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RESEARCH PAPER

Distinguishing ectomycorrhizal and saprophytic fungi using carbon and nitrogen isotopic compositions

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Abstract Ectomycorrhizal fungi, a group of widespread symbiotic fungi with plant, obtain carbon source from trees and improve plant mineral nutrient uptake with their widespread hyphal network. Ectomycorrhizal fungi can be used as inoculants to improve the survival rates of plantation. Saprophytic fungi use the nutrition from the debris of plant or animals, and it is difficult to distinguish the saprophytic and ectomycorrhizal fungi by morphological and anatomic methods. In this research, the differences of stable carbon and nitrogen isotopic compositions of these fungi were analyzed. The results showed that the abundances of ^{13}C of were higher than those of ectomycorrhizal fungi and the abundances of ^{15}N of saprophytic fungi were lower than those of ectomycorrhizal fungi. Such differences of stable carbon and nitrogen isotopic compositions between ectomycorrhizal fungi and saprophytic fungi can be ascribed to their different nutrition sources and ecological functions. These results collectively indicate that stable carbon and nitrogen isotopic compositions are an effective proxy for distinguishing between ectomycorrhizal and saprophytic fungi.

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1. Introduction

Ectomycorrhizal fungi (EMFs) are an important part of forest ecosystem, and almost all trees can form symbiont–ectomycorrhizae with EMFs (Francis and Read, 1994; Markkola et al., 1996). To a great extent, a healthy and stable forest ecosystem rely on the ectomycorrhizal relationship and the community of EMFs for its function in nutrient element cycling (Haselwandter and Bowen, 1996; Lian et al., 2008). Many studies had proved that mycorrhizal fungi obtained carbon source from their vegetable partner (Hodge et al., 2001; Rosling et al., 2004; Hobbie, 2006). In return, EMFs will improve mineral nutrition uptake for plants by weathering on minerals and activating undissolvable nutrition, such as phosphorus (Leyval and Reid, 1991; Dixon and

Hiolhiol, 1992; Marschner and Dell, 1994; Hobbie et al., 2009). Ectomycorrhizal fungi also can assimilate nitric-, ammonium-, and protein-nitrogen with high-efficiency through their widespread hyphal network, providing nitrogen nutrition to plants (Bending and Read, 1996; Keller, 1996; Martin and Lorillou, 1997). EMFs can be used as inoculants to improve the survival rate of plantation (Amaranthus and Perry, 1987) and great economic values existed in the fruit bodies of EMFs, such as *Tuber magnatum*, *Tricholoma matsutake* (Wang and Hall, 2004).

Saprophytic fungi, the largest group of fungi, grow on dead organic matter such as fallen trees, cow patties, dead leaves, and even dead insects and animals. Saprophytic fungi play an important role in decomposition of organic matters and nutrition cycling, especially in the nitrogen cycling by excreting kinds of hydrolase, including proteinase, cellulase, laccase and so on (Mcmillan and Boynton, 1994; Hobbie et al., 1999; Baldrian and Valaskova, 2008; Dinis et al., 2009). EMFs and saprophytic fungi are involved in different ecological functions, but it is difficult to distinguish ectomycorrhizal sporocarps from sporocarps of saprophytic fungi using anatomical and taxonomic methods.

Molecular tools were largely used in identifying EMFs (Bruns and Gardes, 1993). By DNA sequencing targeting fungi partner from mycorrhizae, Bruns et al. (1998) identified a large number of EMFs, and assembled a sequence database for basidiomycetous EMFs. Lian et al. (2007) designed a pair of specific primers to identify *Boletus edulis*, a frequently occurring ectomycorrhizal fungus in Southwest China forest ecosystem. However, the strict methods for distinguishing EMFs are to obtain their marks, such as Internal Transcribed Spacer (ITS) sequences directly from ectomycorrhizas, or inoculate the EMFs to their symbiotic partner. By these ways, it is inevitable that some fungi are assigned to EMFs artificially.

Stable isotopic composition has been widely used in ecological and element cycling studies. The carbon and nitrogen stable isotopic compositions of plant can be affected by species, elevation, humidity, and other environmental factors (Piao et al., 2004; Yang et al., 2007). Taylor and Bruns (1997) found that non-photosynthetic orchid "cheated" carbon and nitrogen source from vicinal plant by mycorrhizal network traced by stable isotopic composition. Thus, due to different trophic manners, do the EMFs vary from saprophytic fungi in carbon and nitrogen isotopic compositions? Can such differences be used to distinguish the EMFs from saprophytic fungi? The aim of this study was to analyze the differences of the stable carbon and nitrogen isotopic compositions of EMFs and saprophytic fungi, and to examine the efficiency of the stable isotopic composition in distinguishing between saprophytic fungi and EMFs.

2. Materials and methods

2.1. Study site

Longli Planted Forest, located in the southwest part of Guizhou Province, China (Fig. 1), ranges in altitude from 1550 to 1700 m, and possesses an annual average temperature of 14.7 °C, a yearly average rainfall of 1100 mm, and relative humidity of above 80%. The weather is affected by north subtropical monsoon climate. The tree species are dominated by *Pinus massoniana* Lamb.

2.2. Materials

Samples were collected in June and August, 2008. All the samples were listed in Table 1. The sporocarps were identified by anatomic method according to a guideline (Wei, 1982). After brought to laboratory, the sporocarps were packed separately and dried at 50 °C for 24 h. Fine roots and ectomycorrhizas were rinsed by distilled water and were then dried at 50 °C for 24 h. The dried samples were ground into powder and then preserved in a dry place for stable isotopic composition analysis.

2.3. Stable isotopic composition analysis

For carbon isotopic composition analysis, 6–10 mg organic sample and 2 g CuO were put in a quartz tube of 25 cm in length and 9 mm in diameter. The tube was vacuumized and sealed with flame on a vacuum system. Then the sample was burnt at 850 °C for 5 h. By this way, the organic carbon was transferred to CO_2. The resulting CO_2 was collected with a vacuum system, and then the stable carbon isotopic composition was analyzed by a gas isotopic mass analyzer (MAT 252, Finnigan). Stable carbon isotopic composition is expressed by $\delta^{13}C(‰) = [(^{13}C/^{12}C_{sample} - {}^{13}C/^{12}C_{standard})/(^{13}C/^{12}C_{standard})] \times 1000$, where $^{13}C/^{12}C_{sample}$ and $^{13}C/^{12}C_{standard}$ are the ratios of the sample and the reference sample (PBD), respectively. For nitrogen isotopic composition analysis, 6–10 mg sample, 2 g CuO and 2 g Cu were added into a quartz tube. The tube was vacuumized and sealed with flame on a vacuum system. Then the sample was burnt at 850 °C for 5 h. The organic nitrogen was transferred to N_2. The stable nitrogen isotopic composition was analyzed by a gas isotopic mass analyzer (MAT 252, Finnigan). Stable nitrogen isotopic composition is expressed by $\delta^{15}N$ (‰) $= [(^{15}N/^{14}N_{sample} - {}^{15}N/^{14}N_{standard})/(^{15}N/^{14}N_{standard})] \times 1000$, where $^{15}N/^{14}N_{sample}$ and $^{15}N/^{14}N_{standard}$ are the ratios of the sample and the reference sample (atmospheric N_2), respectively.

Figure 1 Location of Longli Planted Forest.

Table 1 List of samples used in the study.

Samples	Descriptions of samples
Sporocarps of EMFs	Unaged sporocarps
Sporocarps of saprophytic fungi	Unaged sporocarps, including two sporocarps easy to be mistaken as EMFs (see Fig. 2)
Fine roots and ectomycorrhizae	Underground roots and cut root tip with white hyphae as ectomycorrhizas
Soil and wood	Soil sample under the sporocarps or wood growing sporocarps

3. Results

3.1. Stable carbon and nitrogen isotopic compositions

The stable carbon and nitrogen isotopic compositions of EMFs were significantly different from those of saprophytic fungi (Table 2). The $\delta^{13}C$ values of EMFs ranged from −26.41‰ to −24.22‰ with an average of (−25.16 ± 0.58)‰; the $\delta^{15}N$ values of EMFs ranged from 2.34‰ to 4.54‰ with an average value of (3.79 ± 0.67)‰; The $\delta^{13}C$ values of saprophytic fungi ranged from −23.66‰ to −20.18‰ with an average of (−22.13 ± 1.20)‰; The $\delta^{15}N$ values of saprophytic fungi ranged from −2.02‰ to 0.21‰ with an average value of (−0.78 ± 0.63)‰.

The trophic type is confusing for the Clavariaceae species, usually called coral fungi, which are hard to isolate and/or cultivate. About 150 species of this family were designated as EMFs (Allen, 1992). These species, such as *Clavicorona pyxidata* growing on wood (Fig. 2A), belong to saprophytic fungi according the isotopic composition results. Based on size and morphology of sporocarps, it is also hard to distinguish the sporocarps of *Calvatia craniiformis* from those of species of the order Sclerodermatales, such as *Scleroderma lycoperdoides* and *S. cepa*. And the trophic manner is also confusing for *C. craniiformis*, growing on soil some time or decayed wood in other cases. We collected two sporocarps one each on soil and decayed wood, respectively (Fig. 2B). The isotopic results showed that there was no difference between the $\delta^{13}C$ and $\delta^{15}N$ values of the two sporocarps. The $\delta^{13}C$ and $\delta^{15}N$ values of *Calvatia craniiformis*, *C. pyxidata*, and *Clavulina kunzei* remain in the range of saprophytic fungi (Fig. 3). Taken together, the results showed that all these ectomycorrhizal-like fungi belong to saprophytic fungi. *Calvatia craniiformis* was designated to ectomycorrhizal fungus in some cases (Ma et al., 2008).

Table 2 Stable C/N isotopic compositions of sporocarps of EMFs and saprophytic fungi.

Fungi	$\delta^{13}C$(‰)	$\delta^{15}N$(‰)
EMFs		
Russula crustosa Peck	−25.03	4.54
Russula virescens (Schaeff.) Fr.	−24.76	4.78
Russula vinosa Lindblad	−25.44	2.78
Russula sp.	−26.01	2.90
Tylopilus ballouii (Peck) Singer	−24.22	4.07
Boletinus pinetorum (W.F. Chui) Teng	−24.51	4.24
Strobilomyces confusus Singer	−25.13	3.71
Xerocomus badius (Fr.) Kühner	−25.07	2.34
Boletus ravenelii Berk. & M.A. Curtis	−25.26	3.89
Scleroderma lycoperdoides Schwein.	−25.28	3.22
Scleroderma cepa Pers.	−25.34	3.46
Ramaria mairei Donk	−24.24	4.49
Cantharellus cibarius Fr.	−25.79	3.88
Strobilomyces floccopus (Vahl) P. Karst.	−24.99	4.35
Ramaria rufescens (Schaeff.) Corner	−25.33	3.53
Clavulina cristata (Holmsk.) J. Schröt.	−24.85	3.99
Cantharellus minor Peck	−26.41	4.21
Average of EMFs	−25.16 ± 0.58	**3.79 ± 0.67**
Saprophytic fungi		
Clavulina kunzei (Fr.) Corner	−20.18	−0.83
Ganoderma applanatum (Pers.) Pat.	−20.56	−1.24
Pleurotus ostreatus (Jacq.) P. Kumm.	−22.68	−0.48
Ganoderma lucidum (Curtis) P. Karst.	−22.90	−1.31
Trametes versicolor (L.) Lloyd	−21.70	−2.02
Clavicorona pyxidata (Pers.) Doty	−20.99	−0.50
Calvatia craniiformis (Schwein.) Fr.	−22.50	0.21
Cyathus striatus (Huds.) Willd.	−23.66	−0.32
Copyinds comatus (Muii. Fr) Gray	−23.19	−0.46
Lyophyllum decastes (Fr.) Singer	−22.97	−0.87
Average of saprophytic fungi	−22.13 ± 1.20	−0.78 ± 0.63

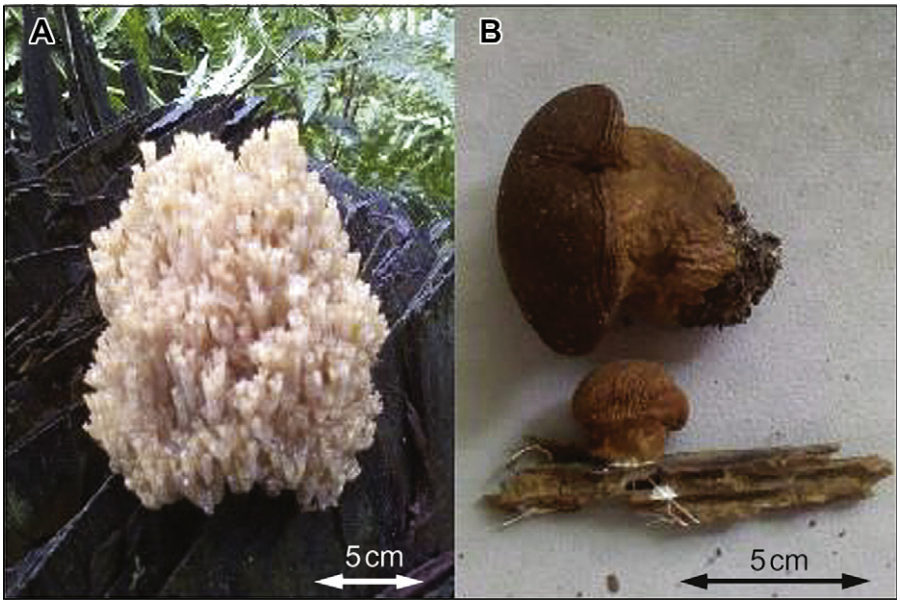

Figure 2 Two saprophytic fungal sporocarps, which are easy mistaken as EMFs. The left one (A) is the sporocarp of *Clavicorona pyxidata* (Pers. Fr.) Doty, and the right one (B) is the sporocarp of *Calvatia craniiformis* (Schw.) Fr. The upper one was found growing on soil, and the lower one was found growing on a decayed wood.

3.2. Fungal discrimination on stable carbon and nitrogen isotopes

The fungal stable carbon and nitrogen isotopic compositions can be affected by trophic types and nutrient sources. Because of carbon and/or nitrogen sources, live root and ectomycorrhizal root usually were depleted in heavy isotopes, whereas decayed wood and soil organic matters usually were enriched in heavy isotopes (Table 3).

The EMFs mainly obtains their carbon source from live trees. Photosynthates, such as glucose and other monosaccharides, are transferred from trees to EMFs through ectomycorrhizae. By this way, more than a half of photosynthates of a seedling and 1%–21% of photosynthates of a mature tree were allocated to their symbiotic partner (Hobbie et al., 1999; Rosling et al., 2004).

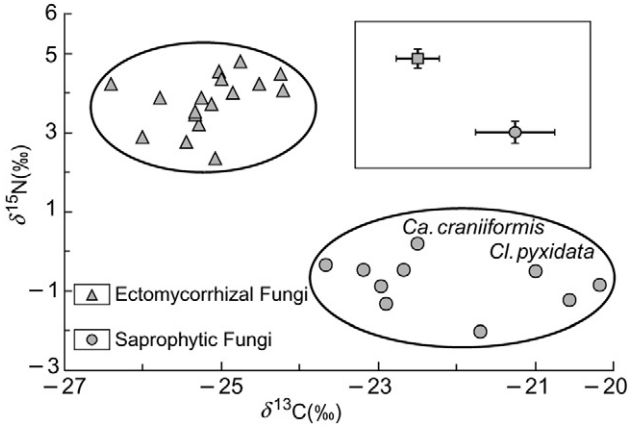

Figure 3 δ^{13}C and δ^{15}N distributions of EMFs and saprophytic fungi; *Ca. craniiformis* and *Cl. pyxidata* were found to grow on rotted wood.

Both EMFs and saprophytic fungi were enriched in heavy carbon isotope (Fig. 4A). In comparison with fine roots, the sporocarps of EMFs were enriched in ^{13}C by (2.45 ± 0.75)‰. Ectomycorrhizae is made up of plant root and fungal mycelia, so the values of δ^{13}C of ectomycorrhizas fall in between those of EMFs sporocarps and plant root. The saprophytic fungi mainly obtained their carbon source from decayed wood and soil organic matters. In comparison with decayed wood and soil organic matters, the sporocarps of saprophytic fungi growing on the wood and soil organic matters were enriched in ^{13}C by (3.90 ± 1.06)‰ and (3.19 ± 0.28)‰, respectively.

Both EMFs and saprophytic fungi obtain their nitrogen nutrition from growing substrates. Additionally, EMFs need to uptake extra nitrogen for their vegetable partners. In comparison with soil nitrogen, EMFs were enriched in ^{15}N by (4.09 ± 0.87)‰. During transferring nitrogen from fungi to plant roots, fine roots tend to obtain lighter nitrogen isotope (^{14}N), and the difference was about (−5.60 ± 0.87)‰. There were no obvious differences of the values of δ^{15}N between saprophytic fungi and their growing substrates, such as soil organic matters, decayed wood (Fig. 4B).

4. Discussion

The stable carbon and nitrogen isotopic compositions were significantly different between EMFs and saprophytic fungi in this study, which is consistent with previous studies (Kohzu et al., 1999; Zeller et al., 2007). Hasselquist et al. (2011) identified the first ectomycorrhizal status of boletes on the Northern Yucatan Peninsula, Mexico by employing nitrogen and carbon stable isotopic compositions. This indicates that the stable carbon and nitrogen isotopic compositions are an effective tool for distinguishing EMFs from saprophytic fungi.

The carbon stable isotopic compositions of EMFs were lighter than that of saprophytic fungi. While the carbon stable isotopic compositions of EMFs carbon source were also lighter than those

Table 3 C/N isotopic compositions of rotted wood, fine root and soil organic carbon.

Samples	Description	δ^{13}C (‰)	δ^{15}N (‰)
Decayed woods			
Wood 1	growing *Clavicorona pyxidata*	−25.52	/[a]
Wood 2	growing *Calvatia craniiformis*	−25.51	/[a]
Wood 3	growing *Pleurotus ostreatus*	−25.41	−1.35
Wood 4	growing *Cyathus striatus*	−26.11	−0.93
Wood 5	growing *Trametes versicolor*	−27.52	−0.14
Average of decayed woods		−26.02 ± 0.89	−0.81 ± 0.62
Fine roots			
Root 1	*Pinus massoniana*	−27.16	−2.14
Root 2	*Pinus massoniana*	−27.36	−2.36
Root 3	*Pinus massoniana*	−27.16	−1.95
Root 4	*Pinus massoniana*	−27.36	−2.27
Average of fine roots		−27.26 ± 0.14	−2.18 ± 0.18
Ectomycorrhizas			
Ectomycorrhizae 1	*Pinus massoniana*	−26.98	−1.89
Ectomycorrhizae 2	*Pinus massoniana*	−26.88	−2.05
Ectomycorrhizae 3	*Pinus massoniana*	−26.36	−2.15
Ectomycorrhizae 4	*Pinus massoniana*	−26.48	−2.12
Ectomycorrhizae 5	*Pinus massoniana*	−26.30	−1.86
Ectomycorrhizae 6	*Pinus massoniana*	−26.89	−1.95
Average of ectomycorrhizas		−26.65 ± 0.31	−2.00 ± 0.12
Soil organic matters			
Soil 1	Under *Pinus massoniana*	−26.45	−0.45
Soil 2	Under *Pinus massoniana*	−25.85	0.27
Soil 3	Under *Pinus massoniana*	−25.84	−0.64
Average		−26.0 ± 0.35	−0.28 ± 0.48

[a] For the sake of low nitrogen content, we failed to obtain these two values of δ^{15}N.

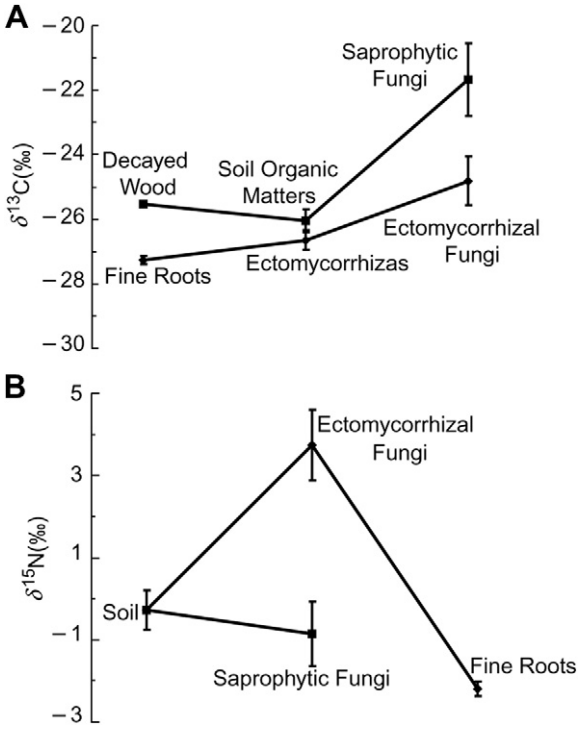

Figure 4 Carbon and nitrogen isotopic differentiations among fungi, plant roots, wood and soil.

of saprophytic fungi, soil organic carbon or decayed wood, by 1.2‰ or 1.7‰, respectively. So, it is evident that the differences of carbon sources are the main causes explaining the variations in the carbon stable isotopic compositions of EMFs and saprophytic fungi.

Sporocarps of EMFs were highly enriched in ^{15}N, in comparison with their nitrogen source (Gebauer and Taylor, 1999; Kohzu et al., 1999). Our study showed that EMFs were enriched in the heavy nitrogen isotope, but saprophytic fungi were a little depleted in the heavy nitrogen isotope compared with their growing substrate. Brearley et al. (2005) found that EMFs were depleted in ^{15}N as they were purely cultivated without mycorrhizal partner. So the symbiotic relationship is probably the reason for the ^{15}N enrichment in EMFs. Most ^{15}N-depleted nitrogen may be transferred to plants. Hobbie and Colpaert (2003) found that fungal biomass and N increased at low N in comparison with high N supply, whereas needle δ^{15}N decreased. Needle δ^{15}N is strongly and negatively correlated with biomass of extraradical hyphae. In our study, the δ^{15}N values of plant roots were much lower than those of EMFs, which also indicated that the nitrogen source was fully supplied in the Longli forest ecosystem.

In conclusion, the different nutrition source and ecological functions result in the fractionation of stable carbon isotopic compositions between EMFs and saprophytic fungi, suggesting that the stable carbon and nitrogen isotopic compositions are an effective proxy for distinguishing between ectomycorrhizal and saprophytic fungi.

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