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SHORT NOTE

Infrageneric variation in partner specificity: multiple ectomycorrhizal symbionts associate with *Gnetum gnemon* (Gnetophyta) in Papua New Guinea

Leho Tedersoo · Sergei Põlme

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Abstract Majority of autotrophic plants and fungi associate with multiple mycorrhizal partners, with notable exceptions being *Gnetum africanum*, *Pisonia grandis*, and *Alnus* spp from the phytobiont perspective. We hypothesized that an understorey tree species *Gnetum gnemon* hosts a narrow range of mycobionts as shown in *G. africanum* and suggested for South American species. Sampling and molecular analysis of *G. gnemon* root tips revealed that besides *Scleroderma* spp. this gymnosperm tree associates with several fungal species from unrelated lineages. However, all *Scleroderma* isolates that associate with *Gnetum* spp. belong to a narrow clade close to *Scleroderma sinnamariense*. Our results demonstrate for the first time that specificity for mycobionts may substantially differ within an ectomycorrhizal plant genus.

Keywords Ectomycorrhizal fungi · Gnetaceae · Host specificity · *Scleroderma*

Introduction

In mycorrhizal symbioses, both the phyto- and mycobiont usually associate with a broad range of partners (Molina et al. 1992; Bruns et al. 2002), but there are numerous exceptions to this rule. In addition to the mycoheterotrophic plants (Orchidaceae, Ericaceae and other families) that have specialized on very narrow groups of fungal species (Bidartondo 2005; Hynson and Bruns 2010), certain fully autotrophic plants from different ecosystems display high specificity towards fungi. In ectomycorrhizal (EcM) associations, such specific interactions are apparent in a tropical liana species *Gnetum africanum* (Gnetaceae; Bechem and Alexander 2012a), a tropical tree species *Pisonia grandis* (Nyctaginaceae; Chambers et al. 2005), and an entire tree genus *Alnus* (Betulaceae; Molina et al. 1992; Tedersoo et al. 2009). *P. grandis* typically inhabits guano-rich soils in coral islands and associates with only one to three species of fungi from the /tomentella-thelephora lineage (Chambers et al. 2005; Suvi et al. 2010; Hayward and Horton 2012). *Alnus* spp. prefer riparian and early successional habitats in subarctic to warm temperate ecosystems, where they associate with both Actinobacteria and a restricted set of EcM fungi (Molina et al. 1992; Pritsch et al. 1997). Specificity for mycobionts in autotrophic plants has been often ascribed to a specific ecological niche or habitat (Molina et al. 1992), but recent findings argue against this notion in several plant taxa. In particular, apart from *Alnus*, other actinorhizal plants such as *Dryas* and *Cercocarpus* (Rosaceae) host a broad range of fungal symbionts that largely overlap with mycobionts of neighboring trees and shrubs (Harrington and Mitchell 2005; Ryberg et al. 2009; Bjorbækmo et al. 2010; McDonald et al. 2010). Other members of the Nyctaginaceae family associate with multiple fungal species from several phylogenetic lineages (Tedersoo et al. 2010b). In *P. grandis*, specificity is evident throughout the steep gradient of soil nitrogen concentration (Hayward and Horton 2012).

The genus *Gnetum* comprises 30–33 species of lianas and understorey trees and has a pantropical distribution (Won and Renner 2006). Within *Gnetum*, EcM symbiosis was first described in *G. africanum* in the Democratic Republic of Congo, where conspicuous yellow root tips were associated with fruit bodies of *Scleroderma* sp. through rhizomorph connections (Fassi 1957). Similar associations were later

L. Tedersoo (✉) · S. Põlme
Institute of Ecology and Earth Sciences, Tartu University,
14A Ravila,
50411 Tartu, Estonia
e-mail: Leho.Tedersoo@ut.ee

L. Tedersoo
Natural History Museum, Tartu University,
Tartu, Estonia

confirmed in varieties of *G. africanum* in Cameroon (Onguene 2000) and other *Gnetum* species in South America (St. John 1980; Singer et al. 1983; Moyersoen 1993). Recently, sequence analysis of EcM root tips from natural and planted *G. africanum* confirmed that only a few closely related species of *Scleroderma* associate with *G. africanum* (Bechem and Alexander 2012a). Experimental inoculation trials revealed that only isolates of *Scleroderma* sp. were able to form EcM with *G. africanum*, whereas the non-native *Pisolithus* and *Paxillus* species failed to infect rooted cuttings (Bechem and Alexander 2009). *Scleroderma* sp. provides mineral phosphorus and nitrogen to the associated *G. africanum* and thereby stimulates growth of the cuttings (Bechem 2011; Bechem and Alexander 2012a, b). Moreover, *G. africanum* cannot establish without its root symbiotic fungi in plantations (Bechem and Alexander 2012b). Leaves of *G. africanum* are very popular and relatively expensive source of food in Equatorial Africa. Because of overexploitation of natural populations and difficulties in establishing plantations, this species has been lost from many natural habitats near human settlements. Unlike *G. africanum*, *G. gnemon* constitutes an understorey tree in lowland rain forests in the Indo-Malay and West Pacific regions. Throughout its range, both the leaves and flowers and fruits of *G. gnemon* are widely used for food (Cadiz and Florido 2001). Cutting trees for access to edible parts has resulted in decline of natural populations of this tree species. The present study addresses diversity of root symbionts of *G. gnemon*. We hypothesized that *G. gnemon* has a very narrow range of mycobionts as observed for *G. africanum* and as suggested for South American *Gnetum* species.

Materials and methods

Root samples of seedlings and saplings of *G. gnemon* were collected in three sites in lowland rain forests of the Morobe Province in Papua New Guinea. Bawituc (6.639° S; 147.909° E; ca 200 m above sea level (a.s.l.)) is a secondary rain forest site located 15 km northeast of the city of Lae. Oomsis (6.712° S; 146.807° E; 150 m a.s.l.) is a selectively cut primary forest located 30 km west of Lae. Kamiali (7.303° S; 147.130° E; 10 m a.s.l.) is a slightly disturbed primary forest located 50 km south of Lae. In all sites, some *G. gnemon* trees were killed by overexploitation and most of the remaining trees displayed signs of recent harvesting of leaves (i.e., partial defoliation). We uprooted nine seedlings or saplings of *G. gnemon* (three in Bawituc, one in Oomsis, and five in Kamiali) and wrapped a 10-cm fragment of a healthy long root with intact short roots in a plastic bag. The roots were washed and examined visually for the presence of a fungal mantle. All clusters of root tips were excised and stored in CTAB buffer (1 % cetyltrimethylammonium bromide, 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, and 20 mM EDTA). DNA was extracted from at least three EcM root tips of each tree individual using a DNeasy 96 Plant Kit (Qiagen, Crawley, UK) as recommended by the manufacturer. PCR was performed with primers ITS-OF and LB-W (Tedersoo et al. 2008) or ITS-OF and ITS4 (White et al. 1990). The PCR included 5 μl of 5× HOT FIREPol Blend Mastermix Ready to Load (Solis Biodyne, Tartu, Estonia), 3 μl of template DNA, 0.5 μl of each 20 μmol/ml primer, and 16 μl dsH_20. PCR reactions were run under the following conditions: an initial 15 min at 95 °C, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final cycle of 10 min at 72 °C. PCR products were separated by electrophoresis through a 1.5 % agarose gel, purified using Exo-Sap enzymes (Sigma, St. Louis, MO, USA) and subjected to sequencing with primers ITS5 and/or ITS4 (White et al. 1990). Sequences were assembled into contigs and checked for quality by use of Sequencher 4.10 software (GeneCodes Corp., Ann Arbor, MI, USA). Species were delimited based on 97.0 % ITS sequence similarity threshold. All unique sequences from each individual plant were submitted to the International Sequence Databases and are available under accessions JX316729-JX316738. To address the phylogenetic placement of *Scleroderma* isolates from the root samples of *G. gnemon* relative to other sources, all 194 ITS sequences of *Scleroderma* that had information about the country of origin were downloaded along with their metadata from the copy of GenBank and UNITE databases over the PlutoF workbench (Abarenkov et al. 2010). Sequences covering <80 % of the ITS region and redundant sequences (identical and originating from the same study and country) were removed. Four sequences of *Pisolithus* spp. were used as outgroups for the alignment in MAFFT server (http://mafft.cbrc.jp/alignment/server/) and subsequent maximum likelihood phylogenetic analysis with fast bootstrapping (using default options) as implemented in RAxML (Stamatakis et al. 2008).

Results

Root samples from seven out of nine *G. gnemon* trees included EcM root tips as revealed by a combination of microscopic examination and molecular identification. Based on microscopic examination, EcM root tips were lacking in two seedlings that were collected from different sites. Sequence analysis of non-ectomycorrhizal root tips revealed colonization of putative endophytes or soil saprotrophs (not shown). In the root samples of other *G. gnemon* trees, only a single EcM fungal species was identified from

each tree individual. This was supported by microscopic observation of a single dominant morphotype on the root tips of each tree. Morphotypes differed from each other by color and the amount of mycelium and rhizomorphs. In addition to white and yellow rhizomorphic types described in Bechem and Alexander (2012a), smooth pale and smooth black morphotypes were observed. From root tips of individual *G. gnemon* trees, two species of *Scleroderma* (/pisolithus-scleroderma lineage; cf. Tedersoo et al. 2010a) and a single species of the /tomentella-thelephora, /russula-lactarius, /sordariales, and /inocybe lineages were recovered from root tips of *G. gnemon* (Table 1). Species of the /inocybe lineage was found from two *G. gnemon* individuals at Bawituc. None of the recovered EcM fungal species were represented in a concurrent sampling effort of >250 specimens of EcM fungi throughout Papua New Guinea.

The maximum likelihood phylogram of the genus *Scleroderma* suggested that the two recovered species are relatively closely related, but not sisters to each other (Fig. 1). The analysis revealed that all isolates from *Gnetum* spp. in Africa, Malaysia, and Papua New Guinea belong to a narrow, well-supported (BS=94) clade of closely related species. *Scleroderma* sp1 (UDB013025) is highly similar and probably conspecific with a root tip isolate of Dipterocarpaceae from Malaysia (GQ268591) that share 98.6 % ITS sequence similarity. *Scleroderma sinnamariense* is the only described species that has available ITS sequences and belongs to the clade that includes root tip isolates of *Gnetum* spp.

Discussion

Contrary to our hypothesis, *G. gnemon* associated with multiple species of EcM fungi that belonged to several phylogenetic lineages such as /pisolithus-scleroderma, /inocybe, /russula-lactarius, /tomentella-thelephora, and /sordariales. Previously, only members of *Scleroderma* spp. were identified from root tips of African and Malaysian *Gnetum* spp. (Bechem and Alexander 2012a). The other fungal species associating with *G. gnemon* belong to lineages that are all commonly ectomycorrhizal with the Dipterocarpaceae and Fabaceae host trees in tropical forests (Tedersoo et al. 2007, 2011; Peay et al. 2010; Smith et al. 2011; Phosri et al. 2012). Our limited sampling effort does not enable us to address whether these fungi are randomly selected from the local pool of EcM fungi that associate with the species of Dipterocarpaceae, *Intsia* (Fabaceae), and Myrtaceae in these lowland rain forests. It is likely that these fungal species have a broad host range because *Scleroderma* spp. that associate with *Gnetum* also establish EcM symbiosis with other EcM host trees (Bechem and Alexander 2012a; see also Singer et al. 1983 and Onguene 2000 for potential mycorrhizal networks involving *Gnetum*).

The two *Scleroderma* species from *G. gnemon* belonged to a narrow clade that includes all sequenced isolates of *Gnetum* symbionts sequenced so far. This indicates that *G. africanum* exhibits high specificity for a few closely related *Scleroderma* spp. in the *S. sinnamariense* group (Bechem and Alexander 2012a), whereas *G. gnemon* hosts a broader range of mycobionts including some of these specific taxa. Because the genus *Gnetum* probably originates from South America and it subsequently dispersed to Africa and Southeast Asia (Won and Renner 2006), information on South American fungi is required to understand the evolutionary ecology and history of host specificity in EcM symbiosis involving *Gnetum*.

Nonetheless, our study indicates that specificity for mycobionts may strongly vary among congeneric plant species. This is in striking contrast with the genus *Alnus*, where all studied plant species share their mycobionts and display high specificity for these (Molina 1981; Põlme et al., unpublished). In the genus *Pisonia*, in-depth molecular investigations of EcM root symbionts are so far restricted to *P. grandis*. These studies recovered only one to three fungal species belonging to the /tomentella-thelephora lineage in coral cays of the Indian and Pacific oceans (Chambers et al. 2005; Suvi et al. 2010; Hayward and Horton 2012).

Table 1 Identification of EcM fungi associated with *G. gnemon* individuals at various sites in Papua New Guinea

Tree	Height (m)	Site	Appearance of morphotype	Associated fungi (genus or lineage)	UNITE accession	INSD accession
Tree #1	0.5	Bawituc	Smooth pale	/inocybe sp	UDB013020	JX316732
Tree #2	0.3	Bawituc	Smooth pale	/inocybe sp	UDB013021	JX316733
Tree #3	0.2	Bawituc	n.d.	n.d.		
Tree #4	1.0	Oomsis	Smooth pale	/russula-lactarius sp	UDB013018	JX316730
Tree #5	2.0	Kamiali	Smooth black	/sordariales sp	UDB013022	JX316734
Tree #6	2.5	Kamiali	Smooth pale	/tomentella-thelephora sp	UDB013023	JX316735
Tree #7	0.3	Kamiali	n.d.	n.d.		
Tree #8	1.0	Kamiali	Hairy white	*Scleroderma* sp1	UDB013025	JX316737
Tree #9	3.5	Kamiali	Hairy yellow	*Scleroderma* sp2	UDB013008	JX316738


UDB007676 Pisolithus Madagascar Unspecified
AY318748 Pisolithus New Zealand Unspecified
UDB007677 Pisolithus Madagascar Eucalyptus
UDB007678 Pisolithus Madagascar Unspecified
FJ840466 Scleroderma Burkina Faso Unspecified
UDB008396 Ectomycorrhiza Benin Unspecified
AM412276 Ectomycorrhiza Seychelles Unspecified
UDB008359 Ectomycorrhiza Benin Unspecified
UDB008381 Ectomycorrhiza Benin Unspecified
FJ840443 Scleroderma dictyosporum Burkina Faso Unspecified
UDB008164 Ectomycorrhiza Benin Isoberlinia doka
UDB007673 Ectomycorrhiza Madagascar Unspecified
FJ840444 Scleroderma dictyosporum Burkina Faso Unspecified
HM237176 Scleroderma citrinum China Unspecified
AB099900 Scleroderma Thailand Unspecified
AM113465 Ectomycorrhiza Guinea Paramacrolobium coeruleum
FM213349 Scleroderma polyrhizum USA Unspecified
FM999606 Ectomycorrhiza USA Fagus grandifolia
UDB004971 Ectomycorrhiza Cuba Coccoloba uvifera
UDB004985 Ectomycorrhiza Cuba Coccoloba uvifera
UDB13027 ectomycorrhiza Cameroon Gnetum africanum
UDB13028 ectomycorrhiza Cameroon Gnetum africanum
UDB13008 ectomycorrhiza PNG Gnetum gnemon
AB453030 Scleroderma sinnamariense Thailand Dipterocarpaceae
UDB13029 ectomycorrhiza Cameroon Gnetum africanum
DQ146378 Ectomycorrhiza Thailand Dipterocarpaceae
UDB13025 ectomycorrhiza PNG Gnetum gnemon
GQ268591 ectomycorrhiza Malaysia Dipterocarpaceae
FRIM161 Scleroderma sinnamariense Malaysia Dipterocarpaceae
IA-S1 Scleroderma sinnamariense Great Britain Unspecified
FM213364 Scleroderma sinnamariense Thailand Unspecified
HQ687222 Scleroderma sinnamariense Thailand Unspecified
FP3058 Scleroderma Malaysia Gnetum gnemon
UDB13026 ectomycorrhiza Cameroon Gnetum africanum
FJ840457 Scleroderma verrucosum Burkina Faso Unspecified
FJ840453 Scleroderma verrucosum Burkina Faso Unspecified
FJ840458 Scleroderma verrucosum Burkina Faso Unspecified
UDB008166 Ectomycorrhiza Benin Isoberlinia tomentosa
AB453029 Scleroderma citrinum Thailand Dipterocarpaceae
GQ478008 Ectomycorrhiza Portugal Quercus
EF644144 Ectomycorrhiza Austria Populus tremula
FJ197962 Soil Austria Unspecified
GU181862 Ectomycorrhiza Italy Unspecified
JF748070 Ectomycorrhiza China Unspecified
GQ240921 Ectomycorrhiza China Castanopsis fargesii
JF273540 Scleroderma China Unspecified
HM105510 Ectomycorrhiza China Quercus
DQ453694 Scleroderma cepa USA Quercus
FM999610 Scleroderma USA Fagus grandifolia
EU784411 Scleroderma cepa Great Britain Unspecified
FM213343 Scleroderma Thailand Unspecified
FM213355 Scleroderma cepa USA Unspecified
GQ205359 Ectomycorrhiza Portugal Unspecified
JF419512 Ectomycorrhiza Poland Unspecified
GU246989 Ectomycorrhiza USA Castanea dentata
HM015474 Ectomycorrhiza Poland Unspecified
EU819518 Ectomycorrhiza USA Quercus
EU819438 Scleroderma areolatum USA Angiospermae
FJ196958 Ectomycorrhiza Mexico Quercus
GQ221643 Ectomycorrhiza USA Unspecified
EU784416 Scleroderma verrucosum Great Britain Unspecified
GU246991 Ectomycorrhiza USA Castanea dentata
EU784407 Scleroderma areolatum Great Britain Unspecified
JF419513 Ectomycorrhiza Poland Unspecified
UDB000044 Scleroderma verrucosum Denmark Unspecified
EU700256 Ectomycorrhiza Germany Unspecified
FM213353 Scleroderma areolatum USA Unspecified
UDB001212 Scleroderma areolatum Sweden Unspecified
EU784415 Scleroderma verrucosum Great Britain Unspecified
EU784412 Scleroderma cepa Great Britain Unspecified
GQ205360 Ectomycorrhiza Portugal Unspecified
FM213354 Scleroderma cepa Spain Unspecified
UDB011607 Scleroderma verrucosum Estonia Unspecified
GQ478009 Ectomycorrhiza Portugal Quercus
Gu256204 Ectomycorrhiza Italy Quercus suber
TU110574 Scleroderma PNG Lithocarpus
TR126-06 Scleroderma PNG Fagaceae
FJ840462 Scleroderma Burkina Faso Unspecified
UDB008165 Ectomycorrhiza Benin Isoberlinia doka
GQ268582 Ectomycorrhiza Malaysia Dipterocarpaceae
UDB004427 Ectomycorrhiza Cameroon Eucalyptus grandis
HM237175 Scleroderma bovista China Unspecified
UDB004641 Ectomycorrhiza Zambia Eucalyptus grandis
HM237170 Scleroderma China Unspecified
UDB004430 Ectomycorrhiza Cameroon Pinus
AB459512 Scleroderma columnare Thailand Dipterocarpaceae
AY756113 Ectomycorrhiza India Dipterocarpaceae
AM412278 Scleroderma Seychelles Unspecified
UDB007666 Ectomycorrhiza Madagascar Unspecified
UDB007663 Ectomycorrhiza Madagascar Uapaca littoralis
AM412277 Ectomycorrhiza Seychelles Unspecified
EU819439 Scleroderma cepa USA Unspecified
FM213348 Scleroderma michiganense USA Unspecified
EU819441 Scleroderma michiganense USA Unspecified
FJ824090 Ectomycorrhiza USA Unspecified
UDB011750 Scleroderma citrinum Estonia Unspecified
GU553366 Ectomycorrhiza USA Castanea dentata
EU784413 Scleroderma citrinum Great Britain Unspecified
FJ816750 Ectomycorrhiza Spain Pinus pinaster
HM189957 Scleroderma citrinum Germany Unspecified
DQ437705 Ectomycorrhiza USA Unspecified
HM146864 Ectomycorrhiza Germany Pinus sylvestris
GQ166907 Scleroderma citrinum USA Unspecified
GU246990 Ectomycorrhiza USA Castanea dentata
JF419511 Ectomycorrhiza Poland Unspecified
AM087282 Ectomycorrhiza Denmark Fagus sylvatica
AY935514 Scleroderma citrinum Spain Pinus
FM213342 Scleroderma USA Unspecified
EU819517 Ectomycorrhiza USA Unspecified
GQ267487 Scleroderma bovista New Zealand Unspecified
FM213340 Scleroderma bovista Spain Unspecified
UDB002179 Scleroderma bovista Sweden Unspecified
UDB001646 Scleroderma bovista Great Britain Unspecified
FM213336 Scleroderma septentrionale Sweden Unspecified
HQ688788 Scleroderma Argentina Unspecified
0.1

◀ **Fig. 1** Maximum likelihood phylogram demonstrating the phylogenetic placement of mycobionts of *G. gnemon* from Papua New Guinea (PNG; *in bold*) among isolates of *Scleroderma.* Bootstrap values >50 are indicated *above branches*. *Bar* indicates 0.1 substitutions per site. The narrow clade associating with *Gnetum* spp. is *highlighted*

Mycological surveys have recovered several EcM fungal species from the /pisolithus-scleroderma and /russula-lactarius lineages under canopies of other *Pisonia* species in Central America (Pegler 1983; Guzman et al. 2004), suggesting that these taxa may not have such a narrow specificity, although reciprocal association with a restricted set of partners seems to be a common phenomenon in the Nyctaginaceae family (Tedersoo et al. 2010b). However, host species require careful identification by molecular identification or tracing of roots because EcM plant species of *Coccoloba* and *Neea* may co-occur with the South American *Pisonia* species, whereas *Intsia bijuga* and some introduced EcM trees may co-occur with *P. grandis* in the Pacific islands.

In conclusion, *G. gnemon* does not, as suggested for *G. africanum* (Bechem and Alexander 2012a), specialize exclusively on *Scleroderma* mycobionts. Our results demonstrate for the first time that congeneric plant species may substantially differ in their specificity for ectomycorrhizal symbionts. Further sampling of *Gnetum* species in South America is required to address the evolution of host specificity in this gymnosperm genus.

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