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Stable isotope evidence for the saprotrophic status of the truffle *Schenella pityophilus*

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Abstract: This research attempts to resolve the mode of nutrition, i.e., saprotrophic or ectomycorrhizal, for *Schenella pityophilus*, a sequestrate hypogeous fungus. While a majority of truffles in both the Ascomycota and the Basidiomycota are ectomycorrhizal, *S. pityophilus* is in the Geastrales in which species are saprotrophic. Stable isotope ratios of C and N have been used to elucidate fungal trophic modes. Stable isotopic ratios of N and C from fruiting bodies of *S. pityophilus* were compared to those of *Rhizopogon* species collected from the same sites and known to be ectomycorrhizal. Fruiting bodies of *S. pityophilus* were depleted in δ15N and enriched in δ13C relative to *Rhizopogon* species. Stable isotope data support the conclusion that *S. pityophilus* is saprotrophic rather than mycorrhizal.

Key words: ectomycorrhizal fungi, Geastrales, saprotrophic fungi, *Schenella pityophilus*, stable isotopes, trophic strategies

Introduction: The fruiting body of *Schenella pityophilus* is hypogeous and sequestrate—a truffle. The majority of truffle species, including those in both the Ascomycota and Basidiomycota, are ectomycorrhizal as detected by molecular methods in surveys of host roots (e.g., Valentine et al. 2004; Smith et al. 2007). However, *S. pityophilus* has not been documented in surveys of ectomycorrhizas based on molecular analyses. Initially, it was classified in the Myxomycota, but was reclassified in the Geastraceae/Geastrales as *Pyrenogaster pityophilus* (Macbride 1911; Malençon and Riousset 1977; Estrada-Torres *et al.* 2005). Further molecular studies put it in a separate family, the Pyrenogastraceae, corrected to the Schenellaceae based on the priority name of the genus (Martin 1961; Estrada-Torres et al. 2005; Hosaka *et al.* 2006). The Geastrales are saprotrophic, but the order is closely related to the Hysterangiales, in which *Hysterangium* is mycorrhizal (Sunhede 1989; Tedersoo et al. 2010). Although *Schenella pityophilus* is associated with ectomycorrhizal hosts, e.g., *Pinus ponderosa*, *P. jeffreyi*, and *Arbutus menziesii* (Domínguez and Castellano 1996), Trappe *et al.* (2009) indicated that it was a "probable saprotroph."

The purpose of this research was to determine the trophic status of *Schenella pityophilus*: mycorrhizal or saprotrophic. Stable isotope ratios of N and C differ between ectomycorrhizal and saprotrophic fungi with the general trend showing mycorrhizal fungi enriched in ^{15}N and depleted in ^{13}C relative to saprotrophic fungi (Hobbie *et al.* 1999; Högberg *et al.* 1999; Hobbie *et al.* 2001; Hobbie & Horton 2007; Whitridge & Southworth 2005; Hobbie and Högberg 2012). Surveys comparing the isotope ratios of saprotrophic and mycorrhizal fungi at particular sites have focused on epigeous fungi with a low fraction of hypogeous species, i.e., 0/45 (Hobbie et al. 2001); 1/135 (Högberg et al. 1999; Taylor et al. 2003); 1/34 (Hobbie and Agerer 2010) and 0/152 species (Trudell et al. 2004). While trophic status might determine the C and N isotopic ratios of both epi- and hypogeous fungi, other factors such as differences in outgassing of volatile compounds, in biomass and nitrogen composition, and in exploration types support the importance of determining isotope ratios for hypogeous fungi (Hobbie and Agerer 2010; D'Auria et al. 2013). Isotope ratios determined from herbarium specimens of 11 species of hypogeous fungi from around the globe were all within the range of epigeous mycorrhizal fungi (Hobbie et al. 2001).

Here we used isotopic ratios of fruiting bodies to determine the nutritional mode employed by *S. pityophilus*. We hypothesize that if *S. pityophilus* were saprotrophic, it would be depleted in $\delta^{15}N$ and enriched in $\delta^{13}C$ relative to ectomycorrhizal *Rhizopogon* species from the same sites or similar habitats to reduce variation in isotopic ratios due to climatic or latitudinal differences (Mayor et al. 2009).

Materials and Methods: A total of 15 hypogeous fruiting bodies, 7 of *Schenella pityophilus* (Malençon & Riousset) Estrada & Lado, 5 of *Rhizopogon truncatus* Linder, and 3 of *R. vinicolor* A.H. Sm., in the Basidiomycota, were collected from Jackson and Josephine Counties, Oregon, at elevations of 290-930 m (Table 1). Samples of 200 to 500 μg from glebal or peridial tissues were combusted in a varioPYRO cube (Elementar, Hanau, Germany). Stable isotope ratios and percent N and C were determined on the IsoPrime100 stable isotope ratio mass spectrometer (Isoprime Ltd., Cheadle, UK) at Southern Oregon University. Stable isotope ratios were calculated as: $\delta^{15}N$ or $\delta^{13}C$ (‰) = $(R_{sample}/R_{standard}) - 1 \times 1000$ where $R = {}^{15}N/{}^{14}N$ or $^{13}C/^{12}C$ for samples and standards. Reference standards were atmospheric N_2 for nitrogen and PeeDee belemnite carbonate for carbon. Sequences of the ITS region (Table 1) were determined by Gladish et al. (2010). Isotope data were analyzed by ANOVA and independent sample *t*-tests using Minitab 15 Statistical Software.

Results and Discussion: In *Schenella pityophilus*, differences in isotopic composition between gleba and peridium were significant for $\delta^{15}N$ ($p = 0.002$), but not for $\delta^{13}C$ ($p = 0.948$). The $\delta^{15}N$ was -3.51 $^{o}/_{oo}$ for glebal tissues and -0.38 $^{o}/_{oo}$ for peridial tissues. In *Rhizopogon* species, differences in isotopic composition between gleba and peridium were not significant for either N or C.

Between *S. pityophilus* and *Rhizopogon* species, isotope ratios of glebal tissues differed for both $\delta^{15}N$ ($p < 0.0001$) and $\delta^{13}C$ ($p < 0.0001$) (Figure 1). *Schenella pityophilus* was lower in ^{15}N (-2.00 $^{o}/_{oo}$) and higher in ^{13}C (-22.29 $^{o}/_{oo}$) than *Rhizopogon* species ($\delta^{15}N$, 4.29 $^{o}/_{oo}$; $\delta^{13}C$, -25.89 ‰). These ratios are consistent with those reported in the literature (e.g., Hobbie et al. 2001; Taylor et al. 2003; Trudell et al. 2004).

The differences in isotopic ratios indicate different modes of nutrition for *Schenella pityophilus* as compared with ectomycorrhizal *Rhizopogon* species. A higher proportion of ^{15}N in *Rhizopogon* relative to *Schenella* supports the hypothesis that *S. pityophilus* is saprotrophic. Several factors may explain the lower $\delta^{15}N$ values in *S. pityophilus (*Hobbie and Högberg 2012). Mycorrhizal fungi may more process more nitrogen-containing compounds or transfer more materials to host roots; thus, isotopic fractionation would leave a higher proportion of ^{15}N in the fruiting body (Hobbie 2005; Hobbie and Högberg 2012). By a similar mechanism, the hyphae of the peridium of *S. pityophilus* may more readily transfer ^{14}N to the developing gleba leaving higher ^{15}N in the peridium than in the gleba. Furthermore, saprotrophic and mycorrhizal hypogeous fungi may access source materials that differ in isotopic ratios (Hobbie and Horton 2007).

Isotopic ratios of C and N provide quantitative evidence for the nutritional mode of sequestrate hypogeous fungi. Other putative saprotrophic truffles may be confirmed by these methods.

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Table 1. Stable isotope ratios of glebal tissue from specimens of *Rhizopogon truncatus* and *R. vinicolor* and *Schenella pityophilus* from southern Oregon.

TAXON	Coll.	OSC	δ15 N (‰)	δ13 C (‰)	GenBank accession	Date	County	Location	Trees
R. truncatus	1073	151375	4.29	-25.26	FJ789620	12-May-07	Josephine	Star Flat	*Pinus ponderosa*, *Quercus garryana*
R. truncatus	1326	151376	4.64	-24.35		30-Apr-10	Jackson	China Gulch	*Q. garryana*, *Pseudotsuga menziesii*
R. truncatus	1502	151377	2.86	-27.96		16-Jul-10	Josephine	Peavine	*P. ponderosa*
R. truncatus	1876	151378	4.74	-27.30		17-Jun-11	Josephine	Limpy Creek siltstone	*P.ponderosa*, *P. lambertiana*, *Ps. menziesii*
R. truncatus	1877	151379	4.07	-27.92		17-Jun-11	Josephine	Limpy Creek siltstone	*P.ponderosa*, *P. lambertiana*, *Ps. menziesii*
R. vinicolor	1035	151380	6.24	-24.69	FJ789616	11-Dec-06	Josephine	Star Flat	*P.ponderosa*, *Q.garryana*
R. vinicolor	1036	151381	6.08	-24.86		11-Dec-06	Josephine	Star Flat	*P.ponderosa*, *Q.garryana*
R. vinicolor	1074	151382	1.38	-24.80	FJ789623	12-May-07	Josephine	Star Flat	*P.ponderosa*, *Q.garryana*
Mean (sd)			4.29 (1.60)	-25.89 (1.55)					
S. pityophilus	1045	151383	-1.16	-21.68	FJ789612	4-Mar-07	Josephine	Star Flat	*P.ponderosa*, *Q. garryana*
S. pityophilus	1052	151384	-0.10	-23.08	FJ789614	4-Mar-07	Josephine	Eight Dollar Mtn	*Ps. menziesii*, *Q. kelloggii*
S. pityophilus	1054	151385	-1.23	-22.15	FJ789613	4-Mar-07	Josephine	Eight Dollar Mtn	*Ps. menziesii*, *Q. kelloggii*
S. pityophilus	1057	151386	-1.77	-22.05		4-Mar-07	Josephine	Eight Dollar Mtn	*Ps. menziesii*, *Q. kelloggii*
S. pityophilus	1323	151387	-1.94	-22.38		30-Apr-10	Jackson	China Gulch	*Q.garryana* , *Ps. menziesii*
S. pityophilus	1828	151388	-5.67	-22.79		29-Apr-11	Josephine	Waldo saddle	*Ps. menziesii*, *Q. kelloggii*
S. pityophilus	1881	151389	-2.12	-21.91		17-Jun-11	Josephine	Limpy Creek Botanical Area	*Q. garryana*, *P. jeffreyi*
Mean (sd)			-2.00 (1.75)	-22.29 (0.49)					

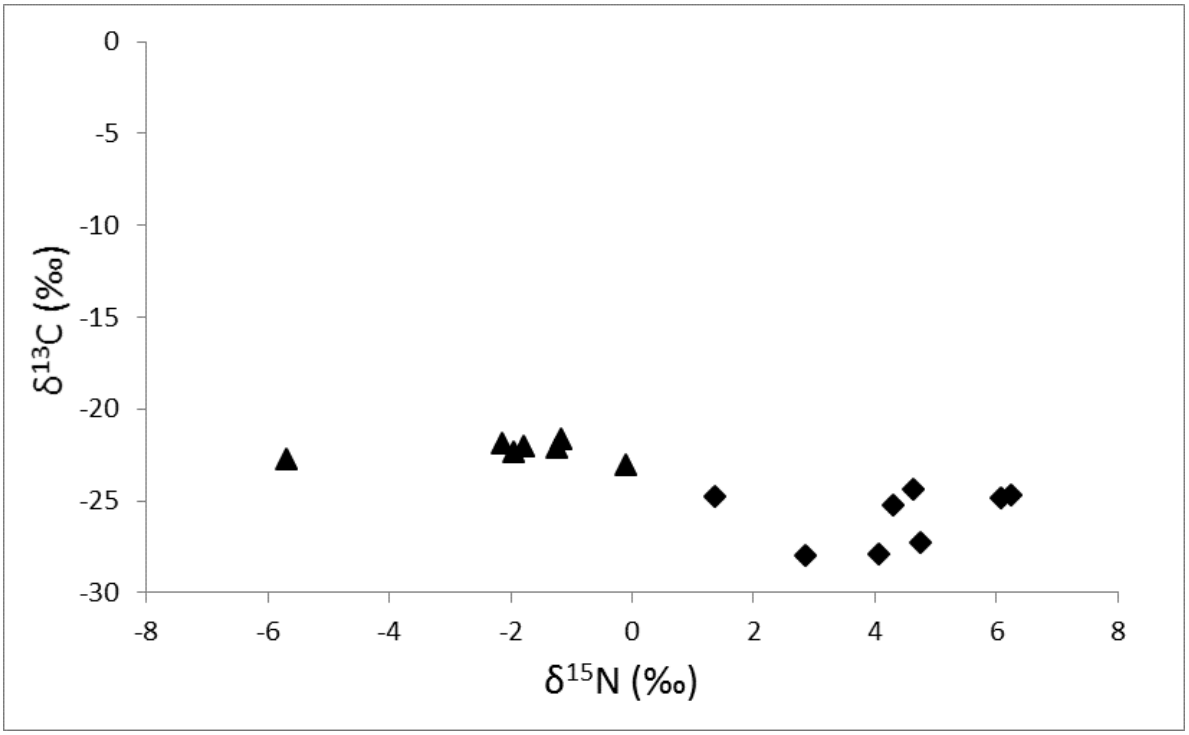

Figure 1. Relationship between stable isotope ratios, δ15N and δ13C, in the glebal tissues of *Schenella pityophilus* (triangles) and *Rhizopogon* species (diamonds).