

## Morphological and Molecular Evidence of Arbuscular Mycorrhizal Fungal Associations in Costa Rican Epiphytic Bromeliads<sup>1</sup>

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### ABSTRACT

Arbuscular mycorrhizal fungi influence the growth, morphology, and fitness of a variety of plant species, but little is known of the arbuscular mycorrhizal (AM) fungal associations of plant species in forest canopies. Plant species' associations with AM fungi are most often elucidated by examining the roots for fungal structures; however, morphological data may provide a limited resolution on a plant's mycorrhizal status. We combined a traditional staining technique with a molecular marker (the 18S ribosomal gene) to determine whether or not a variety of epiphytic bromeliads form arbuscular mycorrhizal fungal associations. Using these methods we show that the epiphytic bromeliad *Vriesea werkleana* forms arbuscular mycorrhizal fungal associations with members of the genus *Glomus*. AM fungal sequences of this plant species formed three distinct clades nested within a larger *Glomus* clade; two of the clades did not group with any previously sequenced lineage of *Glomus*. Novel clades may represent novel species. Although *Vriesea werkleana* is associated with multiple AM fungal species, each individual plant is colonized by a single lineage. The combination of morphological and molecular methods provides a practical approach to the characterization of the mycorrhizal status of epiphytic bromeliads, and perhaps other tropical epiphytes.

*Key words:* cloud forest; Costa Rica; Monteverde; symbiosis; tropical mycorrhizae; VAM fungi.

CANOPY MICROHABITATS DIFFER FROM FOREST FLOOR HABITATS in many ways, including nutrient and water availability and spatial heterogeneity. Because canopy microhabitats are often subject to large temperature fluxes and periods of low water and nutrient availability, the forest canopy is considered an extreme plant environment (Benzing 1995). Epiphytic adaptations for attaining and retaining water and nutrients in this unique environment include water- and litter-trapping leaf arrangements, nutrient-absorbing trichomes, and symbiotic associations with organisms such as nitrogen-fixing bacteria (Benzing 1987, 1995). Arbuscular mycorrhizal (AM) fungi are vital to nutrient absorption in terrestrial plants and may influence the growth (Francis & Read 1995, Wilson & Hartnett 1998), morphology (Streitwolf-Engel *et al.* 1997), and reproductive success (Koide & Shumway 1994) of individual plants. Whereas much is known about the benefits of AM fungi to plants growing on the forest floor, little is known about the associations they form with plants in the forest canopy (Benzing 1987, 1995; Janos 1993; but see Rains *et al.* 2003). Recent work has suggested that some epiphytic plant families are in fact mycorrhizal; specifically epiphytic Araceae and Clusiaceae form AM fungal associations (Rains *et al.* 2003). However, other studies have suggested that mycorrhizal associations are rare and inconsistent in epiphytes, even in plant families (*e.g.*, Piperaceae) in which terrestrial species are mycorrhizal (Lessica & Antibus 1990, Maffia *et al.* 2000).

Epiphytes from the family Bromeliaceae have many adaptations for coping with the canopy environment and are one of the most diverse groups of epiphytes globally with approximately 200 species in Costa Rica alone (Morales 2000). Unique to this family, many species have imbricate leaves that form tanks that retain water and collect detritus. Bromeliads occupy a diverse range of habitats in the tropics, including a large vertical distribution within the upper and lower canopy (Benzing 1995). While much is known about the ecology of these important

epiphytic plants, mycorrhizal associations in bromeliads remain largely uncharacterized (Smith & Read 1997).

Arbuscular mycorrhizal fungal structures have previously been observed in epiphytic bromeliads (Lessica & Antibus 1990, Rabatin *et al.* 1993). Lessica and Antibus (1990) sampled four species of bromeliads and found that only one (*Pitcairnia sp.*) had an association with AM fungi. They suggested that the roots of epiphytic bromeliads may function as a hold fast, rather than being involved in nutrient absorption, and also noted that the one bromeliad species detected in canopy soils was the species that appeared to be mycorrhizal. Rabatin *et al.* (1993) showed that in the drier habitats of Venezuela, epiphytic bromeliads associate with *Glomus tenue*; the AM fungus was identified by collecting spores from around the roots of the plant. In moister regions, bromeliads tended to associate with coarse AM fungi, suggesting that there is variability in the species of AM fungi depending on habitat (Rabatin *et al.* 1993). Allen *et al.* (1993) observed dark septate fungi, and not AM fungi, in the roots of epiphytic bromeliads from Mexico. From these reports, it appears that the presence of AM fungi in epiphytic bromeliads may vary with respect to species, geographic region, and the localized environment (*i.e.*, soil, moisture, etc.).

In these studies, the morphology of structures in or around the root was used to characterize plants as mycorrhizal or non-mycorrhizal; however, morphology provides a limited resolution to the question of whether or not a plant species is mycorrhizal. Within roots, AM fungi are known to make intercellular hyphae, intracellular vesicles, hyphal coils, and arbuscules. Whereas arbuscules are unique to glomalean fungi, they are often rare or absent in field-collected samples (Smith & Read 1997). Many fungi from orders other than Glomales, including parasitic fungi, can form hyphae, vesicles and hyphal coils in plant roots. For these reasons, morphology alone may provide incomplete evidence to the question of whether or not bromeliad species are mycorrhizal.

In this study, our aim was to determine whether or not morphology might be complemented by the use of molecular markers, and particularly when assessing the mycorrhizal status of plants such as bromeliads

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where diagnostic morphological structures may be rare or absent. Are plants without the diagnostic morphology truly mycorrhizal? We collected a variety of epiphytic bromeliad species from the forest canopy of Monteverde, Costa Rica. Mycorrhizal status was assessed by identifying both morphological fungal structures characteristic of AM fungi and by sequencing a molecular marker, specifically the gene for the small subunit of the ribosomal RNA (18S), to confirm the mycorrhizal status of bromeliad roots.

## METHODS

**BROMELIAD SAMPLING AND STUDY SITES.**—Bromeliad root samples were collected during the rainy season (November through December) 2001 from Costa Rican montane cloud forests in Monteverde. The main collection site was the Estacion Biológica (EB) (1450–1650 m, 10°19'25"N 84°47'W). To facilitate species identification, samples were only collected from bromeliads containing inflorescences. Species were identified using Morales (2000). Additional samples were collected near the Monteverde cloud forest preserve (MP) (1460 m, 10°18'N 84°47'W). Canopy heights range from 15 to 32 m, and the forest is composed primarily of broad-leaved evergreens including trees from the Lauraceae, Moraceae, Leguminosae, Sabiaceae, and Meliaceae (Nadkarni *et al.* 1995; Clark *et al.* 1998). The substrate, location and size of the plant were recorded for each bromeliad sampled (data available from first author). Bromeliads were collected from heights ranging from 2 to 20 m (canopy and subcanopy habitats) using climbing techniques described by Perry (1978). In total, the roots of 50 bromeliad plants from 13 different species were collected and stored in 2 percent KOH for no longer than 1 week. Fungal morphology was determined from root samples processed and tested using the techniques described by Read (1993). Briefly, these consist of clearing in 10 percent KOH for 1 h at 90°C and then in 3 percent H<sub>2</sub>O<sub>2</sub> for 1 h at room temperature. The roots were then acidified in 1 percent HCl and stained with 0.05 percent trypan blue and assessed for the presence of AM fungal structures at 100–400 × magnification with a compound light microscope. In most cases the entire plant root was stained and scanned for AM fungal morphology. For larger plants approximately one third was scanned focusing on areas with most blue color. Images were captured using a Zeiss Axiophot photomicroscope.

We targeted three common epiphytic bromeliad species for molecular investigation: *Vriesea werkleana*, *Tillandsia excelsa*, and *Catopsis nitida*. These species were chosen because the individuals of these species possessed AM fungal-like structures inside the roots. DNA was isolated from bromeliad root samples that were collected from the same locations (EB and MP) in December 2002. We sampled 23–30 bromeliads from each of the three species. From each plant three root tips were collected, approximately 1–3 cm in length. The root tips were placed in 300 ml of CTAB buffer (100 mM Tris (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% CTAB cetyltrimethyl ammonium bromide). The samples remained in CTAB at room temperature for approximately 2 weeks and then were stored at –80°C until DNA extraction.

**DNA ISOLATION AND PCR.**—Genomic DNA was isolated from a subset of root samples through repeated freeze-thawing, maceration, and then

through a single chloroform extraction. The DNA was then purified using the QIAGEN Dneasy mini kit (Qiagen inc., Valencia, California). Initially, DNA was extracted from the root tips of eight plants of each of the species collected. Later, we extracted DNA from the rest of the *Vriesea werkleana* (Vw) samples, focusing on this species because the preliminary data suggested that sequences from *V. werkleana* were AM fungal. We amplified the glomalean 18s rDNA region using a combination of the putatively AM-fungal-specific primer AM1 (GTTTCCCCG-TAAGGCGCCGAA, Helgason *et al.* 1998), and the universal eukaryotic primer NS5 (AACTTAAAGGAATTGACGGAAG, White *et al.* 1990). Cycling parameters were 94°C for 3 min, 25–30 cycles of 94°C for 30 sec, 58°C for 1 min, 72°C for 90 sec with a final extension of 10 min at 72°C. The reaction mix consisted of 50 mM of each nucleotide, 0.2 mM of each primer, 0.25U/μL of Taq polymerase in a reaction buffer (0.5M KCl, 0.1M Tris–HCl (pH 8.3), and 25 mM MgCl<sub>2</sub>) diluted 1:4 with ddH<sub>2</sub>O and template.

**DNA SEQUENCING AND ANALYSIS.**—PCR amplicons were purified using QIAGEN QIAquick PCR purification and were then cloned into pCR 2.1 with the Invitrogen TA cloning kit (Invitrogen, San Diego, California). Eight clones from each sample were picked and re-amplified using the NS5/AM1 primer pair, and three of these were then sequenced. Nucleotide sequencing was done using the ABI 3100 Genetic Analyzer using the BigDye version 2.0 chemistry (Applied Biosystems Co., Foster City, California). Sequencing products were precipitated with sodium acetate and ethanol. DNA sequences were analyzed using Sequence Navigator v.1.0.1 (Applied Biosystems Co.). A positive sequence identification of an AM fungal sequence was made when the sample sequence was >90% similar, over more than 90 percent of the sequence, to Glomalean sequences on the GenBank database. Sequences were aligned using Clustal W and then manually aligned with sequences of the complete 18S nuclear rDNA of glomalean fungi and outgroups from Redecker *et al.* (2000). Parsimony and neighbor joining analyses were conducted with PAUP \* 4.0beta8 (Swofford 2002).

TABLE 1. Species of bromeliad containing putative AM fungal structures.

Bromeliad species	Vesicles and hyphal coils	Putative arbuscules	Total plants sampled
<i>Catopsis nitida</i>	4	1	4
<i>Catopsis nutans</i>	3	0	4
<i>Catopsis wagnerini</i>	2	0	2
<i>Tillandsia excelsa</i>	2	1	2
<i>Tillandsia juncea</i>	1	0	1
<i>Tillandsia multicaulis</i>	3	0	5
<i>Tillandsia punctulata</i>	3	0	4
<i>Racinaea spiculosa</i>	2	1	2
<i>Vriesea notata</i>	2	0	2
<i>Vriesea werkleana</i>	4	2	4
<i>Vriesea ororiensis</i>	2	0	2
<i>Vriesea chontalensis</i>	0	0	3
<i>Tillandsia leiboldiana</i>	0	0	4

## RESULTS

Eleven of the 13 different species of bromeliad stained using fungal-specific staining techniques appeared to contain putative AM fungal structures (Table 1). These AM fungal structures included vesicles and hyphal coils (Fig. 1) and although these structures are not exclusive to AM fungi, they did suggest that AM fungal associations are formed

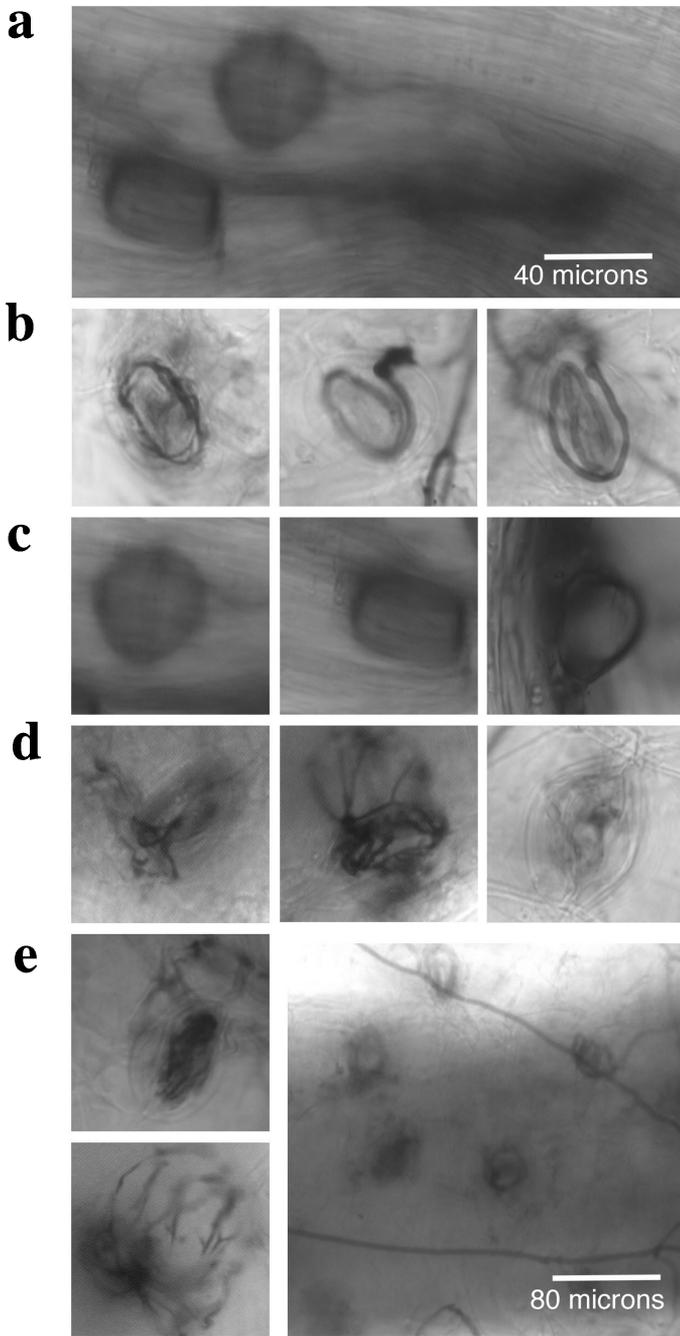


FIGURE 1. Fungal structures characteristic of AM fungi observed in the roots of the epiphytic bromeliad *Vriesea werkleana*: vesicles (a, c) hyphal coils (b, d), and putative arbuscules (e).

TABLE 2. AM fungal sequences obtained from the roots of the epiphytic bromeliad *Vriesea werkleana*. Sample labels include the number of the plant from which the sample was taken, and letter (a, b, c) of the root tip that tested positive. RN represents root nest as described in text. "These sequences were complete enough to positively identify as AM fungal but were not sufficient for the public database and have not been submitted to GenBank."

Sample	Location	Substrate	Height (m)	Sequence
9c	EB	Soil (RN)	3	AY493663, AY493664
41a	MV	Soil	8	a
41c	MV	Soil	8	a
73b	EB	Soil (RN)	10	AY493665, AY493666, AY493667, AY493668
75b	EB	Soil (RN)	2	AY493669, AY493670, AY493671, AY493672
76b	EB	Soil (RN)	11	AY493673, AY493674, AY493675, AY493676, AY493677
76c	EB	Soil (RN)	11	AY493678, AY493679, AY493680, AY493681

by at least some species of epiphytic bromeliad. Structures tentatively identified as arbuscules, which are specific to AM fungi, were observed in at least one individual plant from four of the thirteen species. Based on the morphological data (vesicles, hyphal coils, etc.) of these species, we chose *Vriesea werkleana*, *Tillandsia excelsa*, and *Catopsis nitida* for molecular investigation.

Using a PCR-based identification we were able to conclusively identify AM fungal associations in one of the three species of epiphytic bromeliad we targeted. The primer we used, AM1, has been shown to exclude certain lineages of AM fungi and can also amplify certain members of the ascomycota (Redecker 2000). Nonetheless, we were able to successfully identify glomalean sequences from the bromeliad species *Vriesea werkleana*. Of the 23 *V. werkleana* plants sampled, we were able to amplify AM fungal sequences from at least one root tip of five different bromeliad plants (Table 2). In two of the five plants more than one root tip was AM fungal (Table 2). In the other two species two root tips from eight plants were tested and no AM fungal sequences were amplified.

Comparison of sequences with those of the GenBank sequence database ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)) demonstrated that our sequences were collected from members of the genus *Glomus*, in the order Glomerales (Fig. 2). The sequences form three separate clades within a larger clade that includes only other members from the genus *Glomus*. The sequences form one clade group with an unidentified species of *Glomus*. Sequences of the remaining two clades do not appear to group with any previously sequenced species. These AM fungal sequences are either from the described *Glomus* species that are not yet sequenced, or may represent novel species.

## DISCUSSION

We have positively identified an association between the epiphytic bromeliad *V. werkleana* and AM fungi, using both morphological and

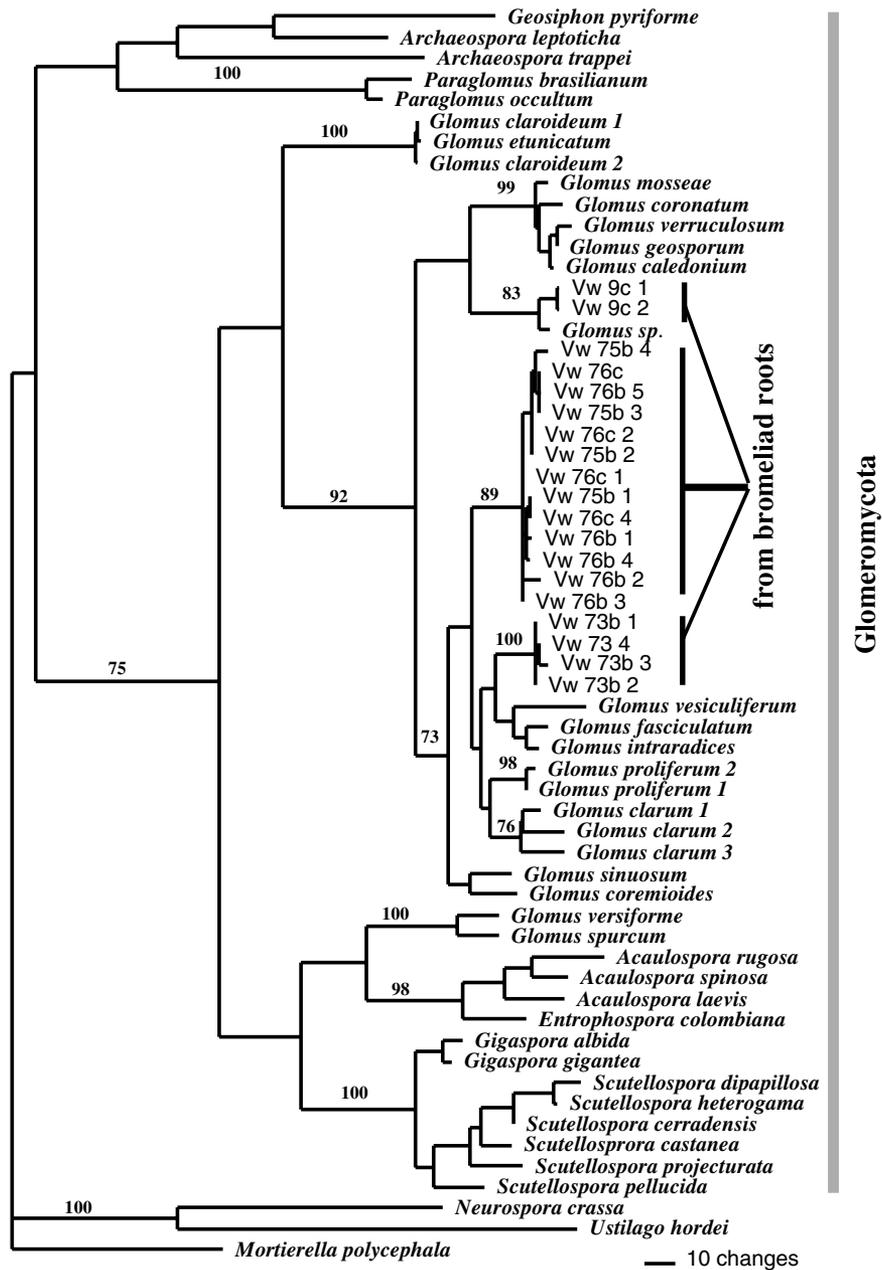


FIGURE 2. Phylogenetic placement of root-associated fungal isolates from *Vriesea werkleana* (Vw) bromeliads in an 18S SSU tree of Glomeromycota obtained from Redecker (2000) and annotated with sequences from GenBank. Sequences are named by species, sample number, and sequence number. Analysis was performed by neighbor-joining with 1000 bootstrap replicates (values of 70% are shown above branches). Representatives of Ascomycota and Basidiomycota were used as outgroups.

molecular approaches. This result suggests that in cases where morphology is not diagnostic of AM fungi (*i.e.*, vesicles and hyphal coils but no or very few arbuscules), molecular markers can be used to determine AM fungal status. Both morphological and molecular methods have advantages and disadvantages. When combined, however, they offer a novel approach to working with this notoriously difficult group of fungi.

The species *V. werkleana* is clearly mycorrhizal; however, we cannot exclude the possibility that *Tillandsia excelsa* and *Catopsis nitida* also form

associations with AM fungi. The primer set that we used (NS5/AM1) is not inclusive of all species of AM fungi, and will not amplify divergent lineages in the glomeromycota (Redecker 2000). Other primer sets have been designed to amplify these lineages; however, when tested on a subset of our samples these primer pairs yielded sequences from Ascomycete lineages (unpublished data). An additional, more general primer set (ITS 1F/ITS 4) amplified fungal sequences from many fungal lineages and of the amplicons sequenced none showed significant homology to the AM

fungal sequences available in GenBank (unpublished data). If we had included additional individuals or greater numbers of root tips from each individual we might have detected AM fungi in these species. Perhaps colonization is particularly rare in *T. excelsa* or *C. nitida*. However, the data suggest that these species are not mycorrhizal, despite the initial discovery of vesicles and hyphal coils in their roots, and the tentative identification of an arbuscule from one individual of each species. As discussed previously, vesicles and hyphal coils are not diagnostic of AM fungi, and may be indicative of infection by other groups of fungi.

Though we cannot rule out the possibility that more than five of the *V. werkleana* tested are mycorrhizal, from the five *V. werkleana* plants that proved to be mycorrhizal we can infer trends related to the location and substrate of plants associated with AM fungi. Most of the *V. werkleana* plants sampled grew on the horizontal surface of a tree trunk, where detritus and soil are limited. To compensate, this particular species traps detritus in the pockets left by the bases of old leaves. *V. werkleana* grows new roots that penetrate and wrap around these humic substances creating a root nest (RN) at the base of the bromeliad. Of the species we tested for AM fungi, only *V. werkleana* has this adaptation. Of the five plants that proved to be mycorrhizal, four made root nests. None of the remaining *V. werkleana* that failed to produce AM sequences contained root nests. We hypothesize that these root nests facilitate AM fungal colonization of roots for plants growing on the vertical surface of the trunk, or in other areas with little to no soil.

From the phylogeny it appears that the plant species can associate with more than one AM fungal species; however, individual plants appear to be colonized by single lineages. Sequences from *V. werkleana* were divided into three distinct fungal clades, but each of the five mycorrhizal plants was associated with only one of the three clades.

It is not surprising that the AM fungal sequences obtained from the roots of *V. werkleana* bromeliads appear to be from as yet unsequenced lineages. It is possible that the lineages are novel species. As additional sequences accumulate in public databases we will be able to use that information for accurate species identification (Pringle *et al.* 2000, 2003). Accurate molecular identification will be especially useful because at present there is no simple way to identify glomalean species other than via spore morphology. AM fungal spores are not formed by all species of AM fungi and are not formed at all times due to seasonality (Morton & Redecker 2001, Pringle & Bever 2002). Furthermore, the spores in the soil surrounding roots may not represent all the species associated with the plant (Redecker 2000).

The difficulties associated with sampling canopy environments and identifying AM fungi have limited our understanding of AM fungal ecology. The ability to conclusively identify AM fungal associations in canopy epiphytes, with both morphological and molecular methods, will facilitate further research. Exploring the ecology of AM fungi in canopy habitats will increase our knowledge of the role of these influential fungi and their impact on tropical habitats.

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