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ORIGINAL PAPER

Edaphic factors do not govern the ectomycorrhizal specificity of *Pisonia grandis* (Nyctaginaceae)

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Abstract *Pisonia grandis* (Nyctaginaceae), a widespread tree of Pacific coral atolls and islands, displays one of the more restrictive ranges of ectomycorrhizal (ECM) fungus associates among autotrophic plants. Only five ECM fungi are currently known associates; our study adds one. In many habitats, *P. grandis* is restricted to large seabird colonies where nitrogen and phosphorus inputs in the form of guano are substantial. It has been suggested that the ECM specificity displayed by *P. grandis* is the result of the unusual nutrient-rich habitat in which *P. grandis* grows. On Rota, Commonwealth of the Northern Mariana Islands, *P. grandis* grows in habitats heavily influenced by guano additions and also in upland forests where seabirds do not roost or nest. To test the hypothesis that the ECM specificity displayed by *P. grandis* is the result of nutrient-related or toxicity-related factors associated with guano inputs, we sampled *P. grandis* growing in both guano-rich and guano-poor habitats on Rota, Commonwealth of the Northern Mariana Islands. We identified ECM symbionts of *P. grandis* from both habitats as well as two symbionts of *Intsia bijuga* (Fabaceae) from nutrient-rich habitats. We identified three ECM symbionts of *P. grandis* from Rota; all three were found in both guano-rich and guano-poor habitats. No differences in community diversity were detected between guano-rich and guano-poor habitats. We also detected two ECM fungal species associating with *I. bijuga* but not associating with *P. grandis* inside guano-rich habitats. From these results, we infer that edaphic factors are not responsible for limiting the ECM community associating with *P. grandis* to its observed level of specificity.

Keywords Ecological specificity · *Pisonia grandis* · Ectomycorrhiza · Guano

Introduction

Pisonia grandis R. Br. (Nyctaginaceae) is a widespread tree distributed on atolls and islands in the Pacific and on the Asian continent. Its range extends from at least 24°S on Great Barrier Reef atolls to at least 21°N in the Pratas Islands near Taiwan, and from Lisianski island in Hawaii to the Jambe Reef, near the Tanzanian coast (St. John 1951; Shaw 1952; Hunt 1967; Burger 2005). Throughout the Pacific, the sticky propagules of *P. grandis* are apparently distributed on the feathers and feet of migratory seabirds (Burger 2005). *P. grandis*, as a dominant canopy species, forms the most important component of the climax plant community throughout most of its range (Walker et al. 1991; Burger 2005).

Unlike most tropical angiosperms, *P. grandis* forms sheathing mycorrhizas (Ashford and Allaway 1982; Ashford et al. 1988). In some cases, these mycorrhizas display unusual morphological features including transfer cells, an underdeveloped or absent Hartig net, and the inclusion of metachromatic phosphorus-rich granules in mantle tissues (Ashford and Allaway 1982, 1985). Some or all of these features have been described in combination as constituting a “pisonioid” mycorrhiza (Brunner and Scheidegger 1995; Imhof 2009). However, *P. grandis* also forms ectomycorrhizas with Hartig nets (Suvi et al. 2010). While it is apparently possible to cultivate nonmycorrhizal *P. grandis* plants (Cairney et al. 1994), no wild populations of *P. grandis* lacking sheathing

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mycorrhizas have been reported; it is, therefore, likely that *P. grandis* is obligately ectomycorrhizal (ECM) in nature.

In the Seychelles (Tedersoo et al. 2007; Suvi et al. 2010) and in Australia (Chambers et al. 2005), *P. grandis* displays one of the more restricted ranges of ECM fungal associations ever observed in nonmycoheterotrophic ECM plants, associating with only five fungal operational taxonomic units (OTUs) in the Thelephoraceae (Suvi et al. 2010; Chambers et al. 1998, 2005; this study adds a sixth). Throughout much of its range, *P. grandis* is reported only from densely populated seabird colonies (see, e.g., Shaw 1952). These habitats are extremely rich in guano, the deposition of which adds more than 100 $\mathrm{gm^{-2}\ year^{-1}}$ nitrogen and 22 $\mathrm{gm^{-2}\ year^{-1}}$ phosphorus to a *P. grandis*-associated seabird colony in Australia (Allaway and Ashford 1984). Mycorrhization and ECM fungal community diversity in temperate and tropical ecosystems are adversely affected by excess nitrogen or phosphorus deposition (Wallenda and Kottke 1998; Treseder 2004). Additionally, the metabolic by-products of guano decomposition include toxic compounds which are likely to inhibit fungal community diversity (Rodgers 1994; Sharples and Cairney 1997, 1998). Cairney et al. (1994), Chambers et al. (2005); Suvi et al. (2010) have suggested that the mycorrhizal specificity of *P. grandis* is ecological *sensu* Molina et al. (1992), presumably resulting from the inability of other potential mycobionts to tolerate the extreme habitat in which *P. grandis* thrives.

The term "ecological specificity" was proposed by Harley and Smith (1983) to refer to the discrepancy between the mycorrhizal specificity displayed by ECM symbionts in monoxenic culture and that found in nature; it was later expanded by Molina et al. (1992). In our discussion of ecological specificity, we mean to include only edaphic factors, i.e., factors not stemming from geographic range constraints or dispersal limitations. Under this usage of "ecological specificity," we mean to imply that, first, the range of ECM symbionts, either plant or fungal, capable of associating with a given ECM taxon is greater than is observed in nature, and second, that this is the case only because that taxon is restricted to a habitat inimical to its symbionts. This hypothesis is intended to be a restatement and clarification of the suggestions of Cairney et al. (1994), Chambers et al. (2005); Suvi et al. (2010), all of whom have suggested that the extreme habitat of *P. grandis* is responsible, at least in part, for its extreme ECM specificity. This hypothesis is also similar to a hypothesis of Branco and Ree (2010) that toxic serpentine soils might restrict the availability of fungal inoculum to ECM plants.

Our ecological specificity hypothesis as it applies to *P. grandis* makes two predictions: first, that very few novel ECM *P. grandis* mycobionts should appear in guano-rich habitats (that is, that guano-rich habitats restrict the availability of inoculum), and second, that *P. grandis* should associate with a diverse group of ECM fungi outside guano-rich habitats (that is, that where inoculum availability is not restricted, *P. grandis* should associate with more ECM fungi). To test these predictions, we sampled the ECM community of *P. grandis* on the island of Rota, in the Commonwealth of the Northern Mariana Islands, from two habitats. We provide evidence that ecological specificity does not explain the observed mycorrhizal specificity of *P. grandis*. We discuss several alternative hypotheses.

Methods and materials

Field site

Rota is an island of approximately 40 $\mathrm{km^2}$ belonging to the Commonwealth of the Northern Mariana Islands. The island is a carbonate cover (i.e., sedimentary overlying igneous and metamorphic bedrocks) island with limestone dating from the Miocene to the Pleistocene (Dickinson 2000). The island consists of three roughly concentric plateaus, each rising from the one below; much of the work on the geology of Rota has defined the island in terms of these "terraces" (Keel et al. 2004). *P. grandis* on Rota is apparently limited to the first and second terraces: the coastal lowlands and the higher inhabited plateau. In addition to *P. grandis*, Rota hosts two ECM species: *Intsia bijuga* (Colebr.) Kuntze, a Caesalpinioid, and *Casuarina equisetifolia* L., a member of the Casuarinaceae (Falanruw et al. 1989). *I. bijuga* is apparently native; it is unclear whether *C. equisetifolia* is native or introduced (Space et al. 2000).

On Rota, the only extant seabird colony is located in Chenchon Park on the eastern end of the island. *P. grandis* is abundant in the Chenchon seabird colony but is also found throughout the island, including areas apparently inhabited for at least a thousand years (Steadman 1999; Leach et al. 2008) and, therefore, unlikely to have hosted seabird colonies for at least that long. In both guano-rich and guano-poor environments, *P. grandis* is interspersed with *I. bijuga*.

Sampling and molecular methods

We collected root tips of *P. grandis* by tracing roots away from the bole of haphazardly selected trees throughout the island of Rota and harvesting fine root material. We sampled a total of 36 trees, of which 11 were growing in guano-rich areas. Limestone forest soils on Rota where *P. grandis* grows consist of shallow pockets of topsoil atop limestone bedrock (Young 1989). We observed no differentiation in soil horizons. We collected approximately 10 cm of fine root material from each tree; no tree was sampled twice. Fine root material was preserved in 2× CTAB buffer (100 mmol/L

Tris–HCl [pH 8.0], 1.4 mmol/L NaCl, 10 mmol/L EDTA, 2 % CTAB [Gardes and Bruns 1996]) at ambient temperature for up to 21 days prior to being returned to the lab. We extracted DNA from at least one ECM root tip selected from each sample; in cases where more than one ECM root possessing unique morphological characters were observed in a sample, DNA was extracted from each morphological type (morphotype).

We sampled root tips from two *I. bijuga* trees growing in guano-rich habitats using the same methodology as above. To establish that ECM inoculum was present outside seabird colonies, we inspected roots of *I. bijuga* and *C. equisetifolia* outside guano-rich areas and preserved roots from four trees (two *I. bijuga* and two *C. equisetifolia*) for molecular analysis. The number of non-*Pisonia* trees sampled was limited by budget constraints.

To quantify the impact of seabird colonies on local edaphic factors, we sampled soils from both guano-rich and guano-poor environments. Because *Pisonia* trees may themselves alter surrounding soils, soil sampling locations were not tied to specific trees. Instead, we selected sampling locations using random GPS coordinates selected within the areas encompassed by either guano-rich habitats (30 samples) or guano-poor habitats (28 samples). We collected approximately 50 g dry weight soil from immediately below the litter layer using a knife to excavate soil blocks. We air-dried the samples at ambient temperature for 1 week and subjected them to bulk C and N analysis using a Thermo Scientific Flash EA 1112 series elemental analyzer (Thermo Scientific, Asheville, NC). For cation analysis, soil samples were extracted using the Mehlich-3 method (Mehlich 1984) and analyzed using a Perkin-Elmer Optima 3300DV Inductively Coupled Plasma-Optical Emission Spectrometer (Perkin-Elmer, Waltham, MA).

We used a modified glassmilk procedure (Vogelstein and Gillespie 1979) to extract DNA from root tips. We ground individual root tips in 300 μL 2× CTAB buffer, incubated at 65°C for 1 h and then vortexed this lysate with 300 μL chloroform. After a 15-min spin at 15,000*g*, we collected the supernatant and added 750 μL 6 M NaI solution (MP Biomedicals, Carlsbad, CA) and 10 μL 325-mesh silica particle suspension. After 15-min incubation at room temperature with constant agitation, the silica particles were pelleted at 15,000*g* for 1 min and the supernatant was discarded. We resuspended the pellet in a wash solution (100 mM NaCl, 1 mM EDTA, 10 mM Tris–HCl, 50 % EtOH; modified from Boyle (1995)), repelleted the silica as above, and discarded the supernatant. This procedure was repeated two more times for a total of three washes. After the final wash and pelleting, we dried the pellet and then resuspended this in 100 μL PCR-grade water. After a final pelleting of the silica pellet, the supernatant was removed; this supernatant became the DNA extract used for PCR.

Table 1 Summary values reflecting variation in nutrient levels between guano-rich and guano-poor environments on Rota. Figures given reflect mean ± standard deviation; *p* values are from two-sample Student's *t* tests comparing the two habitats

Sample origin	Guano-rich	Guano-poor	*p* value
Number of samples	30	28	
Total *N* (ppm)	2,560±690	1,590±710	<.0001
Extractable P (mg/L)	734±148	280±52	0.007
Extractable Ca (mg/L)	18,610±3,923	22,641±6,357	0.005
Extractable K (mg/L)	314.1±97.4	311±146	0.93

We amplified the internal transcribed spacer (ITS) region of the nuclear ribosomal gene cassette using the primers ITS1f (Gardes and Bruns 1993) and NLB4 (Martin and Rygiewicz 2005). PCR conditions were as follows: 3:00 at 94°C, followed by 35 cycles of 35 s at 94°C, 55 s at 53°C, and 45 s at 72°C, adding 5 s/cycle to the extension time, with a final extension period of 10 min at 72°C. We generated restriction fragment length polymorphism (RFLP) profiles using the restriction enzymes *Dpn*II and *Hinf*I (New England Biolabs, Ipswich, Massachusetts) following the procedures of Gardes and Bruns (1996). We grouped ITS-RFLP profiles into three unique types and then sequenced the ITS region of two to three exemplars of each type. We grouped these sequences into OTUs using mothur 1.21.1 (Schloss et al. 2009) using the furthest neighbor method at a cutoff level of 3 %. Exemplars of each OTU were then compared to GenBank sequences using a Mega BLAST search. OTUs were considered conspecific with reported species if their ITS regions differed by less than 3 %; otherwise, OTUs were assigned only to the family level.

Statistics

Statistics, unless otherwise noted, were calculated using Minitab 16 (Minitab Inc., State College, PA). Soil sample means were compared using Student's *t* test. We compared guano-rich to guano-poor habitats using Fisher's exact test. Community richness estimates were calculated using EstimateS 8.2.0 (Colwell 2005).

Table 2 Summary species-richness statistics from EstimateS 8.2.0. Figures given reflect estimate ± 1 standard deviation. All estimates are within 2 % of observed richness

Environment	Both	Guano-poor	Guano-rich
Chao 1 mean	3±0	3±0.01	3±0.04
Chao 2 mean	3±0	3±0.01	3±0.04
1st-order jacknife	3±0	3±0	3±0
2nd-order jacknife	3±0	3±0	3±0
Bootstrap	3±0	3±0	3±0

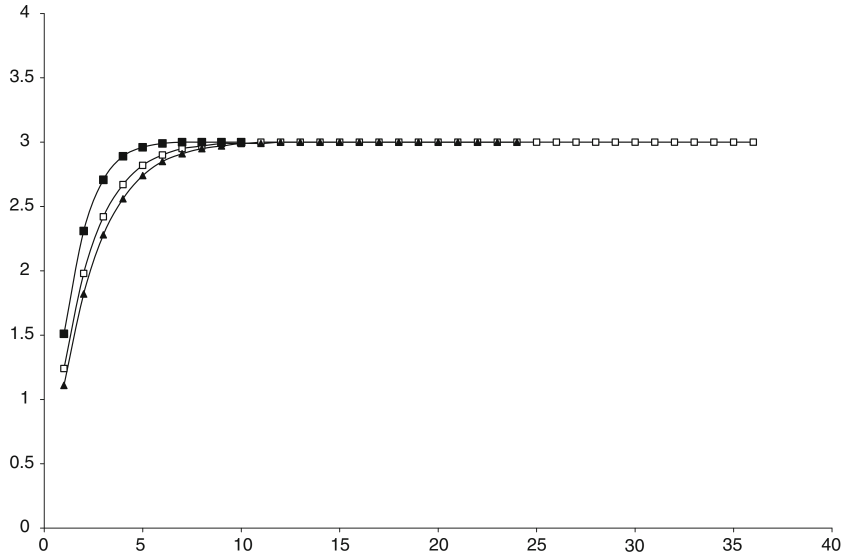

Fig. 1 Cole rarefaction curves for species richness in guano-rich (*closed squares*) and guano-poor (*triangles*) habitats as well as for the island overall (*open squares*). All estimates converge on three species, the detected richness in each habitat

Results

Soil chemistry

Soil nitrogen, calcium, and phosphorus differed significantly between guano-rich and guano-poor environments, while soil potassium did not display significant variation (Table 1). As expected, guano-rich soils contained more N, P, and Ca than guano-poor soils (Table 2); however, intersample variation was high resulting in large standard deviations.

Ectomycorrhizas

All 36 *P. grandis* trees possessed ECM root tips. We selected 161 ectomycorrhizas from the 36 trees sampled for molecular analysis. Of these 161 ectomycorrhizas, 101 yielded clear ITS-RFLP profiles. Because the species accumulation curve (Fig. 1) was entirely saturated, we did not attempt to recover additional ITS-RFLP profiles. From these 101 profiles, we detected three ECM fungal OTUs in association with *P. grandis* on Rota (see Table 3). Two of these had been previously reported: an unnamed species found in association with *P. grandis* on Cousin Island, in the Seychelles, and *Tomentella pisoniae*, found on Cousin and Praslin Islands in the Seychelles. The third OTU, also the most commonly detected in our analysis (present on 21 of 36 trees investigated), has apparently not been reported before. All sequenced exemplars of each OTU were 100 % homologous with each other.

All three OTUs were detected from both guano-rich and guano-poor environments, yielding a Sørensen similarity index of 1 between the two habitat types. A comparison of fungal OTU's detected in guano-rich and guano-poor habitats using Fisher's exact test resulted in $p=0.838$, indicating no significant difference between habitats. We computed Chao1, Chao2, jackknife, rarefaction, and bootstrap estimates for the diversity of species found in guano-rich habitats, in guano-poor habitats, and across the island as a whole, using EstimateS 8.2.0 (Fig. 1; Table 2).

The two *I. bijuga* trees sampled from guano-rich habitats yielded two ECM OTUs with affinities to the Cortinariaceae (GenBank accession number JQ405661; 87 % similar to an *Inocybe* species with accession number FN550932.1) and Russulaceae (GenBank accession number JQ405662; 95 % similar to a *Russula* species with accession number GU222258.1), respectively.

I. bijuga and *C. equisetifolia* growing in guano-poor habitats were ECM, with more than ten morphotypes observed, indicating no lack of ECM inoculum in guano-poor areas of the island. ITS-RFLP analyses of sampled root tips from *I. bijuga* and *C. equisetifolia* growing in guano-poor

Table 3 Sequence information relating to taxa detected in association with *Pisonia grandis* on Rota. 'Guano-rich detections' indicates the number of trees on which a given taxon was detected in guano-rich habitats. 'Guano-poor detections' gives similar information for guano-poor habitats

Taxon	Guano-rich detections	Guano-poor detections	GenBank accession	Closest match	Max score	% Identity
Tomentella OTU1	7	12	JQ405658	FN396394.11	845	97
Thelephoraceae OTU2	8	13	JQ405659	FR852142.1	691	90
Tomentella pisoniae	8	9	JQ405660	FM244910.1	981	98

areas yielded four and five RFLP types, respectively, none of which matched the ITS-RFLP profiles of *P. grandis* mycobionts detected in this study.

Discussion

The failure of *P. grandis* to associate with fungi outside the *Tomentella–Thelephora* group even when growing in habitats unlikely to limit fungal diversity and the presence of ECM fungi not associating with *P. grandis* even within those habitats is in contrast to the predictions made by the ecological specificity hypothesis. The mycorrhizal specificity displayed by *P. grandis*, therefore, cannot be entirely ecologically specific as the term is used here. However, the failure of the ecological specificity hypothesis in explaining the mycorrhizal specificity of *P. grandis* does not mean that the mycorrhizal associations of *P. grandis* are determined entirely by genetic factors. Most obviously, of the six OTUs within the genus *Tomentella* known to associate with *P. grandis*, at most three are known to be present at any single site (Suvi et al. 2010; this study), while only two are known to be present at the Australian site surveyed by Chambers et al. (2005). The apparent inability of any given population of *P. grandis* to associate with more than three fungi is most easily ascribed to the absence of additional compatible symbionts from the sampled regions. While this absence is an environmental factor in the sense that it pertains to the environment of *P. grandis*, it does not fall into the category 'ecological specificity' in the sense used here. It is possible that outside Rota, nitrogen-intolerant but potentially *P. grandis*-compatible ECM fungal taxa exist, but that these taxa are excluded from actual association with *P. grandis* because they are only found in areas where *P. grandis* is restricted to guano-rich zones. This would constitute ecological specificity. Our evidence indicates, however, that no nitrogen-intolerant but *P. grandis*-compatible taxa exist on Rota.

If the mycorrhizal specificity of *P. grandis* is not fully ecological in the sense of "ecological specificity" used here, hypotheses seeking to explain that specificity beyond the presence or absence of compatible taxa must refer to the genetic programs involved in the symbiosis. These, in turn, must make reference to the evolution of the taxa in question. The remaining hypotheses are broadly divisible into two categories: those that propose that mycorrhizal specificity is the ancestral character (see, e.g., Haug et al. 2005) and those that propose it is a derived character.

Vanderplank (1978) argues that specificity is unlikely to evolve among cohabiting generalist mutualists in the absence of disequilibria in the relative fitness benefits accruing to symbiont pairs. It follows that if mycorrhizal specificity is derived, rather than ancestral, one explanation for its existence is the presence of such a disequilibrium: either the plant is reducing the fitness of its mycobionts or the mycobionts are reducing the fitness of their host, resulting in gene-for-gene evolution. This type of ECM specificity is only known from mycoheterotrophic systems (Bruns et al. 2002). We are aware of no evidence suggesting that *P. grandis* is mycoheterotrophic or mixotrophic. Thus, while the possibility of a gene-for-gene interaction between *P. grandis* and its mycobionts is open, it seems unlikely.

The ECM habit in the Nyctaginaceae is a derived condition, apparently unique to the tribe Pisonieae (Haug et al. 2005; Wang and Qiu 2006; Suvi et al. 2010). The degree of mycorrhizal specificity displayed by other ECM plants in the Nyctaginaceae is poorly understood. If the mycorrhizal specificity displayed by *P. grandis* reflects an inherited genetic program of restricted mycorrhizal compatibility, it should be broadly shared among the ECM Pisoniae. *Pisonia zapallo* Griseb. and *Pisonia fragrans* Dum.-Cours. are apparently also ECM and less specific than *P. grandis*, and several *Neea* and *Guapira* species have been shown to associate with members of the Russulaceae (Haug et al. 2005; Suvi et al. 2010; Tedersoo et al. 2010; Jeremy Hayward, unpublished data). However, because selection pressure for generalism is expected to be widespread in mutualistic systems (Harley and Smith 1983; Law and Koptur 1986), these may represent derived generalism.

In that the derived status of mycorrhizal specificity in the Pisoniae is still unclear, differentiating coevolutionary specificity from inherited specificity is impossible at this time. An improved phylogeny of the tribe and better understanding of its mycorrhizal specificity would be of value in determining the actual cause of mycorrhizal specificity in *P. grandis*.

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