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Short communication

Assessment of root-associated fungal communities colonizing two species of tropical grasses reveals incongruence to fungal communities of North American native grasses

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ABSTRACT

This study characterized the root-associated fungal (RAF) communities inhabiting *Eustachys petraea* and *Panicum maximum*, two tropical and weedy grass species on an island off the coast of Honduras, Central America. Media-based analyses revealed that fungi colonizing these grasses exhibited similar morphotypes (albeit in different proportions). Conversely, molecular-based analyses suggested that the community of fungal OTUs were dissimilar, with several OTUs commonly present on only one plant and no AMF sequences present in either plant species. When compared to various datasets of RAF obtained from the North American mainland (with similar methods), the Honduran grasses harbored a different set of fungal OTUs. Interestingly, some cosmopolitan taxonomic clades commonly encountered on the mainland were also isolated in Honduras, though based on ITS rDNA the fungi were only related distantly to those on the mainland. Additional work is necessary to determine how the RAF communities change over geographic distance and whether invasive grasses are constitutionally set up to accept relationships with a different suite of RAF species.

Grasses are an important component of terrestrial ecosystems and constitute the predominant plant form in many areas. Accumulating evidence suggests that many grass species commonly and consistently form symbiotic relationships with fungal endophytes (*sensu* Carroll 1988), particularly within their roots (e.g., Petrini 1996; Jumpponen 2001). Few studies, however, have evaluated whether, and to what extent, common RAF clades extend to other species or to other grasses in less studied geographic areas (e.g., Weiß *et al.* 2011), particularly in the tropics.

In this study, we wanted to characterize the RAF communities of two grass species: *Eustachys petraea* (a warm-season tropical weedy species) and *Panicum maximum* (a cool-season invasive tropical species common around the world). The goal of this descriptive work was to use molecular and media-based identification to determine whether the fungal communities in these two grass species exhibited any congruency with previously described RAF fungal communities colonizing native grasses assessed in a similar fashion but inhabiting other environments. The information, we think,

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would provide additional insights into the host-specificity of some members of the RAF community and characterize the distribution of common RAF clades in other geographic areas (Central America), and in grasses inhabiting different environments.

We collected plants of both grass species on the island of Cayo Cochino Menor (off the coast of Honduras, Central America; Bermingham *et al.* 1998). Additional details concerning the refuge, the island, its climate and biota are available at http://www.cayoscochinos.org/. Both species were found exclusively as co-inhabiting components of the psammophilic vegetation at two locations on the island: the south location near the field station (WGS coordinates $E = 553\,751$ and $N = 1\,764\,057$) and the eastern part of the island ($E = 553\,782$ and $N = 1\,764\,080$). A 50 m linear transect was used at each of the two locations (locations approximately 300 m apart) to select asymptomatic focal plants (10 m away from the nearest focal plant) along the transect. Ten plants (five *E. petraea* and five *P. maximum*) were harvested at each of the two locations (20 plants total) and brought back to the field station for processing.

About 10–15 individual roots (approximately 5 g wet weight) from each plant were chosen to assess the microfungal community. The roots were cleaned, washed and surface sterilized as previously described (Herrera *et al.* 2011b) before being cut in half (transversely; ½ for molecular and ½ for media-based assessment) and transported to Truman State University (USA) for assessment (refrigerated for a total of 3 d with 1 d in a chilled cooler while in transit).

Using standard molecular protocols (and as described by Khidir *et al.* 2010) we obtained DNA from roots of individual plants, and utilizing fungal-specific primers (ITS1F and ITS4; Gardes & Bruns 1993), amplified fungal DNA (approximately 600 basepairs). However, DNA from only 12 plants (six of each plant species) were selected based on the likelihood they would yield sufficient DNA for cloning (assessed with a Nanodrop 2000; Thermo Scientific, Wilmington, DE, and running PCR products on an agarose gel). Once cloned, miniprepped plasmid DNA from individual plants was organized into clone sequence libraries. Edited forward sequences (SEQUENCHER 4.8, Gene Codes Corp., Ann Arbor, MI) were grouped into Operational Taxonomic Units (OTUs) with 97 % similarity and 40 basepair sequence overlap. Any chimeric sequences were identified (as in O'Brien *et al.* 2005) and discarded from the dataset. Randomly selected sequences for each OTU were deposited in GenBank under accession nos., JN802293–JN802329 (provisional identifications in Supplementary Table 1).

In addition, a total of 15 (1 × 1 mm) root segments from each plant (20 plants total) were embedded into plates containing Malt Extract Agar (five segments per plate) with antibiotics (as described by Herrera *et al.* 1997). Morphotypes were subcultured and identified by means of traditional taxonomic methods (e.g., Arnold *et al.* 2007), with the identity of frequent isolates confirmed by molecular techniques. Phenotypic variants of the morphotypes were also assessed with molecular techniques (as described in Herrera *et al.* 2010).

Previous efforts in our laboratory suggested that some closely related clades of the RAF community are cosmopolitan in some native grass species (e.g., Herrera *et al.* 2010) and found in some grass species throughout the year (Porras-Alfaro *et al.* 2008). To assess whether the generality of this assumption could be supported or rejected we used the RAF clone libraries obtained in this study and compared them to RAF clone libraries obtained from native grasses from a variety of locations throughout the US (Supplementary Table 2) utilizing SEQUENCHER at 97 % similarity and 40 basepair overlap. We also included a fungal community obtained from herbivore dung (Herrera *et al.* 2011a) to serve as a more neutral comparison to the RAF communities obtained from grasses.

Clone libraries from a total of 12 plants (six *E. petraea* and six *P. maximum*) yielded a total of 318 good sequences (Table 1), 179 obtained from six *E. petraea* plants (a mean of 29.8 sequences/plant, SD = 13.8) and 139 from six *P. maximum* plants (mean = 23.2 sequences/plant, SD = 10.2). The community of sequences obtained from *E. petraea* appeared to be more diverse compared to those obtained from *P. maximum* (Fisher's $\alpha = 8.36$ vs. 3.88, respectively). However, nearly all of this difference was accounted for by an abundance of singletons in *E. petraea* compared to *P. maximum* (14 vs. 3, respectively). OTUs containing more than one sequence were more equitable (12 vs. 11; and Shannon diversity indices, 2.30 vs. 2.12, respectively).

As with other studies on grasslands, the RAF communities were dominated by Dark Septate Endophytes (DSE; Table 1). The five-most abundant OTUs accounted for 57 % (182/318) of the sequences. Only one of these cosmopolitan dominants was not a DSE (*Psathyrella* sp., represented by 30 sequences). Three of the remaining four were DSE: *Periconia* sp., Xylariales sp., an unknown species within the Diaporthales and *Zopfiella* sp. (with 50, 44, 34, and 24 sequences, respectively). Notable was the absence of sequences allied with arbuscular mycorrhizal fungi (AMF). Although AMFs vary in their incidence rates among several species of grasses, it is likely that, much like other RAF, this fungal group preferentially colonizes some species of grasses and not others. It is difficult to imagine that spores would be absent from our study site given that AMF spores have been found in nearby tropical islands in Panama (Mangan *et al.* 2004). Additional studies focused on host and environmental preferences of AMFs should disclose some of the criteria by which these ubiquitous (and well studied) endophytes choose (or are chosen by) their hosts.

Several OTUs were specific to only one plant (Table 1). For example, 63.6 % (14/22) of non-singleton OTUs were only obtained from one plant, including the first and fourth most numerous OTUs (*Periconia* sp. and *Psathyrella* sp., respectively). In addition, only three of the total 37 OTUs were obtained from both species of grasses (Morisita-Horn similarity index = 0.08).

Unlike the molecular dataset, the assessment of the colony morphotypes indicated that the plants appeared to harbor more similar communities based on the Morisita-Horn (0.68) similarity index. At the same time, Fisher's alpha (8.7 and 7.5, for *E. petraea* and *P. maximum* respectively) and Shannon diversity values (2.53 and 2.57, respectively) suggested that the diversity of RAF communities of both plants were also comparable. A mean of 82.0 % root segments harbored fungi (246 of 300 segments; $n = 20$ plants; SD = 16.1), with those from *E. petraea* yielding slightly more than those of *P. maximum*, 88.0 % (mean of 13.2, SD = 11.2; $n = 10$ plants) vs. 76.0 % (mean

Table 1 – Description of molecular clone libraries and cultural morphotype data bases obtained from 20 plants (10 *E. petreae* and 10 *P. maximum*) collected from Cayo Cochino Menor, Honduras (Central America)

		Molecular data				Cultural data		
Species	Plant	Total sequences in clone library	Of OTUs as contigs[a]	Of singletons	Common sequence identity based on BLAST search[b]	Of segments colonized (from 20)	Of morphotypes	Common morphotypes (based on molecular identity[b])
Eustachys petraea	1	54	2	2	*Periconia* sp. 1 (50)	15	4	Unknown sp. 4 (9) *Paeciliomyces* sp. 1 (3)
	2	37	4	4	*Xylariales* sp. 1 (16) *Monosporascus* sp. 1 (10)	14	3	*Paeciliomyces* sp. 1 (8) Agaricales sp. 1 (5)
	3					17	6	*Paeciliomyces* sp. 1 (8) Nonsporulating sp. 20 (4)
	4	25	4	7	*Zopfiella* sp. 1 (11) *Cercophora* sp. 2 (3)	15	5	*Bipolaris* sp.1 (7) *Paeciliomyces* sp. 1 (4)
	5	15	2	1	*Chaetomium* sp. 1 (11) Pleosporales sp. 3 (3)	15	2	*Paeciliomyces* sp. 1 (14)
	6	25	1	2	*Xylariales* sp. 1 (23)	12	7	*Paraphaeosphaeria* sp. 1 (3)
	7					14	6	Pleosporales sp. 2 (6) Unknown sp. 8 (3)
	8	23	3	0	Pleosporales sp. 2 (10) *Zopfiella* sp. 2 (9)	15	6	Pleosporales sp. 3 (4) *Paraphaeosphaeria* sp. (3)
	9					17	8	*Bipolaris* sp. 1 (7) *Paraphaeosphaeria* sp. 1 (3)
	10					12	9	
Overall		179	12	14	*Periconia* sp. 1 (50) Xylariales sp. 1 (44)	146	25	*Paeciliomyces* sp. 1 (37) *Bipolaris* sp. (18)
Panicum maximum	1					10	7	Agaricales sp. 1 (2) *Cercophora* sp. 3 (2)
	2	16	2	1	*Coprinus* sp. 1 (10) *Zopfiella* sp. 1 (5)	12	5	Nonsporulating sp. 81 (6) *Bipolaris* sp. (2)
	3	15	4	2	*Cercophora* sp. 1 (5) *Cochliobolus* sp. 1 (4)	14	2	*Paraphaeosphaeria* sp. (10) *Paeciliomyces* sp. 1 (4)
	4	23	2	2	Diaporthales sp. 1 (13) *Zopfiella* sp. 1 (8)	12	6	*Paraphaeosphaeria* sp. 1 (4) *Cercophora* sp. 1 (4)
	5	21	1	0	Diaporthales sp. 1 (21)	10	3	*Sordariales* sp. 1 (4) *Paeciliomyces* sp. 1 (4)
	6	21	1	0	Pleosporales sp. 1 (21)	11	4	*Cercophora* sp. 1 (4) Nonsporulating sp. 81 (3)
	7					14	4	*Paraphaeosphaeria* sp. 1(8) Agaricales sp. 1 (4)
	8	43	3	0	*Psathyrella* sp. 1 (30) *Chaetomium* sp. 2 (10)	12	3	*Paraphaeosphaeria* sp. 1 (9)
	9					16	5	Unknown sp. 7 (9) Unknown sp. 3 (3)
	10					6	4	
Overall		139	11	3	Diaporthales sp. 1 (34) *Psathyrella* sp. 1 (30)	117	21	*Paraphaeosphaeria* sp. 1 (32) *Paeciliomyces* sp. 1 (10)

a At least two similar or identical sequences based on 97 % similarity and 40 base overlap using SEQUENCHER.

b Identity of most common instances based on BLAST search. Singletons and doubletons not indicated. Nonsporulating isolates were unable to be sequenced.

of 11.4, SD = 18.4; $n = 10$ plants). Segments from *E. petraea* and *P. maximum* yielded, respectively, a total of 25 and 21 morphotypes (for the purposes of this project, yeasts were considered as one morphotype). The RAF community was dominated by *Chaetomium* spp., *Paraphaeosphaeria* spp. and *Bipolaris* spp. (present in 47, 44 and 22 root segments, respectively).

As in other studies (e.g., Arnold *et al.* 2007) our work suggests that culturing and molecular methods capture different subsets of the fungal community. However, like other studies on grasses using similar methods, we also found that some OTUs are clumped on one or a few plants (Khidir *et al.* 2010). In other words, although there is typically a large diversity of RAF colonizing a population of grasses, many fungal species are spatially clumped (locally) on one or a few plants. A possible explanation for this pattern is that RAF communities within grasses use niche partitioning to parse out localized resources, becoming more or less similar throughout the year (e.g., Mandyam & Jumpponen 2008; Ernst *et al.* 2011).

Comparison of the various datasets that represent several species of grasses on the North American mainland clearly showed that the tropical and weedy grasses from Honduras harbored a different set of fungal species, especially when considering the dominant members of the community. Only two (out of 37) OTUs within Honduras grasses were found in any of the other five datasets assessed. By comparison, nine (of the possible 178) OTUs obtained from dung were also present in the datasets from North American grasses (Fig 1).

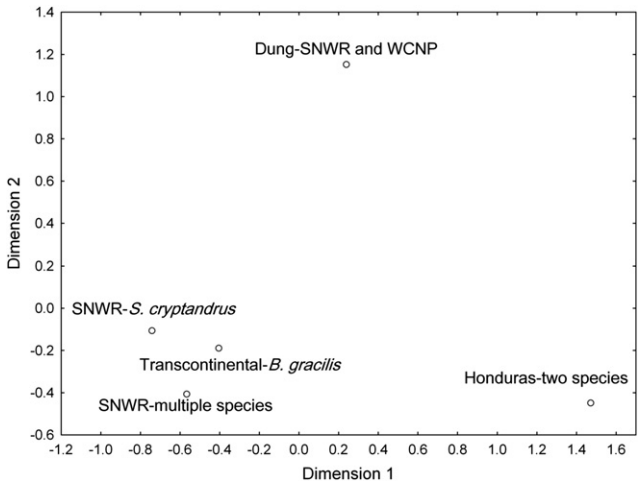

Fig 1 – Multidimensional Scaling (MDS) diagram showing relative similarity in RAF communities of various species of plants and dung at different sites. With the exception of the Honduras site, all sites are on the North American mainland. RAF communities were analyzed using Morisita-Horn similarity indices comparing OTUs created by assessing rDNA of ITS1, 5.8S, and ITS2 regions using 97 % similarity and 40 basepair overlap in SEQUENCHER 4.8. (SNWR = Sevilleta National Wildlife refuge, Socorro, NM; WCNP = Wind Cave National Park, Custer, SD). Transcontinental sites included grasslands National Park, Saskatchewan, Canada; WCNP; Konza Pairie, Manhattan, KS; SNWR; Janos, Mexico; and, Ojuelos, Mexico.

Interestingly, cosmopolitan clades, *Paraphaeosphaeria* spp., *Monosporascus* spp. and *Periconia* spp. commonly encountered in mainland grasses, were also isolated from Honduras grasses (though based on ITS rDNA similarity, the fungi were only distantly related to those on the mainland). Although standard caveats concerning PCR and amplification biases should be considered, taken collectively, we have good evidence to suggest that the RAF communities inhabiting weedy grasses on this Honduran island (and perhaps other islands) are compositionally variable and different from those found on the North American mainland. Additional assessment of RAF communities within grasses at other locations likely will provide needed information about how RAF are influenced by the host, environment and, importantly, how taxonomic proximity to other hosts affects the RAF composition. The fact that cosmopolitan (but distantly related) clades also are present in tropical grasses on islands opens up interesting questions concerning the biogeography and possible evolutionary relationships among the grasses and endophytic fungi. Because we obtained a limited number of sequences and cultures (and because there are seasonal changes), we were unlikely to have fully characterized the RAF community within this habitat. Additional work is necessary to determine how RAF change over time and whether invasive grasses are constitutionally set up to accept relationships with a different suite of RAF species.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.funeco.2012.08.002.

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