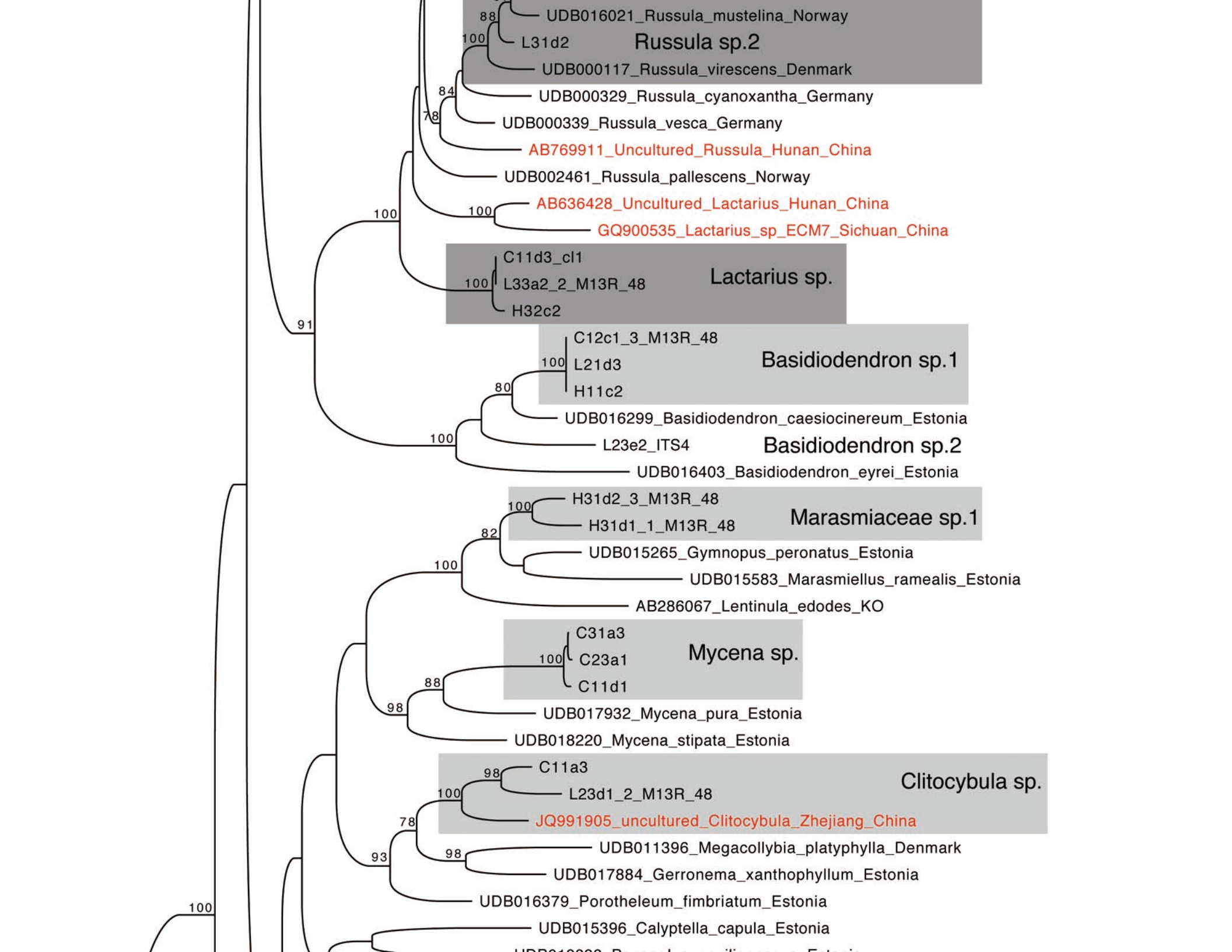
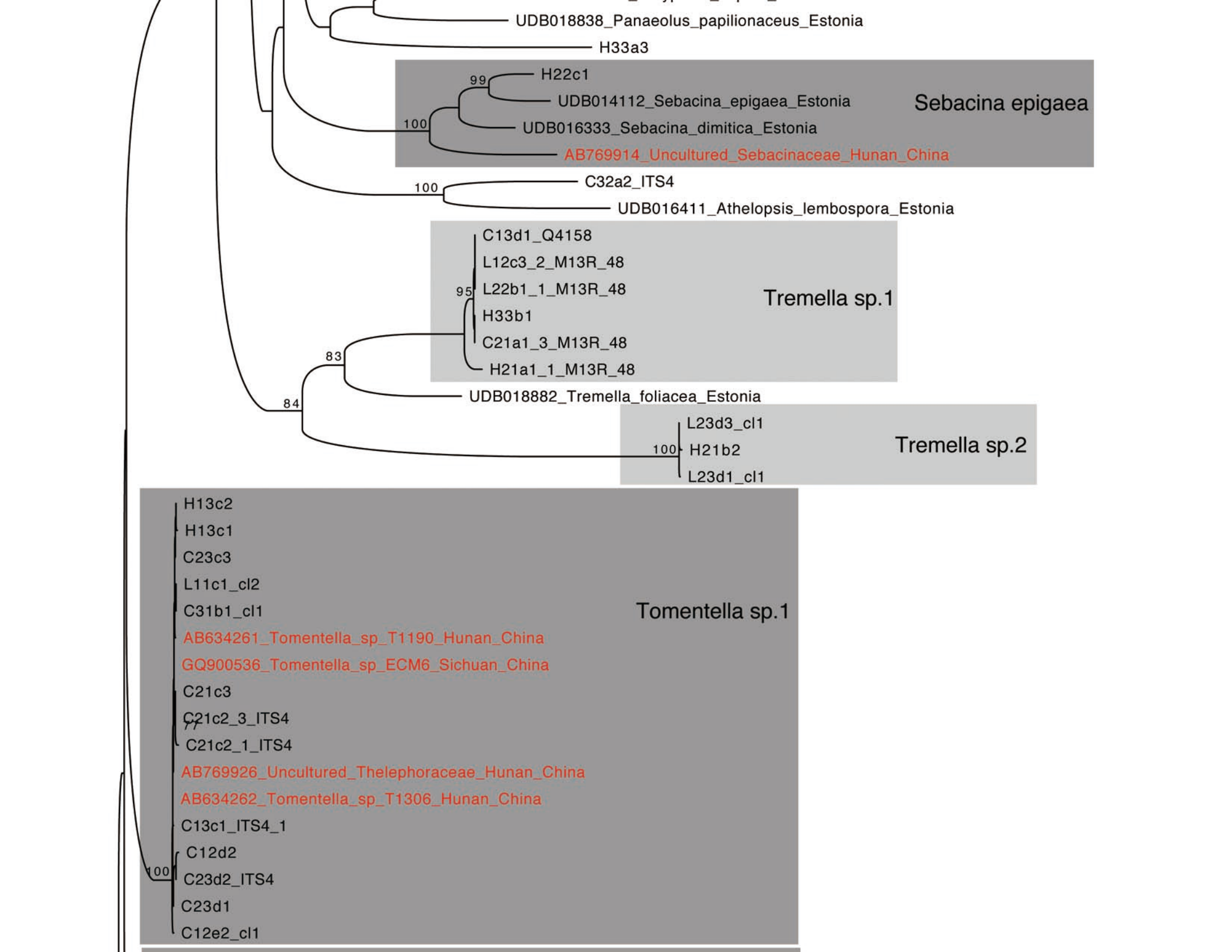


Fig S1. Root tip samples from Pinus elliottii planation, RAxML best tree of Ascomycota species ITS sequencing. Species from our study are indicated by short sample collection number. Numbers adjacent to branches indicated bootstrap statistics. Species names tagged by red color indicate sample from other studies (e.g.) of native tree species masson pine (Pinus massoniana Lamb.) in Hunan (Huang et al., 2012), Jiangxi, and Sichuan province. Maximum likelihood bootstrap percentages are aove the nodes.


C13b3_ITS1F
C13d1_Q4179
L11a3
L11a2
AB634282_Atheliaceae_sp_H353_2_Hunan_China
AB839371_Uncultured_Atheliaceae_Jiangxi_China
C13d3_cl1
AB769885_Uncultured_Atheliaceae_Hunan_China
C13c2_cl1
L11a1
C32a1
AB634281_Atheliaceae_sp_T859_Hunan_China
H23a3_ITS4
UDB008299_Atheliaceae_sp_Estonia
C32c3
C32c1
C32c2
H22d2
H22c2
H11b1_3_M13R_48
H22d3_ITS4
Atheliaceae sp.1
L21d1_ITS4
AB769886_Uncultured_Atheliaceae_Hunan_China
Atheliaceae sp.2
UDB002469_Tylospora_asterophora_Germany
UDB008303_Amphinema_byssoides_Estonia
AB636414_Uncultured_Agaricomycetidae_Hunan_China
AB636467_Uncultured_ECM_Hunan_China
AB636415_Uncultured_Agaricomycetidae_Hunan_China
AB839407_Uncultured_Piloderma_Jiangxi_China
H11d3
C13e1_ITS4
C13e2_ITS4
H11d1
C13e3
Scleroderma citrinum
H12c1_ITS4
Scleroderma sp.2
80
100
81
100
68
94
99
100
100
87


AB769913_Uncultured_Scleroderma_Hunan_China
UDB011750_Scleroderma_citrinum_Estonia
UDB002179_Scleroderma_bovista_UK
UDB015889_Scleroderma_sinnamariense_UK
AB769902_Uncultured_Pisolithus_Hunan_China
AB839390_Uncultured_Rhizopogon_Jiangxi_China
AB769904_Uncultured_Rhizopogon_Hunan_China
AB636448_Uncultured_Suillus_Hunan_China
AB839396_Uncultured_Suillus_Jiangxi_China
H21c1
C21c1
C21c2_2
H12c2
L13b3
L23a1
L23a2
C12d1
L11b2
H21c3_ITS4
H12c3_ITS4
L23a3
Ceratobasidium sp.
UDB013314_Ceratobasidiaceae_sp_Australia
UDB017026_Ceratobasidium_cornigerum_Estonia
UDB016424_Tubulicrinis_gracillimus_Estonia
L21d2
L22c3
L22c2_ITS4
Russula sp.1
H33d2_cl1
H33d1_ITS4
Russula sp.3
UDB016027_Russula_pallidospora_Finland
AB769906_Uncultured_Russula_Hunan_China
AB636419_Uncultured_Russula_Hunan_China
UDB000353_Russula_caerulea_Germany
AB636423_Uncultured_Russulaceae_Hunan_China
AB769909_Uncultured_Russula_Hunan_China
100
92
100
95
89
100
100
100
100
93
46
100
100
99
86
100
90
100
97


UDB016021_Russula_mustelina_Norway
L31d2
Russula sp.2
UDB000117_Russula_virescens_Denmark
UDB000329_Russula_cyanoxantha_Germany
UDB000339_Russula_vesca_Germany
AB769911_Uncultured_Russula_Hunan_China
UDB002461_Russula_pallescens_Norway
AB636428_Uncultured_Lactarius_Hunan_China
GQ900535_Lactarius_sp_ECM7_Sichuan_China
C11d3_cl1
L33a2_2_M13R_48
H32c2
Lactarius sp.
C12c1_3_M13R_48
L21d3
H11c2
Basidiodendron sp.1
UDB016299_Basidiodendron_caesiocinereum_Estonia
L23e2_ITS4
Basidiodendron sp.2
UDB016403_Basidiodendron_eyrei_Estonia
H31d2_3_M13R_48
H31d1_1_M13R_48
Marasmiaceae sp.1
UDB015265_Gymnopus_peronatus_Estonia
UDB015583_Marasmiellus_ramealis_Estonia
AB286067_Lentinula_edodes_KO
C31a3
C23a1
C11d1
Mycena sp.
UDB017932_Mycena_pura_Estonia
UDB018220_Mycena_stipata_Estonia
C11a3
L23d1_2_M13R_48
JQ991905_uncultured_Clitocybula_Zhejiang_China
Clitocybula sp.
UDB011396_Megacollybia_platyphylla_Denmark
UDB017884_Gerronema_xanthophyllum_Estonia
UDB016379_Porotheleum_fimbriatum_Estonia
UDB015396_Calyptella_capula_Estonia


UDB018838_Panaeolus_papilionaceus_Estonia
H33a3
H22c1
99
UDB014112_Sebacina_epigaea_Estonia
Sebacina epigaea
100
UDB016333_Sebacina_dimitica_Estonia
AB769914_Uncultured_Sebacinaceae_Hunan_China
C32a2_ITS4
100
UDB016411_Athelopsis_lembospora_Estonia
C13d1_Q4158
L12c3_2_M13R_48
95
L22b1_1_M13R_48
Tremella sp.1
H33b1
C21a1_3_M13R_48
83
H21a1_1_M13R_48
UDB018882_Tremella_foliacea_Estonia
84
L23d3_cl1
100
H21b2
Tremella sp.2
L23d1_cl1
H13c2
H13c1
C23c3
L11c1_cl2
C31b1_cl1
Tomentella sp.1
AB634261_Tomentella_sp_T1190_Hunan_China
GQ900536_Tomentella_sp_ECM6_Sichuan_China
C21c3
77
C21c2_3_ITS4
C21c2_1_ITS4
AB769926_Uncultured_Thelephoraceae_Hunan_China
AB634262_Tomentella_sp_T1306_Hunan_China
C13c1_ITS4_1
C12d2
100
C23d2_ITS4
C23d1
C12e2_cl1

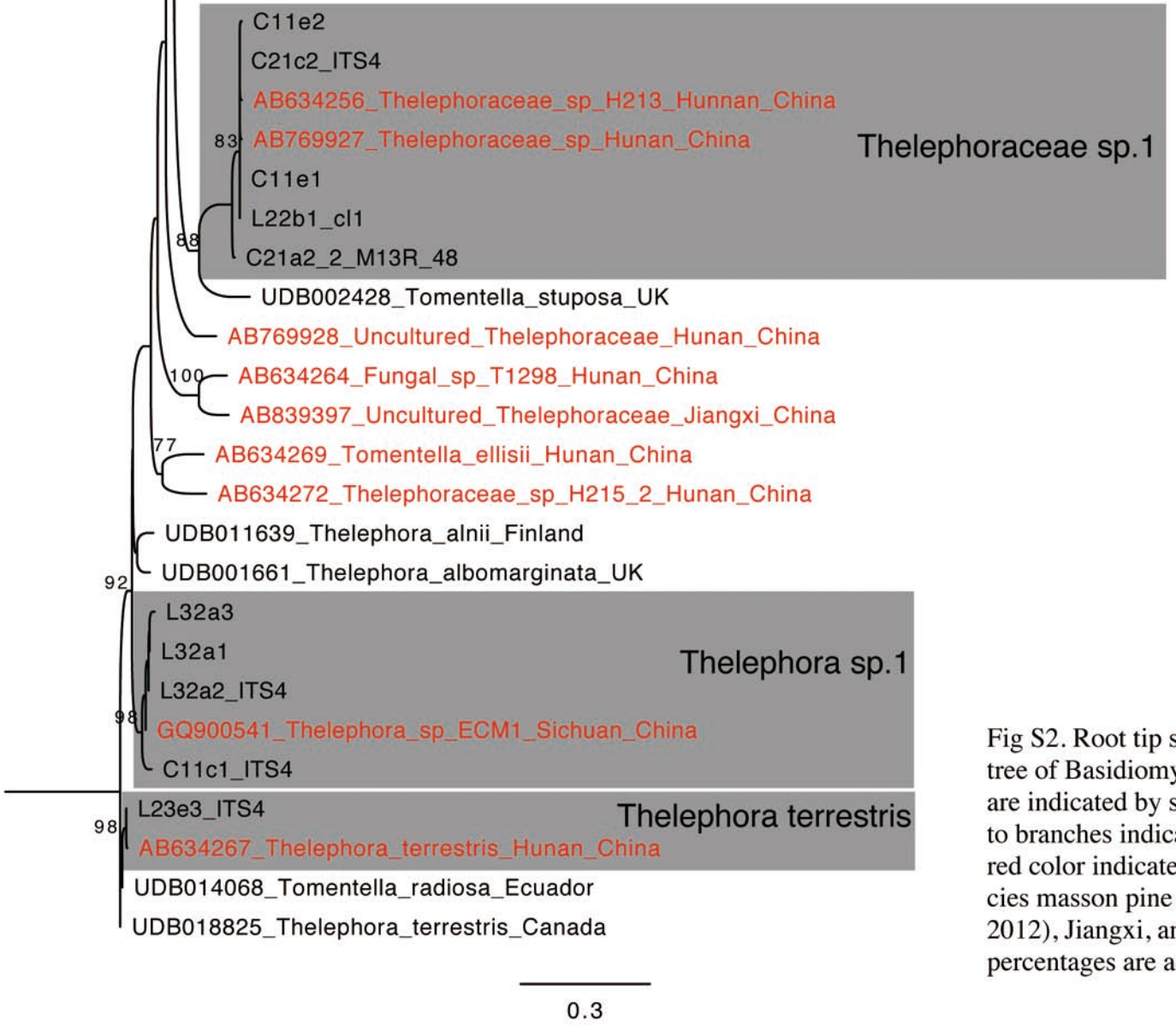


Fig S2. Root tip samples from Pinus elliottii planation, RAxML best tree of Basidiomycota species ITS sequencing. Species from our study are indicated by short sample collection number. Numbers adjacent to branches indicated bootstrap statistics. Species names tagged by red color indicate sample from other studies (e.g.) of native tree species masson pine (Pinus massoniana Lamb.) in Hunan (Huang et al., 2012), Jiangxi, and Sichuan province. Maximum likelihood bootstrap percentages are aove the nodes.