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First report of the ectomycorrhizal status of boletes on the Northern Yucatan Peninsula, Mexico determined using isotopic methods

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Abstract Despite their prominent role for tree growth, few studies have examined the occurrence of ectomycorrhizal fungi in lowland, seasonally dry tropical forests (SDTF). Although fruiting bodies of boletes have been observed in a dry tropical forest on the Northern Yucatan Peninsula, Mexico, their occurrence is rare and their mycorrhizal status is uncertain. To determine the trophic status (mycorrhizal vs. saprotrophic) of these boletes, fruiting bodies were collected and isotopically compared to known saprotrophic fungi, foliage, and soil from the same site. Mean $\delta^{15}N$ and $\delta^{13}C$ values differed significantly between boletes and saprotrophic fungi, with boletes 8.0‰ enriched and 2.5‰ depleted in ^{15}N and ^{13}C, respectively relative to saprotrophic fungi. Foliage was depleted in ^{13}C relative to both boletes and saprotrophic fungi. Foliar $\delta^{15}N$ values, on the other hand, were similar to saprotrophic fungi, yet were considerably lower relative to bolete fruiting bodies. Results from this study provide the first isotopic evidence of ectomycorrhizal fungi in lowland SDTF and emphasize the need for further research to better understand the diversity and ecological importance of ectomycorrhizal fungi in these forested ecosystems.

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Introduction

Numerous studies have demonstrated that mycorrhizal symbioses are ubiquitous features of terrestrial plant communities (Allen et al. 1995; Smith and Read 2008). Of the two most common mycorrhiza types, arbuscular mycorrhizal (AM) fungi are widespread, forming symbiotic associations with roughly 85% of all terrestrial plants (Smith and Read 2008). Ectomycorrhizal (EM) fungi, on the other hand, are restricted to a smaller number of host plant species (Allen et al. 1995) and are best known from studies in northern temperate and boreal ecosystems.

In contrast to EM-rich temperate and boreal forests, tropical forests had long been thought to be impoverished in fungal species forming EM associations (Dennis 1970; Pirozynski 1980). This generalization was based on surveys that demonstrated the prevalence of AM trees in wet tropical forests and the concomitant dearth of tree species known to form EM associations (Janos 1983; Redhead 1968; St. John 1980). However, recent mycological studies in wet tropical forests have shown a wide diversity of EM fungi (Henkel et al. 2002 and references therein). Currently, only a few EM studies have been conducted in lowland, seasonally dry tropical forests (SDTF) (Brundrett et al. 1996; Högberg 1992; Thoen and Ba 1989), and to our knowledge, no studies have examined the occurrence of EM fungi in the highly diverse, lowland SDTF of the Yucatan Peninsula and Northern Central America.

Fruiting bodies of boletes (Boletaceae species) have previously been observed at El Eden Ecological Reserve, a lowland SDTF reserve located on the Northern Yucatan

Peninsula, Mexico (Allen and Rincon 2003). However, the production of bolete fruiting bodies is extremely sporadic in this area. During 15 years of research at El Eden Ecological Reserve, we have observed fruiting bodies of boletes only twice, during the rainy season in 2001 and 2005. Because nearly all bolete species are obligate ectomycorrhizal symbionts, it seems reasonable to expect that EM fungi occur in this forested ecosystem. However, it has been difficult to evaluate the existence of EM fungi in this forested ecosystem because of a previous lack of putative EM fruiting bodies, the highly interspersed distribution of potential host trees, and the complex mixing of root systems. For instance, despite examining plant roots from soil cores taken throughout the Reserve, Allen and Rincon (2003) did not observe readily discernible EM root tips.

Many putative EM taxa have proven difficult to culture, and therefore, their mycorrhizal status cannot be directly proven according to the methods of Koch's postulates. However, differences in carbon and nitrogen isotope ratios ($\delta^{13}C$ and $\delta^{15}N$ values) among fungal fruiting bodies of EM and saprotrophic fungi provides a way of determining trophic strategies (ectomycorrhizal vs. saprotrophic) for a broad range of fungi, including fungi whose trophic strategies is uncertain (Hobbie et al. 2001). In general, EM fungi are enriched in ^{15}N and depleted in ^{13}C relative to co-occurring saprotrophic fungi (Henn and Chapela 2001; Hobbie et al. 1999, 2001; Taylor et al. 2003: Trudell et al. 2004). In this study, we compared the carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic signature of bolete fruiting bodies to known saprotrophic fungi, foliage, and soil from the same site in order to determine the trophic status of bolete species in a highly diverse, lowland SDTF on the Northern Yucatan Peninsula.

Methods

Study-site This study was conducted at the El Eden Ecological Reserve (see Allen et al. 2003), a 2500-ha SDTF reserve located in the northeastern corner of the Yucatan Peninsula, Mexico (21°12′61″N, 87°10′93″W). The mean annual temperature of the site is 24.2°C. The region averages 1,500–2,000 mm annual precipitation, mostly falling during June to December. The landscape is flat, with an elevation of 6 m asl. The soils at the reserve are extremely thin (often only a few centimeters) and overlie limestone bedrock. Soils are further characterized by ca. 30% soil organic matter, pH 7.5, bulk density of 0.35 g/cm^3, and the lack of a well-developed mineral soil layer (Allen et al. 2003). The reserve is characterized by high plant diversity (Schultz 2005), and among these species are a number of angiosperm genera known to form EM associations in other tropical forested ecosystems, including *Guapira*, *Neea*, *Pisonia*, and *Coccoloba* (Haug et al. 2005; Lodge 1996; Tedersoo et al. 2010). Although these genera are rare at the reserve, they are evenly distributed throughout the forest.

Sample collection In October 2005, fruiting bodies of boletes and saprotrophic fungi were collected in a 1-ha area. Collected specimens were classified, using both local, as well as broader taxonomic keys (Bessette et al. 2000; Guzman 2003; Halling and Mueller 1999; Ortiz-Santana et al. 2007). Additionally, a small piece of the cap (~5 g) was removed from each boletes fruiting body and stored in a 1.5-ml microcentrifuge tube (SealRite, Germany) containing cetyltrimethylammonium bromide (CTAB) buffer for molecular analyses. The remaining fruiting body tissue was oven-dried (60°C, 24 h) for stable isotope analyses.

Molecular protocols DNA was extracted from bolete fruiting bodies using a modified CTAB method (Gardes and Bruns 1993). The internal transcribed spacer (ITS) region of the ribosomal DNA was sequenced using the primer IST1F in various combinations with primers ITS4 and LR3. The general protocol was 94°C for 5 min, followed by 25 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, followed by 7 min at 72°C. The PCR products were visualized on 1.5% agarose gels with ethidium bromide. PCR products were cut from gels and cleaned with Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, CA, USA) before being sequenced. Sequencing was performed with the same primers as above at the Core Instrumentation Facility (CIF) of the University of California at Riverside's Institute of Integrative Genome Biology. Sequences were edited using Sequencher (version 4.6, Gene Codes Corporation, Ann Arbor, MI, USA). Once individual samples were grouped according to sequence type, a general BLAST search was performed in order to aid in the identification of the obtained sequences.

Stable isotope analyses In addition to bolete fruiting bodies, we also collected fruiting bodies of saprotrophic fungi in October 2005. Although fruiting bodies of saprotrophic fungi were abundant, we only report isotopic values for a subset of wood-decomposing and litter-decomposing taxa (n=6 species of wood-decomposers and n=13 species of litter-decomposing fungi). Wood-decomposing fungi included *Cookeina speciosa* (Fr.) Dennis, *Cyathus colensoi* Berk., *Scutellinia scutellata* (L.) Lambotte, *Trametes villosa* (Sw.) Kreisel, *Xylaria coccophora* Mont., and *Marasmiellus cubensis* (Berk & M.A. Curtis) Singer. Litter-decomposing fungi included *Leucocoprinus birnbaumii* (Corda) Singer, *Marasmius haematocephalus* (Mont.) Fr., *Agaricus spp*. (n=4 species), *Coprinus spp*. (n=3 species), *Pholiota spp*. (n=2 species),

and *Psathyrella spp.* (n=2 species). For this study, we also determined the isotopic composition of fruiting bodies of archived boletes collected in 2001 (n=10). Bolete species gathered in 2001 were collected in a similar way as described above, with the exception that no material was taken for molecular analyses.

Leaf and soil samples were also collected from the same area for isotopic analysis. Sunlit leaf samples were collected from six of the dominant tree species at our site (*Caesalpinia gaumeri* Greenm., *Coccoloba diversifolia* Jacq., *Esenbeckia pentaphylla* (Macfad.) Griseb., *Lonchocarpus castilloi* Standley, *Lysiloma latisiliquum* (L.) Benth., and *Vitex gaumeri* Greenm.). Live leaf samples were collected from the canopy of three trees of each species giving a total of 18 samples. For each individual tree, five mature leaves were collected from the top canopy using a pole tree pruner and pooled into a single sample. Soil samples were randomly collected within the 1-ha area by inserting a 4.5-cm diameter metal corer until we encountered the limestone bedrock (usually <10 cm in depth) for a total of 18 samples. Roots were carefully removed from individual cores. Both leaf and soil samples were dried at 70°C for 48 h prior to stable isotope analyses.

Dried samples of entire fungal fruiting bodies, foliage, and soil were ground with a Thomas Wiley mini-mill (Thomas Scientific, Swedesboro, NJ, USA) and analyzed for $\delta^{13}C$ and $\delta^{15}N$. Analyses of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope ratios were performed using an elemental analyzer (Model ANCA-SL, Europa Scientific, Ltd., Crewe, UK) connected to a continuous flow isotope ratio mass spectrometer (Model 20/20, Europa Scientific) at the Center for Stable Isotope Biogeochemistry of the University of California, Berkeley. Stable isotope abundances are reported as: $\delta^{15}N$ or $\delta^{13}C$ (‰)=(R_{sample}/ $R_{standard}$ −1) × 1,000, where R=$^{15}N/^{14}N$ or $^{13}C/^{12}C$. The sample ratio is relative to the Vienna PeeDee Belemnite (VPDB) standard for carbon and N_2 atmospheric gas for nitrogen.

Student t-tests were used to determine significant (P< 0.05) differences in isotopic ratios between boletes collected in 2001 and 2005. Isotopic data from fungal fruiting bodies, plant foliage, and soil were subjected to a one-way ANOVA followed by post hoc LSD tests to determine significant (P<0.05) differences among different ecosystem compartments. Statistical analyses were conducted using SPSS statistical software (v.16.0; SPSS, 2007).

Results

We observed a total of 21 bolete fruiting bodies in 2005, yet many of these fruiting bodies were severely colonized by a *Hypomyces* species. We excluded fruiting bodies that were visibly colonized by *Hypomyces* from this study, and therefore, analyzed a total of 11 boletes for molecular phylogeny and isotopic values. In general, bolete fruiting bodies tended to be moderate in size and relatively slender (Fig. 1).The caps ranged in size from 3 to 7 cm, and the stipes were no greater than 2 cm in diameter. Pores were yellow at maturity, relatively large, and nearly adnate or somewhat depressed around the stipe apex. Stipe was relatively slender, lacked a bulbous base, and non-reticulated. If stipe was reticulated, then only in the upper part with elongated meshes, sometimes taking the appearance of longitudinal ribs. Spore print was brown with olive tinges.

We obtained sequences from all 11 bolete fruiting bodies, and based on sequence similarities, we indentified four distinct taxa. Two taxa consisted of single collections, whereas the other two taxa consisted of three and six independent collections. The closest match for boletes spp. one and four was with an uncultured *Boletus* species found on root tips of *Quercus* spp. in a tropical cloud forest in central Mexico (Morris et al. 2009). Based on morphology, all four taxa appeared to belong to the genus *Xerocomus* (Fig. 1), yet the closest matches in GenBank were less than 90%.

Among the boletes, there was no significant difference in $\delta^{13}C$ and $\delta^{15}N$ values between samples collected in 2005 and archive samples (P>0.05 for both; Fig. 2). Fruiting bodies of boletes and saprotrophic fungi, on the other hand, differed significantly in both $\delta^{15}N$ and $\delta^{13}C$, with boletes 8.0‰ enriched and 2.5‰ depleted in ^{15}N and ^{13}C, respectively, relative to saprotrophic fungi (P<0.001 for both; Table 1; Fig. 2).

The $\delta^{13}C$ values of wood-decomposing fungi ranged from −23.3‰ to −22.6‰, which was slightly higher than the range for litter-decomposing fungi (−25.0 to −23.3‰). On the other hand, the $\delta^{13}C$ values of boletes ranged from −26.7‰ to −25.2‰, with a mean value of −26.0±0.1‰ (Fig. 2). The mean $\delta^{13}C$ differed significantly among the different ecosystem compartments ($F_{4,71}$=175.5, P<0.001) and increased in the order: foliage < soil < boletes < litter-decomposing fungi < wood-decomposing fungi (Table 1).

The $\delta^{15}N$ values of wood-decomposing fungi ranged from 0.5‰ to 2.4‰, with a mean value of 1.5±0.3‰. On the other hand, the $\delta^{15}N$ values of boletes varied from 6.2‰ to 14.2‰, with a mean value of 9.3±0.5‰. Litter-decomposing fungi had $\delta^{15}N$ values more similar to wood-decomposing fungi and ranged from −1.8‰ to 3.4‰ (Fig. 2), with a mean value of 1.2±0.3‰. As observed for $\delta^{13}C$, mean $\delta^{15}N$ differed significantly among the different ecosystem compartments ($F_{4,71}$=92.9, P<0.001). However, the mean $\delta^{15}N$ ranking was different to that for $\delta^{13}C$ and increased in the order: litter-decomposing fungi < foliage < wood-decomposing fungi < soil < boletes (Table 1).

Fig. 1 Fruiting bodies of bolete species found in a seasonally dry tropical forest on the Northern Yucatan Peninsula. **a** Bolete sp. 1, **b** Bolete sp. 2, **c** Bolete sp. 3, **d** Bolete sp. 4

Discussion

Contrary to the traditional belief that tropical forests are impoverished in fungal species forming EM associations, recent mycological studies in tropical forests have revealed a wide diversity of EM fungi. For instance, the Dipterocarpaceae forests in Southeast Asia have been shown to be associated with various EM fungal genera (Natarajan et al. 2005; Smits 1992; Watling and Lee 1995) as have forests dominated by EM leguminous trees in western Guyana (Henkel et al. 2002). However, the majority of these studies have been conducted in wet tropical forests and/or in forest patches that are characterized by a single tree species that occupies 80% or more of the trunk basal area (Connell and Lowman 1989; Henkel et al. 2002; Natarajan et al. 2005; Newbery et al. 1988). Few studies have been conducted in lowland SDTF (Brundrett et al. 1996; Högberg 1992; Thoen and Ba 1989), and to our knowledge, no studies have examined the occurrence of EM fungi in the highly diverse, lowland SDTF on the Yucatan Peninsula. Despite the lack of similarity to known species, the low $\delta^{13}C$ and high $\delta^{15}N$ signature of bolete fruiting bodies relative to co-occurring saprotrophic fungi were highly suggestive of an ectomycorrhizal trophic status for these boletes. Our results not only provide the first isotopic evidence of the ectomycorrhizal status of boletes on the Northern Yucatan Peninsula, they also complement the recent review by Mayor et al. (2009) by providing the first isotopic data for EM fungi in a SDTF in general.

Given the limited number of mycological studies in this area, it is not surprising that we were unable to identify these boletes to known species. It is estimated that 7,000–

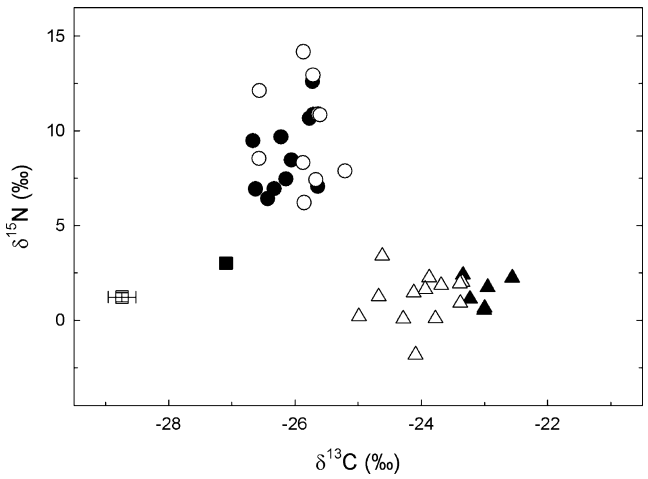

Fig. 2 Nitrogen and carbon isotope values for fungal fruiting bodies collected in a seasonally dry tropical forest on the Northern Yucatan Peninsula, Mexico. *Open circles*, boletes collected in 2001 (n=10); *closed circles*, boletes collected in 2005 (n=10); *filled triangles*, wood-decomposing fungi (n=6); *open triangles*, litter-decomposing fungi (n=13); *open square*, mean (±SE) foliage (n=18); *closed squares*, mean (±SE) soil (n=18)

Table 1 Mean (±SE) $\delta^{15}N$ and $\delta^{13}C$ values for foliage, soil, bolete, wood-decomposing, and litter-decomposing fungi collected at El Eden Ecological Reserve, Mexico in 2005

	$\delta^{15}N$ (‰)	$\delta^{13}C$ (‰)
Bolete fungi (11)	8.77±0.61	−26.12±0.11
Bolete sp. 1 (1)	9.66	−26.22
Bolete sp. 2 (1)	6.41	−26.43
Bolete sp. 3 (3)	7.61±0.45	−26.27±0.18
Bolete sp. 4 (6)	9.60±0.92	−25.97±0.17
Wood-decomposing fungi (6)	1.45±0.32	−23.01±0.11
Cookeina speciosa	0.65	−23.00
Cyathus colensoi	0.54	−23.01
Marasmiellus cubensis	1.13	−23.23
Scutellinia scutellata	2.38	−23.34
Trametes villosa	2.24	−22.56
Xylaria coccophora	1.74	−22.95
Litter-decomposing fungi (13)	1.17±0.32	−24.01±0.14
Agaricus sp. 1	2.25	−23.88
Agaricus sp. 2	0.20	−24.99
Agaricus sp. 3	1.46	−24.12
Agaricus sp. 4	0.07	−24.28
Coprinus sp. 1	2.01	−23.35
Coprinus sp. 2	1.64	−23.94
Coprinus sp. 3	1.94	−23.39
Leucocoprinus birnbaumii	1.85	−23.68
Marasmius haematocephalus	0.08	−23.77
Pholiota sp. 1	3.39	−24.62
Pholiota sp. 2	−1.83	−24.09
Psathyrella sp. 1	1.25	−24.68
Psathyrella sp. 2	0.91	−23.38
Foliage (18)	1.22±0.24	−28.74±0.22
Caesalpinia gaumeri (3)	0.21±0.19	−28.25±0.65
Coccoloba diversifolia (3)	1.42±0.11	−29.63±0.34
Esenbeckia pentaphylla (3)	2.68±0.37	−29.41±0.52
Lonchocarpus castilloi (3)	1.82±0.06	−28.32±0.52
Lysiloma latisiliquum (3)	1.28±0.36	−28.30±0.67
Vitex gaumeri (3)	−0.11±0.18	−28.55±0.28
Soil (18)	3.01±0.25	−27.09±0.10

Values in *parentheses* represent the number of independent collection for each taxon

10,000 species of fungi form EM associations worldwide (Taylor and Alexander 2005), many of which remain undescribed and likely inhabit poorly studied tropical ecosystems, such as lowland SDTF. For instance, recent mycological studies throughout the Caribbean region have found a number of previously undescribed EM species (Halling and Mueller 1999; see Ortiz-Santana et al. 2007). Additionally, the erratic nature in fruiting body production makes it difficult to study putative EM taxa. During 15 years of research at our site, we have only observed bolete fruiting bodies twice. Thus, the combination of few mycological studies, along with the sporadic nature of boletes fruiting body production, may help explain why we were unable to identify these boletes to known species using local taxonomical keys (Guzman 2003) as well as molecular techniques.

Bolete fruiting bodies were highly enriched in ^{15}N compared to other ecosystem compartments. On average, the $\delta^{15}N$ signature of bolete fruiting bodies was 6.3‰ higher than that of soil, which is similar to the 6.2‰ enrichment of EM fungi relative to soil reported by Kohzu et al. (1999). The $\delta^{15}N$ signature of boletes was also significantly higher compared to plant foliage and saprotrophic fungi (Table 1). Fractionation against the heavier isotope during the assimilation and transfer of nitrogen from EM fungi to host plants has been proposed to explain why EM fungi are often enriched in ^{15}N relative host plant foliage (Högberg et al. 1999b; Kohzu et al. 2000; Taylor et al. 1997). Additionally, mycelium morphology and repeated cycling of nitrogen may help explain high $\delta^{15}N$ values in EM fungi (Hobbie and Agerer 2010; Trudell et al. 2004). In contrast, the absence of nitrogen transfer to plants by saprotrophic fungi causes them appear $\delta^{15}N$ depleted relative to co-occurring EM fungi. Thus, the highly enriched $\delta^{15}N$ signature of bolete fruiting bodies relative to both saprotrophic fungi and tree foliage (Table 1) strongly supports an ectomycorrhizal trophic status for these boletes species.

Carbon isotopes have also been used to determine the trophic status for unknown species and are thought to be a more reliable indicator of the fungal trophic status than nitrogen isotopes (Hobbie et al. 2001). Differences in the $\delta^{13}C$ signature of EM and saprotrophic fungi are mainly attributed to fundamental differences in carbon sources (e.g., plant photosynthate vs. detritus) (Henn and Chapela 2001; Högberg et al. 1999a). Because EM fungi receive carbon from their host plant, the $\delta^{13}C$ signature of EM fungi tends to be more similar to the $\delta^{13}C$ signature of plant foliage relative to saprotrophic fungi, which are often enriched in ^{13}C relative to both plant foliage and EM fungi. Although bolete fruiting bodies were on average 2.8‰ enriched in ^{13}C relative to plant foliage, they were significantly depleted in ^{13}C relative to co-occurring saprotrophic fungi. Previous studies have shown EM fungi to be enriched in ^{13}C relative to plant foliage (Hobbie et al. 1999; Högberg et al. 1999a), and this enrichment has been attributed to the transfer of isotopically enriched sugars from the plant to EM fungi (Hobbie et al. 1999) and/or the respiration of isotopically light CO_2 by EM fungi (Boutton 1996). In contrast, saprotrophic fungi were on average 2.4‰ enriched in ^{13}C relative to bolete fruiting bodies, which is similar to the reported 2.3‰ enrichment of saprotrophic fungi relative to EM fungi across a global range of ecosystem types (Mayor

et al. 2009). Thus, the depleted $\delta^{13}C$ signature of bolete fruiting bodies relative to co-occurring saprotrophic fungi provides further evidence for an ectomycorrhizal trophic status of these boletes.

In conclusion, despite the lack of similarity to known species, isotopic analyses strongly support an ectomycorrhizal trophic status for these boletes. Our results not only provide the first isotopic evidence of EM fungi in a highly diverse SDTF on the Northern Yucatan Peninsula, they also highlight the need for further research to better understand the diversity and ecological importance of EM fungi in these forested ecosystems.

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