Sporocarp δ15N and use of inorganic and organic nitrogen in vitro differ among host-specific suilloid fungi associated with high elevation five-needle pines

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A B S T R A C T

Widespread decline of whitebark and limber pines in the northern Rocky Mountains (USA) has created an imperative to understand functional diversity in their ectomycorrhizal associates. Because suilloid fungi are likely important in successful reestablishment of pines the nitrogen-related functional traits of 28 high-elevation suilloid isolates were examined. Radial growth, mass accumulation and mycelial density were measured for isolates on six different nitrogen sources. The $\delta^{15}N$ values of suilloid sporocarps used as sources for pure cultures were compared against growth parameters to investigate a possible link between these N-related functional traits. Isolates grew poorly on nitrate and BSA and grew well on glutamine, alanyl-glutamine and ammonium phosphate, with somewhat slower growth on alanine. Isolates and species varied considerably in their growth response to different nitrogen sources. Effective use of nitrate and BSA was uncommon and associated with isolates with high inherent growth rates. Sporocarp $\delta^{15}N$ was negatively correlated with relative growth on alanine of the corresponding isolates. Our results suggest strong similarities in N source use patterns of suilloid fungi of whitebark pine origin and those of another high-elevation five-needle stone pine, the Swiss stone pine.

1. Introduction

In conifer-dominated ecosystems, nitrogen often limits growth (Hawkins, Jones, & Kranabetter, 2015), and ectomycorrhizal (ECM) fungi play an essential role in accessing forms of nitrogen considered unavailable to nonmycorrhizal hosts (Smith & Read, 2008). Mutualistic ECM fungi may be of particular benefit at higher latitudes and elevations where nitrogen is predominantly present in organic forms (Read & Perez-Moreno, 2003). Under such conditions of short growing seasons and low soil temperatures, fungi are well suited to circumvent the mineralization-immobilization-turnover route traditionally considered to precede N uptake (Polacco & Todd, 2011; Schimel & Bennett, 2004).

Five-needle pines are a prevalent and functionally significant life form at the alpine-subalpine ecotone from a global perspective (Arno & Hammerly, 1984). In the northern Rocky Mountains of North America, *Pinus albicaulis* Engelm. (whitebark pine = WBP) and *Pinus flexilis* E. James (limber pine = LP), often grow in pure stands on rocky soils at high elevations where they associate with a unique set of host-selected or host-specific suilloid fungi (Cripps & Antibus, 2011; Mohatt, Cripps, & Lavin, 2008). These suilloid fungi, including species such as *Suillus sibiricus* (Singer) Singer studied herein, are of importance because they are specific to five-needle pines (Bruns, Bidartondo, & Taylor, 2002), associate with both seedlings and mature trees (Rainer et al., 2015), and play a role in nitrogen uptake (Cripps & Jenkins, 2015). These suilloid fungi are also important to restoration of WBP and LP as these pines are currently suffering range-wide die-offs from blister rust and mountain pine beetles (Keane et al., 2012, p. 106; Lonergan, Cripps, & Smith, 2014; Tomback, Achuff, Schoettle, Schwandt, & Mastrogiuseppe, 2011). In a recent review, Karst, Randall, and Gehring (2014) noted that loss of host pines at the landscape level, in this case WBP or LP, can lead to loss of host-specialist ECM fungi. The potential loss of suilloid species or genotypes and its effects create a critical need for studies aimed at understanding the

extent and variation in functional traits of host-associated ECM (Karst et al., 2014).

Generally, suilloid fungi have played an essential part in studies characterizing N-related functional traits of ECM fungi. Pioneering studies employing *Suillus bovinus* (L.) Roussel and *Rhizopogon roseolus* (Corda) Th. Fr., associated with two-needle *P. contorta* Douglas ex Loudon, demonstrated *in vitro* the ability of these species to use simple organic N sources and demonstrated their resynthesized ECM to provide N to hosts from bovine serum albumin (BSA) (Abuzinadah & Read, 1986a, b). Subsequent studies and field observations led to functional classifications into nitrophilic, protein, and nonprotein ECM fungi (Hawkins et al., 2015), and fostered suggestions that fungi from sites with lower or higher N availability differ in their use of different N sources (Lilleskov, Hobbie, & Horton, 2011; Talbot & Treseder, 2010). Most studies however have focused on a few species or employed only a small number of isolates (Cairney, 1999). Intraspecies trait variation is high in fungi (Crowther et al., 2014) and is expected to be critical in a community dominated by a small number of keystone species (Johnson, Martin, Cairney, & Anderson, 2012), as is true of WBP and LP stands. Intraspecies variation allows greater species coexistence and more complete resource use (Albert et al., 2010), with Kranabetter (2014) suggesting that site-adapted ECM fungi and non-redundant functional diversity are key elements to forest fitness and productivity. Intra- and interspecific variation both enhanced nitrogen use and ECM fungal community production in microcosm studies (Wilkinson, Solan, Taylor, Alexander, & Johnson, 2010; 2012). Similarly, intra- and interspecific diversity yielded complex but generally positive impacts on plant and ECM production with *Pinus sylvestris* L. seedlings (Hazard, Kruitbos, Davidson, Taylor, & Johnson, 2017). In the most comprehensive study of *Suillus* isolates to date Rineau et al. (2016) demonstrated widespread protease production *in vitro* with a range of species and species isolates. Most species demonstrated enhanced protease activity when sourced from sites with increased soil organic N availability, however exceptions were observed. In contrast, Keller (1996) studied three high elevation *Suillus* species and was unable to link protein use with stand age or soil development.

Genomic methods currently provide a broad-spectrum analysis of fungal communities and functional traits, however physiological studies are needed to confirm results (Branco et al., 2015). In pure culture studies, fungal traits are examined under standard conditions, allowing cross study comparison and illuminating patterns that foster hypothesis generation (Aguilar-Trigueros et al., 2015). Crowther et al. (2014) noted that isolated fungi are necessary to match traits to individuals or ecotypes. Here we evaluate both inter- and intraspecific variation in N-related functional traits of suilloid fungi tissue-cultured from sporocarps collected in WBP and LP forests and grown *in vitro* on different N sources.

An ongoing challenge has been relating differences in ECM fungal functions *in vitro* with ECM activity in soils. Studies of the natural abundance of ^{15}N provide time-integrated measures on the functional roles of ECM (Mayor et al., 2015). The examination of nitrogen stable isotope ratios (^{15}N:^{14}N, expressed as $\delta^{15}N$ values) of ECM sporocarps may provide complimentary insight into nutritional differences among these fungi (Hobbie et al., 2014). For example, work on Alaskan ECM fungi by Lilleskov, Hobbie, and Fahey (2002) demonstrated that protein use by pure cultures was positively correlated with high $\delta^{15}N$ in sporocarps, however few studies have tested the generality of this observation. A better understanding of how isolate physiology and sporocarp $\delta^{15}N$ link functionally for whitebark and limber pine ECM fungi will enhance our knowledge of their ecology and could provide an additional tool for identifying fungal species or strains of high conservation interest.

Pellitier and Zak (2017) recently highlighted a need for greater biogeographic coverage in studies dealing with nitrogen physiology of ECM fungi. To date Keller's study (Keller, 1996) on the European five-needle pine Swiss stone pine (*Pinus cembra* L.) remains the only such work on high-elevation ECM forests. In that study, inorganic and organic nitrogen use by *P. cembra* associates varied among species and individual strains, including several suilloid fungi. However, several of these isolates had been in culture for more than two decades, which can complicate interpretation (Anderson, Chambers, & Cairney, 2001). Here we examine *in vitro* inorganic and organic nitrogen use and further examine $\delta^{15}N$ of sporocarps from which the cultures were obtained. Our aim is to probe the linkage of these traits as well as the diversity in N-related functional traits of high-elevation suilloid fungi. Pure cultures of newly isolated suilloid fungi were used to test *in vitro* the following hypotheses: 1) ammonium is the preferred nitrogen source of these fungi; 2) protein use will be well-developed and widespread among these fungi; 3) interspecific differences among *Suillus* species will be robust to high levels of intraspecific variation; 4) protein use of pure cultures and $\delta^{15}N$ of the corresponding sporocarp will positively correlate.

2. Materials and methods

2.1. Collections and sites

The fungal isolates employed in the present study, with three exceptions, were obtained during the 2009 field season from stands dominated by either *P. albicaulis* or *P. flexilis* primarily in Montana (Table 1). Stands varied in tree age, slope, aspect and parent materials. Although all stands were on rocky soils; soil development varied and understory species when present included primarily grasses or ericaceous shrubs. Additional stand details for select sites are given in Mohatt (2006) and Mohatt et al. (2008). Fungal sporocarps were collected, identified and dried following standard practices accepted for macrofungi (Lodge, Ammirati, O'Dell, & Mueller, 2004). Voucher specimens of dried sporocarps were deposited in the MONT Herbarium fungal collection at Montana State University, Bozeman, Montana.

2.2. Culture and experiment

Isolations of fungi into pure culture were attempted within 24–36 h of sporocarp collection. Tissue was removed aseptically from sporocarp context tissue and placed on sterile MMN (modified Melin-Norkrans) agar (Molina & Palmer, 1982) supplemented with ampicillin and tetracycline at 50 mg l^{-1}. Parafilm-sealed petri dishes were incubated at room temperature (22–25 °C) until visible signs of growth appeared at which point they were transferred to modified MMN medium lacking antibiotics and malt extract while containing biotin at 1.0 μg per liter. Stock cultures were maintained on slants of this medium at 4 °C. A list of fungi employed in nitrogen source and stable isotopes analysis studies along with site of origin is given in Table 2.

Nitrogen source experiments were conducted within months of initial isolation. In addition to controls (no N source) treatments included, ammonium phosphate [$(NH_4)_2PO_4$], potassium nitrate (KNO_3), amino acids (L-alanine and L-glutamine), dipeptide (alanyl-glutamine) and protein (bovine serum albumin). All organic N sources exceeded 96% purity. The base media were MMN salts with glucose reduced to 5 g l^{-1} and ammonium phosphate removed from all but the ammonium treatment. Phosphorus for treatments lacking ammonium phosphate was supplied at equimolar concentrations with KH_2PO_4. For all treatments except nitrate the potassium concentrations were held constant by addition of KCl. A trace

Table 1
Sites from which fungal isolates were obtained. MT = Montana, WY = Wyoming.

Site	Abbreviation	Mountain Range	Elevation (m)	County, State
Avalanche Lake	AL	Madison	2750	Beaverhead, MT, USA
Beartooth Highway	BT	Absaroka– Beartooth	2890	Carbon, MT, USA
Crown Mountain	CM	Front	2020	Lewis and Clark, MT, USA
Dunraven Pass	DP	Absaroka	2700	Park, WY, USA
Fox Meadows	FM	Gallatin	2190	Gallatin, MT, USA
Golden Trout Lake	GTL	Gallatin	2590	Gallatin, MT, USA
Gravelly Mountains	GM	Gravelly	2590–2630	Madison, MT, USA
New World	NW	Absaroka–Beartooth	2590–3105	Park, MT, USA
Red Mountain	RM	Lewis	2320–2370	Lewis and Clark, MT, USA
Sacajawea	SA	Bridger	2700	Gallatin, MT, USA
Storm Lake	SL	Anaconda– Pintler	2560–2900	Deerlodge, MT, USA
Waterton Park	WP	Canadian Rockies	2300–2900	Alberta, CAN

Table 2
Isolates examined for response to nitrogen source with site and associated host plant information.

Species	Collection number	Location	Host plants	Year isolated
Rhizopogon sp.	Hyp1	WP	LP	2007
Rhizopogon evadens A.H. Sm.	CLC 2035	NW	WBP	2004
	CLC 2451	NW	WBP	2009
Rhizopogon milleri A.H. Sm.	CLC 2544	BT	WBP	2009
Rhizopogon roseolus (Corda) Th. Fr.	CLC 2469	BT	WBP	2009
	CLC 2489	AL	WBP	2009
	CLC 2475	CM	LP	2009
	CLC 2536	GTL	WBP	2009
Suillus discolor (A.H. Sm., Thiers & O.K. Mill.) N·H. Nguyen[a]	CLC 2422	FM	WBP-LP	2009
	CLC 2433	FM	WBP-LP	2009
	CLC 2441	GM	WBP	2009
	CLC 2500	SL	WBP	2009
	CLC 2510	DP	WBP	2009
	CLC 2539	BT	WBP	2009
Suillus sp.[b]	CLC 2199	DP	WBP	2005
	CLC 2467	BT	WBP	2009
	CLC 2480	RM	LP	2009
Suillus cf. *placidus* (Bonorden) Singer	CLC 2473	CM	LP	2009
Suillus sibiricus (Singer) Singer[c]	CLC 2421	SA	LP	2009
	CLC 2449	NW	WBP	2009
	CLC 2450	NW	WBP	2009
	CLC 2484	SA	LP	2009
	CLC 2540	BT	WBP	2009
Suillus subalpinus M.M. Moser	CLC 2347	DP	WBP	2006
	CLC 2487	AL	WBP	2009
	CLC 2505	SL	WBP	2009
	CLC 2508	SL	WBP	2009
	CLC 2533	GTL	WBP	2009
	CLC 2536	GTL	WBP	2009

[a] Older name *Suillus tomentosus* var. *discolor* A.H. Smith, Thiers & O.K. Mill. See Nguyen, Vellinga, Bruns, and Kennedy (2016).
[b] This group consists of morphologically similar fungi that do not fit descriptions of known species.
[c] Synonym *Suillus americanus* f. *sibiricus* (Singer) Klofac.

element solution was added to give final concentrations of: $CuSO_4 \cdot 5H_2O$ (0.13 mg l^{-1}), KI (7.5 mg l^{-1}), $FeSO_4 \cdot 7H_2O$ (3 mg l^{-1}), $MnCl_2 \cdot 4H_2O$ (5 mg l^{-1}), H_3BO_3 (1.5 mg l^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.0024 mg l^{-1}) and $ZnSO_4 \cdot 5H_2O$ (2 mg l^{-1}). Media were adjusted to pH 5.5 with either HCl or NaOH prior to autoclaving (20 min at 121 °C). Gellan (Phytagel, Sigma Life Sciences, St Louis, USA) was used at 6.5 g l^{-1} as a solidifying agent (Dickie, Koide, & Stevens, 1998). Gellan was chosen as it is solubilized by weak acids allowing dry mass determinations at the end of experiments. Organic N sources were filter-sterilized (0.2 μm) and added after autoclaving to media cooled to 55–60 °C. Final pH was verified once media had gelled. All treatments were adjusted to contain a final nitrogen concentration of 64 mg l^{-1} of N resulting in C:N values from 30:1 to 32:1.

Inoculum fungi in N source work were grown in plates with 20 ml MMN medium lacking malt extract at 22 °C for at least 21 d. Inorganic and organic nitrogen source plates were aseptically center-inoculated with one disk obtained from the active colony margin with a sterile 6 mm cork borer and sealed with parafilm. Three replicate plates were established for each treatment. Twenty 6 mm cork borer disks were placed on pre-dried and weighed filter paper and dried overnight at 60 °C. The average dry weights of these disks were used to remove the contribution of agar plugs from final mycelial dry weights. Plates were subsequently incubated at 22–25 °C. Plates were examined weekly and radial growth measurements were obtained in two directions at right angles. Data were recorded on colony morphology (Keller, 1996). At the end of the growing period plates were photographed by ambient natural light with lids removed on a Dreadnaught gray (# 6099 Daler-Rowney, Berkshire, UK). Mycelial areas were analyzed from digital images with Image J (Rasband, 2009).

Dry weights for density calculations were collected when the

fastest growing nitrogen treatment had covered approximately two-thirds of the plate surface (90 × 15 mm dishes), for our isolates this ranged from 21 to 42 d. Colonies were removed by cutting around the margins with a scalpel and transferring this to a weighing dish to obtain a fresh weight. Colonies were then cut into pie-shaped wedges and placed in plastic drink cups. Citrate buffer (pH 6.0) was then added to give a solution volume to mycelium-gel weight ratio of 10:1; the covered cups then stood 24–48 h prior to filtration and rinsing to remove Gellan (Dickie et al., 1998). Released mycelium on pre-dried filter paper was weighed after 24 h at 60 °C.

2.3. Stable isotopes

Stable isotope analyses were conducted on dried fruiting bodies from the same collection used for axenic culture isolations. Whole single fruiting bodies dried at 60 °C for 24–48 h were analyzed at the University of New Hampshire Stable Isotope Lab following protocols described by Ouimette, Guo, Hobbie, and Gu (2013). Stable isotope abundance is reported as $\delta^{15}N = (R_{sample}/R_{standard} - 1)$ in parts per thousand (‰) deviation from the standard, where $R = {}^{15}N/{}^{14}N$ of either samples or the standard of atmospheric N_2.

2.4. Data analysis

Linear growth rates were estimated by regressing average radius measurements against time after subtraction of controls (growth on media lacking nitrogen). Final dry masses were calculated following subtraction of masses of agar disks and control growth; these correspond to dry mass growth (*Gd*) (Keller, 1996). Mycelial density was calculated as dry weight per unit area corresponding to Keller's *Ga* (Keller, 1996). Direct comparisons of mass among isolates were not possible as values were determined after different periods of growth (to achieve similar mycelial areas). Dry mass growth rates were determined by dividing final mass by time, however, it cannot be assumed that mass growth rates were linear over this period. Relative growth on specific substrates was calculated as a percentage by dividing the mass growth on that substrate by the average mass growth across all six substrates and multiplying by 100%.

Linear regression, analysis of variance (ANOVA) and Pearson product-moment correlations were performed in R (v2.13.1; R Development Core Team, 2011). Prior to statistical testing, data were evaluated for compliance with relevant assumptions and transformed appropriately when necessary (Verzani, 2005). The effect size of treatments was estimated using the omega squared (ω^2) statistic according to Lakens (2013).

3. Results

3.1. Pure culture studies

Results of two-factor analysis of variance (ANOVA) employing all isolates indicated a significant effect on linear growth of species ($F_{(8,446)} = 37.351$, $p < 0.001$) and nitrogen source ($F_{(5,446)} = 40.963$, $p < 0.001$). The presence of a significant interaction effect ($F_{(40,446)} = 2.820$, $p < 0.0001$) precluded further testing for differences among species or N treatment. ANOVA of mycelial density indicated significant differences among species ($F_{(8,446)} = 39.543$, $p < 0.001$) and N source ($F_{(5,446)} = 22.421$, $p < 0.001$) as well as a significant interaction effect ($F_{(40,446)} = 3.742$, $p < 0.001$). No species comparisons were made for final dry mass values as isolates grew for different periods. However, estimated dry mass growth rate demonstrated significant species ($F_{(8,446)} = 26.177$, $p < 0.001$), N source ($F_{(5,446)} = 94.464$, $p < 0.001$) and interaction effects ($F_{(40,446)} = 2.800$, $p < 0.001$).

Linear growth, averaged across all isolates (Table 3), was greatest on glutamine (Gln), the dipeptide alanyl-glutamine (Ala-Gln) and ammonium (Amm). Linear growth on alanine (Ala) was lower than the aforementioned sources but significantly greater than for BSA and nitrate (Nit). Across all isolates and N sources linear growth was positively correlated with final dry mass ($r = 0.63$, $p < 0.001$, n = 498) and mass growth rate ($r = 0.79$, $p < 0.001$, n = 498) but negatively correlated with mycelial density ($r = -0.30$, $p < 0.001$, n = 498). Final dry mass and mass growth rates (Table 3) were greatest on glutamine, alanyl-glutamine and ammonium. Average dry mass on alanine was lower than on the aforementioned sources again significantly greater than with BSA and nitrate (Table 3). Mycelial density, calculated by dividing the final colony area by dry mass at harvest, was greatest on alanine. Growth on glutamine, ammonium and alanyl-glutamine resulted in similar densities, whereas nitrate and BSA resulted in substantially lower average densities (Table 3).

Visual evaluation of the patterns of linear growth and density associated with genus, species, isolate and N source are presented in Tables 4 and 5. Overall linear growth rates were greatest in *Suillus discolor* (A.H. Sm., Thiers & O.K. Mill.) N.H. Nguyen (a five-needle pine associate), *S. sibiricus*, *Suillus* sp. and a single isolate of *Rhizopogon* sp. Taxa with slower linear growth included *S. subalpinus* M.M. Moser, *R. evadens* A.H. Sm. and a single isolate of *R. milleri* A.H. Sm. Significant differences in density are evident in Table 5. Species with lower linear growth, *S. subalpinus, R. evadens* and *R. milleri,* produced mycelium with higher masses per area. Differences in density were evident when dishes were examined visually. *Rhizopogon* isolates, as a group, isolates produced denser mycelium than *Suillus* isolates.

The impact of N source on linear growth of species and isolates is evident in Table 4. Data demonstrate that, under the conditions of this experiment (temperature, pH, C:N), ammonium is not always a superior N source for radial growth compared to simple amino acids. Linear growth in many of our suilloid isolates on alanine (10 of 28) or glutamine (18 of 28) equaled or exceeded that on ammonium. Several *Suillus* and *Rhizopogon* isolates grew as well on the dipeptide alanyl-glutamine as on ammonium. Many isolates grew poorly with nitrate and BSA. Isolates differed greatly in their inherent linear growth rate based upon how quickly the original isolate grew on MMN agar, these inherent differences appear to explain some nitrogen source patterns. Strains of *S. discolor,* one of the fastest growing species, typically grew much better on glutamine than on ammonium. High overall growth rates of individual isolates of *S. discolor* and *S. sibiricus* corresponded with marked growth on BSA in these species as well. *Suillus subalpinus,* typically characterized by slower growing isolates, grew very poorly on nitrate. In fact, several isolates grew more slowly on nitrate than on controls lacking nitrogen, resulting in negative values when controls were subtracted (Table 4).

Mycelial density, a measure integrating mass and radial growth, was altered by N source with virtually all isolates demonstrating highest densities on alanine, glutamine and the dipeptide. Densities were noticeably lower on BSA than on other N sources in faster growing species like *S. sibiricus* and *S. discolor*, whereas *R. roseolus* isolates produced consistently higher densities on this source. The high variability in growth on nitrate and BSA compared to other N sources is evident in an examination of coefficients of variation (CV) across all isolates. The CV for density on nitrate was 72%, BSA 80%, ammonium 47% and 53–55% for the remaining organic N sources. Among *Suillus* isolates CV on nitrate was 81%, BSA 64%, ammonium 33% and 30–38% for remaining organic N sources.

We performed separate two-factor ANOVAs on species

Table 3
Summary growth measures for all isolates on various nitrogen sources. [a] mean ± standard error.

Nitrogen source	*n*	Linear growth (mm day^{-1})	Mass (mg)	Mass growth rate (μg day^{-1})	Density (mg cm^{-2})
Ammonium	84	0.84 ± 0.051[a]	24.9 ± 0.742[a]	729.2 ± 30.63[a]	1.97 ± 0.101[a]
Nitrate	82	0.21 ± 0.046	5.0 ± 0.763	155.9 ± 23.63	1.61 ± 0.127
Alanine	83	0.62 ± 0.058	20.3 ± 0.995	584.7 ± 34.24	2.41 ± 0.142
Glutamine	83	0.97 ± 0.066	27.7 ± 0.834	812.9 ± 37.66	1.96 ± 0.118
Alanyl−glutamine	84	0.85 ± 0.063	25.5 ± 0.810	743.8 ± 33.22	2.15 ± 0.127
Bovine serum albumin	84	0.34 ± 0.034	4.4 ± 0.706	122.6 ± 20.78	1.07 ± 0.093
Overall mean		0.64 ± 0.025	18.0 ± 10.543	525.7 ± 17.69	1.86 ± 0.052

Table 4
Growth rates of ectomycorrhizal fungi on different nitrogen sources: Amm = $(NH_4)_2PO_4$, Nit = KNO_3, Ala = L-alanine, Gln = L-glutamine, Ala-Gln = alanyl-glutamine and BSA = bovine serum albumin. The shaded bars represent growth of the isolate on different nitrogen sources. Numbers to the right represent average growth (mm day^{-1}) of each fungal strain represented in the bar at the left. CLC = isolate number.

Species	CLC	Amm	Nit	Ala	Gln	Ala-Gln	BSA
Rhizopogon evadens	2035	0.62	0.09	0.15	0.27	0.26	0.10
Rhizopogon evadens	2451	0.24	0.33	0.23	0.47	0.33	0.37
Rhizopogon milleri	2544	0.21	-0.04	0.38	0.37	0.34	0.07
Rhizopogon roseolus	2469	0.88	0.08	0.76	1.10	1.23	0.28
Rhizopogon roseolus	2475	0.30	0.23	0.22	0.26	0.44	0.32
Rhizopogon roseolus	2489	0.63	0.09	0.35	0.58	0.48	0.18
Rhizopogon sp.	Hyp1	1.70	0.26	0.52	1.64	1.83	0.29
Suillus cf. *placidus*	2473	0.92	-0.07	0.83	1.60	1.34	0.56
Suillus discolor	2422	1.11	0.12	0.33	0.64	0.42	0.16
Suillus discolor	2433	0.22	0.28	0.70	1.32	0.87	0.11
Suillus discolor	2441	1.39	0.90	1.89	2.11	1.81	1.37
Suillus discolor	2500	0.98	0.10	1.58	1.53	1.45	0.51
Suillus discolor	2510	0.92	1.55	1.60	1.91	1.31	0.65
Suillus discolor	2539	0.92	0.19	1.05	1.41	0.81	0.88
Suillus sibiricus	2421	1.17	0.82	1.17	1.18	1.14	0.63
Suillus sibiricus	2449	1.22	0.50	1.21	1.35	1.06	0.66
Suillus sibiricus	2450	0.73	0.00	0.33	0.96	1.01	0.55
Suillus sibiricus	2484	1.27	-0.03	0.85	1.52	1.48	0.19
Suillus sibiricus	2540	0.65	-0.13	0.15	0.30	0.16	0.15
Suillus sp.	2199	1.08	0.11	0.08	0.89	1.23	0.08
Suillus sp.	2467	1.06	0.10	0.93	0.96	0.85	0.34
Suillus sp.	2480	2.07	0.90	0.69	2.06	2.10	0.42
Suillus subalpinus	2347	0.80	-0.04	0.67	0.58	0.56	0.29
Suillus subalpinus	2487	0.40	-0.12	0.22	0.39	0.25	0.12
Suillus subalpinus	2505	0.36	-0.05	0.12	0.24	0.16	0.03
Suillus subalpinus	2508	0.35	-0.10	0.20	0.44	0.19	0.05
Suillus subalpinus	2533	1.11	0.13	0.20	0.97	0.68	0.32
Suillus subalpinus	2536	0.35	-0.09	0.18	0.33	0.14	0.07

represented with more than five isolates. A goal being to understand how intraspecific variation contributed to the patterns in response to N source. The results of separate ANOVAs of *S. discolor*, *S. sibiricus* and *S. subalpinus* are presented in Supplementary Table S1. In each species, there were both significant isolate and N source effects as well as significant interaction effects ($p < 0.001$). These results applied to both linear growth rate and mycelial density. The relative contributions of isolate and N source were evaluated by calculation of omega squared (ω^2) to estimate effect size (Table S1). Isolate-related properties across the three species of *Suillus* explained 30–40% of the variation in linear growth and 6–30% of the variation in density. Differences in N source accounted for 28–48% of the variation in linear growth and 14–47% of the variation in density. *Suillus subalpinus* growth responded more strongly to N source and less strongly to isolate-related properties than the other two species examined. This observation is explained by poor nitrate and BSA use by all *S. subalpinus* isolates, which is not true for the other two species. Several conspecific isolates were collected at different locations or times within the same site. Isolates CLC 2505 and CLC 2508 of *S. subalpinus* both collected at Storm Lake (Table 4) behaved similarly with respect to growth rate and N source response, as did isolates CLC 2449 and CLC 2500 of *S. sibiricus* from New World. In contrast, isolates CLC 2422 and CLC 2433 of *S. discolor* from Fox Meadows – a mixed WBP-LP stand – responded differently to several N sources.

3.2. Stable isotope studies

We examined sporocarp $\delta^{15}N$ values of 19 source collections of the pure cultures examined here. Our goal was to establish relationships between overall organic N use (especially protein) and sporocarp ^{15}N enrichment. Examined sporocarps were all enriched in ^{15}N with a $\delta^{15}N$ sample mean of 10.6‰ (±2.73 SD) and values ranging from 5.6 to 14.3‰. Average $\delta^{15}N$ values for *Rhizopogon* 11.0‰ (±3.49 SD) and *Suillus* 10.5‰ (±2.54 SD) were similar. Linear growth on alanine, glutamine, dipeptide and BSA was inversely

Table 5
Densities of ectomycorrhizal fungal mycelia on different nitrogen sources: abbreviations follow those in Table 4. Average density of each strain (mg cm^{-2}); numbers to the right represent average density of each fungal strain.

Species	CLC	Amm	Nit	Ala	Gln	Ala-Gln	BSA
Rhizopogon evadens	2035	3.5	1.5	6.4	5.7	5.5	0.7
Rhizopogon evadens	2451	3.2	2.1	4.4	2.5	3.4	1.1
Rhizopogon milleri	2544	4.5	4.1	3.2	3.9	4.0	3.1
Rhizopogon roseolus	2469	2.2	2.5	2.0	1.6	1.4	3.4
Rhizopogon roseolus	2475	1.3	1.8	1.8	1.4	1.6	1.5
Rhizopogon roseolus	2489	1.8	2.4	3.2	2.4	2.8	1.2
Rhizopogon sp.	Hyp1	1.6	3.8	3.8	1.5	1.4	2.7
Suillus cf. *placidus*	2473	2.7	1.8	2.8	1.3	1.7	0.9
Suillus discolor	2422	1.3	0.3	2.4	2.3	2.4	0.6
Suillus discolor	2433	2.3	0.7	1.7	1.0	1.2	0.4
Suillus discolor	2441	1.6	1.5	1.0	1.2	1.5	1.2
Suillus discolor	2500	1.6	0.3	1.1	1.2	1.0	0.6
Suillus discolor	2510	2.1	0.9	1.4	1.5	1.5	0.3
Suillus discolor	2539	2.0	0.9	2.1	1.3	2.1	0.4
Suillus sibiricus	2421	1.1	1.0	0.8	0.9	0.9	0.3
Suillus sibiricus	2449	1.6	1.2	1.4	1.5	1.9	0.4
Suillus sibiricus	2450	1.5	0.6	2.2	1.1	1.1	0.5
Suillus sibiricus	2484	2.0	4.1	1.9	1.6	1.3	1.9
Suillus sibiricus	2540	1.4	1.2	3.2	2.1	3.4	0.9
Suillus sp.	2199	2.0	2.3	1.0	2.6	2.0	0.9
Suillus sp.	2467	0.6	0.5	0.9	0.8	0.9	0.3
Suillus sp.	2480	0.8	1.4	2.0	1.2	1.1	0.5
Suillus subalpinus	2347	1.7	1.7	1.9	2.3	2.4	1.9
Suillus subalpinus	2487	2.9	3.0	3.1	2.5	2.8	1.0
Suillus subalpinus	2505	2.0	0.6	3.0	2.6	2.8	0.5
Suillus subalpinus	2508	2.0	1.4	2.8	2.3	2.7	1.5
Suillus subalpinus	2533	0.9	0.8	3.0	1.5	1.7	0.7
Suillus subalpinus	2536	3.1	1.2	3.4	2.9	3.6	0.7

Table 6
Summary of Pearson correlation coefficients (r) and associated probabilities relating growth in pure culture on different N sources with $\delta^{15}N$ of fruiting body from which isolate was obtained (n = 19). Probabilities are given above and to the right of blank diagonal spaces of each table whereas correlation coefficients are below left of blank diagonal spaces in each table. Significant correlations ($p < 0.05$) are shown in bold letters. Abbreviations follow those used in Table 4.

	Amm	Nit	Ala	Gln	Ala-Gln	BSA	$\delta^{15}N$
Linear growth							
Amm		**0.029**	**0.035**	**0.000**	**0.000**	**0.034**	0.213
Nit	0.499		**0.008**	**0.004**	**0.014**	**0.027**	0.437
Ala	0.486	0.591		**0.000**	**0.001**	**0.000**	**0.007**
Gln	0.794	0.628	0.790		**0.000**	**0.000**	**0.006**
Ala-Gln	0.817	0.552	0.699	0.932		**0.003**	**0.019**
BSA	0.488	0.505	0.750	0.756	0.646		**0.035**
$\delta^{15}N$	−0.300	−0.190	−0.597	−0.604	−0.531	−0.486	
Dry mass							
Amm		0.068	**0.000**	**0.000**	**0.000**	0.503	0.112
Nit	0.280		0.787	0.094	0.068	0.457	0.788
Ala	0.776	0.066		**0.000**	**0.002**	0.600	**0.002**
Gln	0.878	0.395	0.840		**0.000**	0.452	**0.014**
Ala-Gln	0.854	0.428	0.667	0.875		0.565	**0.029**
BSA	−0.164	0.182	−0.128	−0.184	0.141		0.885
$\delta^{15}N$	−0.377	0.066	−0.660	−0.554	−0.500	−0.036	

correlated with the $\delta^{15}N$ of sporocarps from which cultures were obtained (Table 6). *Rhizopogon* isolates with the highest average sporocarp $\delta^{15}N$ typically demonstrated the lowest linear growth rates. No significant correlations were observed for mycelium density and $\delta^{15}N$ (results not shown). We then compared $\delta^{15}N$ values against relative growth on the six different N forms, where relative growth was expressed as the percentage biomass accumulation per day for a given N source divided by the mean biomass accumulation per day for the six different N forms. In a stepwise multiple regression with species, the six nitrogen forms, %C, and the natural log of C:N as factors, the best-fit model only included species, %C, and relative growth on alanine as factors, as shown in Supplementary Tables S2a and S2b and Supplementary Fig. S1, with $\delta^{15}N$ negatively correlated with relative growth on alanine.

4. Discussion

4.1. N source use by pure culture studies

Whereas ammonium is generally assumed to be the preferred N source for ECM fungi (Smith & Read, 2008), we observed, contrary to our first hypothesis, that growth on amino acids often equaled or exceeded that on ammonium. We evaluated our results following Keller (1996) wherein he defined excellent N source use as achieving mass growth 50% or better than observed with ammonium phosphate for *P. cembra* ECM fungi. All our isolates met Keller's criterion for excellent growth on glutamine and 19 of 28 did for alanine. Furthermore growth exceeded that on ammonium in 19 of 28 isolates with glutamine and 12 of 28 isolates with alanine.

Use by ECM fungi of small peptides, compared to single amino acids, has received little attention (Chalot & Brun, 1998). Several of our five-needle associates used alanyl-glutamine at least as well as ammonium. By Keller's criterion all isolates had excellent growth on alanyl-glutamine and 17 of 28 isolates grew better on alanyl-glutamine than on ammonium. Better use of the dipeptide than inorganic sources by several fungi suggests this source either reduces the energy cost of N metabolism or supplements growth by providing carbon skeletons to central metabolism (Chalot, Kytöviita, Brun, Findlay, & Söderström, 1995).

Nitrate proved to be a poor N source for our set of fungi. Keller (1996) came to a similar conclusion for *P. cembra* associates, as have other *in vitro* studies (Smith & Read, 2008). Mycelial density was reduced by growth on nitrate. In contrast to our suilloid fungi, a LP-associated isolate of *Hebeloma* grown as part of this study did grow well on nitrate and BSA demonstrating increased densities on these sources (data not shown). Most of the fungi we have examined do grow on nitrate and belong to genera possessing genes for nitrate metabolism (Nygren et al., 2008).

Bovine serum albumin (BSA) as a substrate to examine protein use has been widely employed and better defined than alternative sources, permitting cross-study comparisons (Guidot, Verner, Debaud, & Marmeisse, 2005). Our BSA results are contrary to our hypothesis that protein use would be widespread in high-elevation suilloid fungi. Average growth of our isolates was poor on BSA. Average mass accumulation on BSA was similar to that on nitrate, but linear growth was higher, resulting in colonies with lower densities on BSA. Keller (1996) observed similar responses in at least some isolates of *S. sibiricus*, however Dickie et al. (1998) found BSA use to increase density in other ECM fungi.

Our results and Keller's contrast with those of Rineau et al. (2016). These authors demonstrated growth, protein degradation, and protease activity on BSA in a wide range of *Suillus* species. They also confirmed that isolates from sites with older soils and greater organic N accumulation produced higher protease activities than ones from younger soils. Our isolates came from mature or climax stands, although well-developed soil horizons are not a common feature in WBP forests. It would prove instructive to conduct further studies on isolates from sites with well-characterized but differing degrees of litter and understory development — unfortunately a dearth of healthy WBP stands remain.

4.2. Intra- and interspecies differences

As hypothesized, our results confirm interspecific differences robust to intraspecific variation in N-related functional traits among *Suillus* species. Significant interaction effects between species and N sources, across all isolates and with individual *Suillus* species, indicate that species respond in different ways to N sources. Wilkinson et al. (2010; 2012) have shown *in vitro* that both intra- and interspecific variation in ECM fungi can lead to increases in community mycelium production and metabolism. The work of Cripps and Grimme (2011) supports *in vivo* the importance of variation observed within and among different isolates of the suilloid species used in the current study. Greenhouse studies with mycelial inoculum demonstrate *S. sibiricus* and *S. discolor* colonized WBP and LP seedlings more quickly than did *S. subalpinus* and *Rhizopogon* species (Cripps & Grimme, 2011). The findings correlate with *in vitro* innate growth rate differences of species discussed below. Root colonization by *S. sibiricus* increased biomass and total N in needles of WBP seedlings under low nitrogen conditions in the greenhouse, confirming potential benefits of suilloid fungi (Jenkins, 2017).

Our data suggest an intriguing link between the inherent linear growth rate *in vitro* and the range of N sources used. *Suillus sibiricus* and *S. discolor* on average grew faster than *S. subalpinus* and made effective use of more forms of N. Inherently slower growing species *S. subalpinus* used fewer N sources which translates to higher ω^2 effect values; meaning N source explains a greater fraction of observed variation than it does for faster growers. Linear growth varied more among conspecific isolates of faster growing species than in slower growing species; again, reflected in ω^2 effect values (Supplementary Table S1). Likewise, faster growing *S. sibiricus* isolates in Keller (1996) appear better able to make use of nitrate and BSA than slower growing isolates. In slow growing species like *S. subalpinus* virtually none of the isolates grew on nitrate and few on BSA. As seen for linear growth N source explained a very large fraction of variation (high ω^2) in density. Changes in hyphal density with different N sources are generally linked with foraging strategies including N extraction from soil organic matter (Dickie et al., 1998). Our results, and those of Keller (1996), suggest growth of *Suillus* on a soluble protein source (BSA) is less dense or more explorative than growth on simpler organic N sources.

Contrary to our hypothesis significant use of BSA was uncommon in *Rhizopogon* species examined. *Rhizopogon* isolates grew more slowly than those of *Suillus* and produced quite dense mycelium. The differences, as in *Suillus*, were apparent upon visual inspection of colony morphology.

4.3. Stable isotope studies

Our data failed to support our hypothesis that protein use by pure cultures would positively correlate with $\delta^{15}N$ of associated sporocarp collections. All suilloid sporocarps examined were enriched in ^{15}N; an observation consistent with observations of many ECM genera across a range of environments (Mayor et al., 2015). However, linear growth on BSA was negatively correlated with sporocarp $\delta^{15}N$; cumulative mass growth and mycelial density were not significantly correlated with $\delta^{15}N$. Our findings indicating that isolates obtained from high $\delta^{15}N$ fungi (*Suillus* and *Rhizopogon*) grew poorly on BSA contrast directly with the findings of Lilleskov et al. (2002) wherein cultures of high $\delta^{15}N$ fungi grew well on BSA, although no overlap in genera occurs between the studies. Our findings do not eliminate the possibility that these suilloid fungi use other proteins or use BSA under different experimental settings. In addition, and contrary to our expectations, sporocarp $\delta^{15}N$ significantly and negatively correlated with linear growth on amino acids and the dipeptide. Fungi grew faster on these organic N sources if they were obtained from fruiting bodies with lower $\delta^{15}N$. When normalized by growth rates within individual strains, growth on alanine correlated strongly and negatively with sporocarp $\delta^{15}N$ (Supplementary Tables S2a and S2b and Supplementary Fig. S1). This pattern currently lacks a mechanistic explanation, although we note that relative growth on alanine is also negatively correlated with relative growth on BSA ($r = -0.52$, $p = 0.0238$, $n = 19$, Supplementary Fig. S1) so good relative growth on alanine may to some extent serve as a marker for taxa that are not as effective at growing on protein, although we are aware of potential limitations of BSA as a surrogate for soil proteins. Mayor et al. (2015) stated that differences in fungal physiology explain patterns in $\delta^{15}N$ of sporocarps independent of environmental factors. However, the $\delta^{15}N$ of field-collected sporocarps is dependent on both source-related factors (Hobbie et al., 2014) and the internal cycling of N within the ECM and fungal mycelium (Hobbie, Sánchez, & Rygiewicz, 2012; Lilleskov et al., 2002). Future studies could be conducted with ^{15}N-labelled compounds on pure cultures and resynthesized ECM to track the fate of nitrogen allocation in different fungal species or strains, although many common ECM fungi cannot currently be cultured.

4.4. Nitrogen source use variation

Studies of N-related functional traits of ECM fungi assume that species occurrence in the field reflects N form and availability (Cox, Barsoum, Lilleskov, & Bidartondo, 2010; Kranabetter, Friesen, Gamiet, & Kroeger, 2009). We started with the expectation that protein would serve as an important component of N nutrition for WBP and LP associates. Whereas this idea is plausible, knowledge of forms and distribution of soil proteins is scant and little is known regarding N economies in high-elevation WBP and LP forests. An

unexpected observation was the high capacity for use of amino acids and dipeptides among N forms examined. Examination of N source trait variation, by analysis of CVs related to hyphal densities, indicates amino acid and dipeptide use is more constrained in our fungi than is BSA or nitrate use. An analysis of Keller's (1996) density data supports this observation; his CV for nitrate is 189%, BSA 150%, asparagine 54% and glycine 69%. We don't know whether this degree of genetic conservation or expression is linked to a basic function like nutrient transfer in the ECM or an adaptation to the regular presence of amino acids in soil. Amino acids are prevalent in the diffusive flux of boreal forest soils (Inselsbacher & Näsholm, 2012), and under conditions of low litter input and slow nutrient cycling, typical of WBP and LP stands, competition could be significant for organic N monomers (Schimel & Bennett, 2004).

5. Conclusions

The importance of N as a limiting element has driven interest in the diversity of N-related functional traits of ECM fungi and degree to which these reflect local N economies (Kranabetter, 2014). Our results are based on a set of freshly isolated, host-filtered, high-elevation suilloid fungi and broadly support the existence of significant intra- and interspecific variation. Greenhouse experiments strongly support these elements of our *in vitro* results (Cripps & Grimme, 2011). Contrary to our hypothesis and work of Rainer et al. (2016) we did not observe widespread use of the protein BSA among our isolates. High inherent isolate growth rates were related to use of more forms of potentially available N. Contrary to expectations we failed to establish a positive link between sporocarp $\delta^{15}N$ and the *in vitro* use of protein by the derived culture. Finally, we demonstrate high levels of intraspecific variation in *Suillus* species especially for nitrate and protein use. Intraspecific variation in function is likely critical to the survival of fungi under stress such as ECM associates of WBP (Karst et al., 2014). Variation among species and isolates is hypothesized to permit greater species coexistence and more complete resource use, however *in vitro* results should be corroborated further with *in situ* mycorrhiza activity.

Disclosure

All necessary permits and permissions were obtained for the described field studies. Permission for field studies and sample collection was granted by the National Park Service, United States Department of Interior.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.myc.2017.11.007.

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