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SHORT NOTE

Ectomycorrhizal species associated with *Pinus radiata* in New Zealand including novel associations determined by molecular analysis

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Abstract Ectomycorrhizal (ECM) associates of the exotic plantation species *Pinus radiata* were investigated above and below ground over two years in the North Island of New Zealand. ECM species were identified using morphological and molecular (restriction fragment length polymorphism and DNA sequencing) analysis. Eighteen ECM species were observed fruiting above ground; 19 ECM species were identified below ground. In the above ground study, *Wilcoxina mikolae*, *Rhizopogon pseudoroseolus* and *Inocybe sindonia* were noted for the first time as ECM associates of *P. radiata* in New Zealand. Below ground, the species *W. mikolae, R. pseudoroseolus, Rhizopogon luteorubescens, Pseudotomentella* sp., *Pseudotomentella tristis* and *Tomentella* sp. were found as new associates of *P. radiata* in New Zealand. Additionally, six ECM types were found that could not be identified with molecular analysis. The putative ECM taxa *Tricholoma pessundatum*, *Laccaria laccata* and *Hebeloma crustuliniforme* were examined by molecular analysis, and species identifications were proposed to be changed to *Tricholoma* sp., *L. laccata* and *Hebeloma* sp. for specimens associated with *P. radiata* in New Zealand. The species identity of *I. sindonia*, previously unidentified to species level, was determined with direct sequencing.

Keywords Ectomycorrhiza · *Pinus radiata* · Exotic plantation forest · New Zealand · RFLP · DNA sequence analysis

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Introduction

Planting of exotic forest trees to increase timber production is a common practise in many parts of the world (Barroetaveña et al. 2005). In New Zealand, there are 1,790,000 ha of production forest (NZFOA 2009). *Pinus radiata* (D. Don 1836) was first introduced in 1850s (Shepherd 1990) as a shelter belt tree and is now the most important exotic plantation species in New Zealand. Today almost 90% of New Zealand's plantation estate is made up of *P. radiata*. Due to New Zealand's mild climate, *P. radiata* can grow almost continuously throughout the year without a dormancy stage, allowing a rotation of 25–30 years (Burdon 2002).

Species in the Pinaceae are ectomycorrhizal (ECM). The symbiosis with these fungal partners is fundamental for tree establishment, growth and survival (Smith and Read 1997). *P. radiata* was introduced to New Zealand into cultivation, both as seed and seedlings, on multiple occasions from California, England and Australia between 1859 and 1872 (Shepherd 1990). It does appear that fungal associates of the species were brought to New Zealand with the seedlings and adhering soil, yet it is unknown if ECM fungi were intentionally imported during this time.

Significant research on ECM of exotic plantation trees in New Zealand was carried out in the late 1970s–1990s by

Chu-Chou and Grace (Chu-Chou 1979, 1980; Chu-Chou and Grace 1983a, b, 1984a, b, 1987, 1988, 1990). However, mycorrhizal associations have not been a research focus since then. This previous research focused mainly on above ground ECM diversity as techniques necessary to identify below ground ECM species were limited to morphological identification and culturing from root tips, methods which left many ECM species present on root tips unidentified. As it is the below ground fungal communities that benefit the host, it is of importance to have an understanding of this community. The research presented here resumes the work on ECM of *P. radiata* in New Zealand, aiming to expand existing data and explore, especially below ground, ECM communities more rigorously by using methods such as molecular fingerprinting (restriction fragment length polymorphism, (RFLP)) and DNA sequencing. Therefore, a nursery and four *P. radiata* stands of varying age in Kaingaroa Forest, North Island of New Zealand, were examined, and sporocarp as well as soil core surveys were conducted in 2005 and 2006.

Material and methods

The research was conducted in monoculture *P. radiata* plantation sites in 2005 and 2006. Sites investigated were a nursery in Rotorua and four plantation stands in Kaingaroa Forest (2, 8, 15 and 26 years of age in 2006). All sites were located in the interior volcanic plateau of the North Island, New Zealand. All stands were *P. radiata* plantations in their third rotation and located within 2 km of each other. At all study sites, a 1-ha plot was established approximately in the middle of each stand (stand size ranging from 56 to 144 ha). Within each plot, sporocarps were surveyed along five permanent 100-m long, randomly positioned, parallel transects with the exception of the 15-year old site which had only two transects. The nursery site at Te Ngae was initially set up for surveying as 100 × 60 m in 2005, which was increased to 100 × 100 m in 2006.

Sporocarp surveys were carried out over two consecutive fruiting seasons in two to three weekly intervals from March to June each year, and specimens were identified based on macromorphology. Soil core collections were undertaken in June 2005, December 2005 (no sampling in the nursery) and May/June 2006, and a total of 84 soil cores were analysed. Soil cores (50 mm diameter, 400 mm length) were collected 60 mm from the tree base, placed in a plastic bag and stored at 4°C. In the nursery site, the whole seedling was extracted from the soil due to the small root system. Samples were processed within 1–2 weeks by soaking in distilled water overnight, followed by gentle washing with tap water over a 2-mm sieve, then colonised root tips were removed under a dissecting microscope (Zeiss, Jena, Germany) using forceps. ECM root tips were categorised into ECM morphotypes based on mantle colour and texture, root branching pattern, root tip shape and the morphology of mycelial strands and emanating hyphae (Agerer 1987; Goodman et al. 2003). Three to five representatives from each ECM morphotype from each soil core were chosen randomly for DNA extraction.

Initially, DNA was extracted from sporocarps, and ECM colonised root tips using the FastDNA® Kit and the FastPrep® Instrument (Qbiogene Inc., Valencia, CA, USA), following manufacturer's instructions; however, polymerase chain reaction (PCR) amplification rate of DNA from colonised roots was 50% only. The use of the CTAB extraction method (Gardes and Bruns 1996), a modified CTAB & Phenol extraction method (Sambrook et al. 1987) and the DNeasy® Plant Mini Kit (Qiagen Inc., Hilden, Germany), did not increase the PCR success rate. A change to the plant DNA extraction kit REDExtract-N-Amp™ Plant PCR kit (Sigma, St. Louis, Missouri, USA) increased the PCR success rate to 100%. For sporocarp DNA extraction, the manufacturer's instructions were followed. For extraction of DNA from ECM root tips, the manufacturer's instructions were modified as follows: 50 µl of extraction and 50 µl of dilution solution were added to a sample, and mycorrhizal root tips were broken into pieces with a pipette tip when adding the extraction solution (Avis et al. 2003).

The internal transcribed spacer (ITS) regions of the recombinant DNA were amplified using the fungal specific primer combination of ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993). PCR was performed as described in Walbert (2008). PCR products were purified using the GenElute PCR Clean-Up Kit (Sigma). Only PCR products consisting of a single band were used for sequencing and RFLP analysis. At least two representatives of an ECM morphotype were used for a preliminary sequence analysis screening; all representatives of an ECM morphotype were used for RFLP analysis. Where putative basidiomycete mycorrhizae produced more than one PCR product, the DNA was amplified with the basidiomycete primers ITS1F and ITS4B (Bruns and Gardes 1993), before using a nested reamplification with ITS1F and ITS4 to allow subsequent comparison of RFLPs and sequences (Genney et al. 2006). RFLP patterns were generated with *Alu*I (Roche Applied Science, Penzberg, Germany), *Hinf*I (Roche Applied Science) and *Mbo*I (Invitrogen, Carlsbad, USA) as described in Walbert (2008) and compared using the spreadsheet-based freeware GERM (Good-Enough RFLP Matcher; Dickie et al. 2003). A representative of the ITS from each ECM-RFLP type was cloned using the pGEM-T® Easy Vector System (Promega Corporation, Madison, USA) as outlined in Walbert (2008). DNA sequences were edited and aligned using Sequencher version 4.7 (GeneCodes Corp. Ann Arbor,

MI, USA), and identities were determined by BLAST (Altschul et al. 1990) searching of GenBank and UNITE (Kõljalg et al. 2005) nucleotide databases. For identification, a minimum of 95% sequence identity to an ITS sequence of at least 450 bp from a known specimen in the database was required. Those samples with 97–100% identity match to a known species were considered a match and named to the species level. Those sequences with 96% or lower identity to known sequences were named to the genus, family or order. Samples that had no ITS sequence match were referred to as unknown 1, 2, etc. (Ashkannejhad and Horton 2006).

Results

During the sporocarp surveys in 2005 and 2006, 18 ECM taxa were observed (Table 1). Out of these, *Wilcoxina mikolae*, *Rhizopogon pseudoroseolus* and *Inocybe sindonia* were noted for the first time as ECM associates of *P. radiata* in New Zealand. *Lactarius rufus* is known to occur in New Zealand; however, no formal collections from *P. radiata* have been officially noted to date. The three soil core assessments revealed the presence of 19 distinct ECM morphotypes. A total of 31,520 ECM root tips were counted in the 84 analysed soil cores (Table 2). From the below ground study, *W. mikolae*, *R. pseudoroseolus*, *Rhizopogon luteorubescens*, *Pseudotomentella* sp., *Pseudotomentella tristis* and *Tomentella* sp. were found to be unreported ECM associates of *P. radiata* in New Zealand.

Comparison of RFLP patterns from ECM morphotypes with unknown identity to sporocarps of known identity positively identified *Amanita muscaria* and *Hebeloma* sp. only (Table 1). RFLP patterns from *Inocybe* sp., *Thelephora terrestris*, *Rhizopogon rubescens* and *R. pseudoroseolus* ECM could only tentatively be matched to respective sporocarps RFLPs, but confirmed with direct sequencing of the cloned material and *in silico* RFLP patterns (Table 1).

Assumed *Tricholoma pessundatum* specimens collected throughout the study and reference material obtained from the New Zealand Fungal Herbarium (PDD) were not positively identified to species level using either morphological or molecular methods; hence, specimens were noted only to genus level. *Hebeloma* specimens collected were positively identified as *H. crustuliniforme* based on morphological characteristics; however, sequence analysis of collected and reference material from PDD did not confirm this identity and could not resolve the species identification. The organism label *Hebeloma* sp. was applied accordingly. *Laccaria* specimens collected during this study were morphologically identified as either *Laccaria proxima* or *L. laccata*, but sequencing confirmed all specimens analysed as *L. proxima*.

Discussion

This research presents a comprehensive study of ectomycorrhizal species associated with *P. radiata* in a plantation forest in New Zealand using both morphological and molecular criteria. This approach has not been used for this plantation species in New Zealand before. The methods employed in this study increased the knowledge on ECM species colonising *P. radiata* in New Zealand and clarified the identities of several species. Chu-Chou and Grace's extensive work on mycorrhizal associates of *P. radiata* in New Zealand in the late 1970s–1990s (Chu-Chou 1979, 1980, Chu-Chou and Grace 1983a, b, 1984a, b, 1987, 1988, 1990) identified 17 taxa as ECM partners of *P. radiata* (Table 3). Species recorded by Chu-Chou and Grace were not observed in this study, may be due to the duration of this study or absence from the sites that were investigated. During this study's sporocarp surveys, *I. sindonia* (synonym *Inocybe eutheles* (Berk. & Broome) Sacc.), *R. pseudoroseolus* and *W. mikolae* were observed for the first time in New Zealand as ECM associates of *P. radiata*. Although these species are known mycorrhizal associates (Molina and Trappe 1994; Yu et al. 2001; Cairney and Chambers 1999), these are new reports to New Zealand.

Through the belowground surveys and molecular analysis, the following species were identified as previously unreported ECM symbionts of *P. radiata* (in New Zealand): *Pseudotomentella* sp., *Pseudotomentella tristis*, *Tomentella* sp., *R. pseudoroseolus*, *R. luteorubescens* and *W. mikolae*. All of these are new reports to New Zealand with the exception of *Tomentella* sp. This species as well as other resupinate thelephoroid fungi have been considered to be mainly saprotrophs in New Zealand for a long time. Kõljalg et al. (2000) demonstrated the symbiotic nature of several *Tomentella* spp. and highlighted that tomentelloid fungi are common ECM symbionts in boreal and temperate forests. This present study showed for the first time that this group is also associated with *P. radiata* in New Zealand.

The identity of the putative ECM taxa *T. pessundatum*, *Laccaria laccata* and *Hebeloma crustuliniforme* was reassessed with direct sequencing. These three taxa are reported ECM associates of *P. radiata* in New Zealand (Chu-Chou 1979, 1980; Chu-Chou and Grace 1988). Putative representatives were collected during this study; however, sequence analysis of this material did not confirm the suggested species names. In the case of both *T. pessundatum* and *H. crustuliniforme*, sequence analysis of collected material as well as reference material from the New Zealand Fungal Herbarium (PDD; Maanaki Whenua-Landcare Research, Private Bag 92170, Auckland 1030, New Zealand) did not resolve species classification; hence, specimens associated with *P. radiata* collected in this

Table 1 ECM taxa/morphotypes observed above and below ground from different aged *P. radiata* stands and a nursery (2005 and 2006)—accession number of sequences from this study, closest match to NCBI or UNITE database and Internal transcribed spacer (ITS)—*in silico* restriction fragment length polymorphism (RFLP) generated by restriction digest with enzymes *Alu*I, *Hinf*I and *Mbo*I of ectomycorrhizal fungi from root tip (R) or sporocarps (S)

ECM taxa/ morphotype	Source	Accession	Closest match	Match (NCBI/ Unite)	Uncut (bp)	*Alu*I fragment length[a] (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Amanita muscaria[b] (L.) Lam. (1783)	S	GQ267469	AB080983	98	711	19, 105, 197, 390	8, 347, 356	60, 97, 224, 330
Amanita muscaria	R	GQ267468	AB080983	98	711	19, 104, 198, 390	8, 347, 356	60, 97, 224, 330
Cenococcum geophilum Fr. (1829)	R	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Chalciporus piperatus (Bull.) Bataille (1908)	S	GQ267470	AF335357	99	731	59, 727	8, 62, 111, 165, 205, 235	10, 53, 60, 76, 280, 307
Hebeloma sp.	S	GQ267472	EF411103	98	731	191, 211, 329	8, 331, 392	60, 260, 411
Hebeloma sp.	R	GQ267471	EF411103	98	733	190, 213, 330	8, 331, 394	60, 262, 411
Inocybe lacera (Fr.) P. Kumm. (1871)	S	GQ267473	AY750157	99	722	182, 214, 326	8, 74, 258, 382	20, 60, 230, 412
Inocybe sindonia[d] (Fr.) P. Karst. (1879)	S	GQ267474	UDB002392[e]	n/a	731	144, 587	5, 8, 187, 209, 322	60, 269, 402
Inocybe sp.	S	GQ267476	DQ974812	88	745	154, 181, 410	8, 200, 214, 323	60, 101, 102, 180, 302
Inocybe sp.	R	GQ267475	DQ974812	88	745	154, 181, 410	8, 200, 214, 323	60, 101, 102, 180, 302
Laccaria proxima (Boud.) Pat. (1887)	S	GQ267477	DQ068958	99	730	15, 94, 96, 128, 397	8, 191, 202, 329	60, 261, 409
Lactarius rufus[d] (Scop.) Fr. (1838)	S	GQ267478	EF685089	100	763	79, 162, 522	8, 108, 264, 383	60, 106, 134, 206, 257
Pseudotomentella sp.[d]	R	GQ267479	DQ377428	96	721	16, 68, 75, 96, 193, 273	8, 349, 364	60, 232, 429
Pseudotomentella tristis[d] (P. Karst.) M. J. Larsen (1971)	R	GQ267480	AJ889968	100	788	19, 32, 43, 64, 69, 96, 149, 316	8, 366, 414	60, 64, 170, 234, 260
Rhizopogon luteolus Fr. (1817)	S	GQ267481	AF062936	97	889	67, 221, 601	7, 8, 128, 159, 228, 359	60, 62, 74, 78, 254, 361
Rhizopogon luteorubescens[d] A.H. SM. (1966)	R	GQ267482	AJ810038	99	762	94, 668	11, 23, 112, 137, 236, 243	60, 62, 161, 234, 245
Rhizopogon pseudoroseolus[d] A. H. Sm. (1996)	S	GQ267484	AJ810042	99	765	94, 277, 394	8, 23, 112, 144, 235, 243	60, 62, 160, 238, 245
Rhizopogon pseudoroseolus	R	GQ267483	AJ810042	99	765	94, 277, 394	8, 23, 112, 144, 235, 243	62, 62, 160, 238, 245
Rhizopogon rubescens (Tul. & C. Tul.) Tul. & C. Tul. (1844)	S	GQ267485	AF158018	95	737	94, 251, 392	8, 11, 23, 113, 129, 210, 243	60, 62, 135, 234, 246
Rhizopogon rubescens	R	GQ267486	AF158018	95	738	94, 252, 392	8, 11, 23, 113, 129, 211, 243	60, 62, 136, 234, 246
Scleroderma bovista[b] Fr. (1829)	S	GQ267487	AB099901	100	699	18, 59, 212, 410	8, 37, 116, 252, 286	12, 60, 124, 152, 173, 178
Suillus sp.	S	GQ267488	AY880932	99	723	109, 614	8, 11, 23, 37, 74, 92, 124, 129, 225	60, 62, 69, 141, 157, 234
Thelephora terrestris Ehrh. (1787)	S	GQ267490	U83486	98	701	30, 70, 601	8, 100, 241, 352	56, 60, 220, 365
Thelephora terrestris	R	GQ267489	U83486	98	700	30, 69, 601	8, 100, 240, 352	55, 60, 220, 365
Tomentella sp.[d]	R	GQ267491	DQ990851	95	700	30, 54, 67, 91, 458	8, 144, 177, 177, 194	55, 60, 363
Tricholoma sp.	S	GQ267492	AF458435	96	737	40, 77, 117, 503	8, 336, 393	60, 175, 241, 261
Tuber sp.	R	GQ267493	AY748861	99	688	no cut site	8, 70, 101, 147, 362	49, 60, 230, 349

Table 1 (continued)

ECM taxa/ morphotype	Source	Accession	Closest match	Match (NCBI/ Unite)	Uncut (bp)	*Alu*I fragment length[a] (bp)	*Hinf*I fragment length (bp)	*Mbo*I fragment length (bp)
Wilcoxina mikolae[d] (Chin S. Yang & H.E. Wilcox) Chin S. Yang & Korf (1985)	R	GQ267499	DQ069000	98	636	no cut site	8, 161, 183, 284	49, 60, 97, 212, 218
Unknown Basidiomycete	R	GQ267494	AB211143	98	722	10, 15, 94, 95, 128, 380	8, 328, 386	60, 121, 133, 408
Unknown 2	R	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Unknown 8[c]	R	GQ267496	AY641466	91	769	183, 586	93, 142, 171, 363	60, 188, 231, 290
Unknown 9	R	GQ267497	AM1096	99	636	no cut site	8, 66, 241, 321	49,, 58, 60, 130, 339
Unknown 10	R	GQ267498	AM901986	90	632	183, 449	66, 248, 318	49, 50, 60, 185, 288
Unknown 12	R	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Primer pairs ITS1F and ITS4 were used for the PCR amplification of the ITS region

n/a not applicable, ECM type failed to amplify or identification based on morphology only

[a] *Alu*I gave rise to many partial digests

[b] Sequences appeared to be from mixed PCR product. To gain full sequence for *in silico* RFLP analysis, the sequence from the ITS1F and ITS4 primer was aligned with the GenBank result, and consensus bases assigned

[c] Reverse sequence only

[d] New report as ECM associate of *P. radiata* in New Zealand

[e] UNITE specific accession number, no percent match applicable from this database

study were identified to genus level only. It is interesting that in New Zealand, *Hebeloma* sp. associated with *P. radiata* have never been observed fruiting in a plantation forest (Chu-Chou 1979; Walbert 2008). Furthermore, colonisation of root tips was only observed during the first year of outplanting (Walbert 2008). This is in contrast to reports from Western Australia where the taxon is widely distributed in exotic plantations and was associated with pines up to 60 years of age (Dunstan et al. 1998). Phylogenetic studies on the genus *Hebeloma* (Aanen et al. 2000; Boyle et al. 2006) suggest that there has been a rapid recent speciation within the genus. This could imply that *Hebeloma* species associated in *Pinus* sp. in Australia are different to the ones observed in New Zealand and consequently explain the difference in the presence of *Hebeloma* sp. in older forests. Specimens of *Laccaria* collected in this study were found to be the taxa *L. proxima*, not *L. laccata*, even though the latter species was reported to be the only *Laccaria* species as a mycorrhizal partner of *P. radiata* in New Zealand in Chu-Chou and Grace's research (Chu-Chou 1979; Chu-Chou and Grace 1983a, 1985, 1988, 1990). The high plasticity within the *Laccaria* genus makes it difficult to distinguish species like *L. proxima* and *L. laccata* morphologically, where only minor differences in spore size, shape and ornamentation are discriminating (Gardes et al. 1990). It is suggested that the *Laccaria* species associated with *P. radiata* in New Zealand is *L. proxima* and not *L. laccata*.

Chu-Chou and Grace's work covered a wide range of nurseries and plantation sites in both the North and South Island of New Zealand. The studies were undertaken over a period of 20 years and identified 17 taxa as ECM associates of *P. radiata* in New Zealand (Table 3). In contrast, this study investigated one nursery site and one plantation forest over the course of 2 years and found 28 ECM taxa (above and below ground surveys combined)

Table 2 Below ground surveys of *P. radiata* nursery and stands of different age (2005 and 2006): Total number of soil cores analysed, average observed richness and average number of root tips/soil core counted

	Total number of soil cores analysed	Average observed richness	Average number of root tips/soil core counted
Nursery	11	5.0	1163
2 years	26	3.3	159
8 years	17	6.7	197
15 years	15	7.7	270
26 years	15	9.3	414

Table 3 List of ECM taxa found, associated as sporocarps with *P. radiata* in New Zealand identified in Chu-Chou and Chu-Chou and Grace publications, and ECM taxa found as sporocarps and as colonising root tips in the present study

ECM taxa	Chu-Chou and Chu-Chou & Grace publications (year of publication)	Present in this study
Amanita muscaria	1979, 1983 a,1987, 1988	+
Cenococcum geophilum	–	+
Chalciporus piperatus	1979	+
Hebeloma sp.[a]	1979, 1983 a, 1985, 1987, 1988, 1990	+
Endogone flammicorona	1983a, 1983b, 1984,1988	–
Inocybe lacera	1979, 1983a, 1987, 1988	+
Inocybe sindonia	–	+
Inocybe sp.	–	+
Laccaria proxima[b]	1979, 1983a, 1985, 1987, 1988	+
Lactarius rufus	–	+
Lycoperdon gunnii	–	+
Lycoperdon perlatum	1983b	–
Lycoperdon sp.	–	+
Pseudotomentella sp.	–	+
Pseudotomentella tristis	–	+
Rhizopogon luteolus	1979, 1983a, 1983b, 1985, 1988, 1990	+
Rhizopogon luteorubescens	–	+
Rhizopogon pseudoroseolus	–	+
Rhizopogon rubescens	1979, 1983a, 1983b, 1985, 1987, 1988, 1990	+
Scleroderma aurantium	1979, 1983a	–
Scleroderma bovista	1983b, 1987	+
Scleroderma verrucosum	1979, 1983a, 1983b, 1987	–
Suillus luteolus	1979, 1983a, 1987, 1988, 1990	–
Suillus granulatus	1979, 1988	–
Suillus sp.	–	+
Thelephora terrestris	1979, 1983a, 1988, 1990	+
Tomentella sp.	–	
Tricholoma sp.[c]	1983a, 1988	+
Tuber sp.	1983b, 1984, 1987, 1988,1990	–
Wilcoxina mikolae	–	+
Unknown Basidiomycete	–	+
Unknown 2	–	+
Unknown 8	–	+
Unknown 9	–	+
Unknown 10	–	+
Unknown 12	–	+

[a] *Hebeloma* sp. listed as *Hebeloma crustuliniforme in* Chu-Chou and Grace's publications

[b] *Laccaria proxima* listed as *Laccaria laccata* in Chu-Chou and Grace's publications

[c] *Tricholoma* sp. listed as *Tricholoma pessundatum* in Chu-Chou and Grace's publications

associated with *P. radiata*. The assessment of both, the above and below ground mycorrhizal system with molecular identification has increased our knowledge of fungal species associated with this exotic host. Still, several morphological types remained unidentified, and these could potentially be native ECM species as sequence data from native ECM species is limited. To fill gaps in this area of research, more work on both native and exotic ECM is required.

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