PRESENCE AND DISTRIBUTION OF FUNGAL SYMBIOSIS IN
CHEILANTHES FEEI AND CHEILANTHES LANOSA IN SOUTHEASTERN
MISSOURI AND SOUTHERN ILLINOIS

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PRESENCE AND DISTRIBUTION OF FUNGAL SYMBIOSIS IN CHEILANTHES FEEI AND CHEILANTHES LANOSA IN SOUTHEASTERN MISSOURI AND SOUTHERN ILLINOIS

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ABSTRACT

_Cheilanthes feei_ (Polypodiaceae) is a xerophytic fern that grows exclusively on dry, exposed calcareous rocks and rocky outcrops. _C. feei_ is unusual in this regard since ferns typically inhabit wet environments, but the physiological basis for this is unclear. I hypothesized that _C. feei_ survives its arid environment in part with the help of mycorrhizal associations, which enhance water and nutrient uptake in host plants. Although fungal symbioses rarely occur in members of the Polypodiaceae, most plants do have mycorrhizal associations. In this study, light microscopy was used to document the presence of vesicular-arbuscular mycorrhizae in _C. feei_ and _C. lanosa_ sporophytes. Results indicated that an endosymbiotic relationship exists in the sporophyte generation of these two species. This is the first report of mycorrhizal associations within the genus _Cheilanthes._
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INTRODUCTION

Cheilanthes is a genus of small (10-20 cm), evergreen, lithophytic, perennial ferns that inhabit warm, dry and rocky regions. They are commonly referred to as the Lip Ferns, since the sori are borne at the margins of the pinnules and the leaf margins themselves are often reflexed to form false indusia that protect the sori. Many of the species are apogamous and most of them are xerophytic.

The term "xerophytic fern" is somewhat of an oxymoron since most pteridophytes inhabit moist environments. Ferns generally lack some of the more typical adaptations of xerophytes, such as a thick hydrodermis, sunken stomata and CAM photosynthesis (Raven et al. 1992). These adaptations moderate moisture loss and maintain cellular integrity and function (Fahn, 1990). In addition to the lack of typical xerophyte morphology, ferns also require water for reproduction. Fern gametes are free-swimming, and at least a film of water is necessary for the male gametes to travel from the antheridia into the neck of the archegonia to fertilize the eggs (Raven et al. 1992).

However, cheilanthoid ferns have alternate reproductive strategies that allow them to persist in their xeric environments. For instance, many cheilanthoid ferns are capable of apogamy, which allows for reproduction in the absence of adequate water (Raven et al. 1992). Apogamous outgrowths form directly from the gametophyte and produce a new sporophyte without syngamy (Steil, 1939). In this manner, reproduction is achieved in spite of a water deficit.

Although apogamy is an adaptation that enables reproduction in xeric environments, there is no evidence that there are any physiological or strong morphological adaptations that would explain their survival. Cheilanthoid ferns do possess some common
xeromorphic adaptations which prevent water loss and give them an environmental advantage. One specific example is poikilohydry, in which the fronds curl up during periods of water stress (Mohlenbrock, 1999, Gratani et al. 1998). Cheilanthoid ferns also exhibit microphyllly, in which the leaves are diminished in size. Their stomata are located on the undersurface of the blades, which possess a large number of trichomes. These adaptations purportedly prevent water loss and drought necrosis and reduce overheating. (Daubenmire, 1959; Hevly, 1963; Martin, 1943; Gratani et al., 1998).

Although ferns of the genus *Cheilanthes* may possess some or all of these adaptations, research suggests that the genus *Cheilanthes* is less desiccation tolerant than other xerophytic pteridophytes. For example, when compared to two other desert species, *Selaginella lepidophylla* and *Notholaena sinuata* var. *cochisensis*, *Cheilanthes tomentosa* was found to be the least tolerant of extended desiccation (Harten and Eickmeier, 1987). In addition, an aridity gradient exists for the cheilanthoid ferns, in which they frequently occupy more humid niches. They occupy niches at higher elevations than other xeric taxa (Hevly, 1963; Harten and Eickmeier, 1987). Harten and Eickmeier (1987) suggested that there is a correlation between the presence of *Cheilanthes* species at higher altitudes in deserts and increased humidity in these environments.

Other evidence suggests that *Cheilanthes* ferns are less desiccation tolerant than other xerophytic pteridophytes. Spore germination requirements for *Cheilanthes* do not appear to differ significantly from their more mesic counterparts (Nondorf et al., 2001; Swatzell et al., 2001). *C. feei* spores germinate in varying light qualities and also in darkness. They germinate in a range of pH levels and in widely varying moisture levels.
This is in contrast to other xerophyte spores, which specifically require red light and appreciable moisture to germinate (Hevly, 1963). From the data it may be inferred that these ferns might not be true xerophytes, yet they somehow survive in arid environments. The physiological basis for this phenomenon remains unclear.

One potential mechanism is the utilization of fungal symbionts in the form of mycorrhizal associations. The mutualistic relationship between a fungus and the absorptive organs of a plant are called mycorrhizae, from the Greek *myko* meaning fungus and *rhiza* meaning root. In this relationship, the fungus obtains photosynthates from the plant and the plant obtains various nutrients from the fungus (Cox and Tinker, 1976; Mendoza and Borie, 1998). Mycorrhizal fungi also improve the water relations of host plants (Harley and Smith, 1983, Al-Karaki 1998).

Mycorrhizae have often been grouped into three broad classifications: ectomycorrhizae, ectendomycorrhizae and endomycorrhizae. Ectomycorrhizae have hyphae that do not penetrate the cell walls of their host plants. Their hyphae surround the cortical cells to form a Hartig net and their extramatrical hyphae form a mantle around the root. Mycelial strands extend from the mantle into the soil and greatly increase the surface area available for nutrient and water absorption (Harley and Smith, 1983). Ectendomycorrhizal hyphae penetrate the root cortical cells and form a mantle around the root. The majority of the fungus remains in the extraradical matrix. Ectomycorrhizal and ectendomycorrhizal colonization of pteridophytes has not been documented.

Endomycorrhizae penetrate root cortical cells and do not form a Hartig net. The most common type of mycorrhizae are known as the vesicular-arbuscular mycorrhizae (VAM). VAM hyphae penetrate the host cell walls and colonize the cortical region in an
intercellular, as well as an intracellular, manner. VA mycorrhizae produce intercellular vesicles, which store lipids, and branching arbuscules, which provide a large surface area for plant/fungal interface and the exchange of water and nutrients (Bonfante-Fasolo, 1984; Figure 1). The extraradical contingent in the soil matrix consists of hyphae, vesicles, and spores that are either borne singly or grouped in sporocarps.
Figure 1. Sporophyte root with vesicular-arbuscular mycorrhizal colonization.

Arbuscules (A) and intraradical vesicles (IV) are the diagnostic characteristics of vesicular arbuscular mycorrhizae (VAM). Coiled hyphae (CH) are common but not omnipresent. The fungus can enter the root cortical cells directly from an appressorium (AP) or enter through root hairs (not shown). Note that the fungal mycelium (H) penetrates the cell wall (CW) and prompts callose deposition (arrow) but does not penetrate the host plasma membrane (arrowhead). In addition, the VAM mycelium does not penetrate meristematic tissues or the vascular cylinder (VT). Spores (S) are essential for identification to species. Extraradical vesicles (EV) and intraradical vesicles (IV) are lipid storage organs. Modified from Swatzell (1995).
Boullard (1979) correlates fungal colonization in pteridophytes to their evolutionary status. He suggests that endosymbiosis is obligatory in the gametophytes and sporophytes of less highly evolved pteridophytes, but that the relationship shifts with evolutionary advancement and becomes facultative and exclusive to the sporophyte in the higher pteridophytes. He lists mycorrhizae as absent in the gametophytes of the Polypodiaceae and rare or absent in the sporophytes.

Mycorrhizae have since been documented in most plant families (Raven et al. 1992). A few known exceptions include the Cruciferae, Chenopodiaceae, Caryophyllaceae and Cyperaceae (Powell and Bagyaraj, 1984). Moreover, mycorrhizae are geographically and ecologically ubiquitous. They occupy virtually every biome, at almost every latitude, longitude, and altitude (Allen, 1991). Mycorrhizae are also non host-specific. Any mycorrhizae can potentially colonize any host plant (Allen, 1991). Studies have documented the ability of VA mycorrhizae to foster drought tolerance in plants (Sieverding, 1979: quoted in Cooper, 1984; Nelsen and Safir, 1982; Aboul-Nasr, 1998). Furthermore, some VAM species are themselves xerophytic (Allen, 1991). In light of this, I hypothesize that Cheilanthes ferns are in fact mycorrhizal.

*Cheilanthes feei* and *Cheilanthes lanosa* are the two fern species chosen for this study. *Cheilanthes feei* is the Slender Lip Fern (Figure 2). It grows in the crevices of exposed, calcareous cliffs and dry rocky slopes. It has a short scaly rhizome, fronds that reach 25 cm, a linear-lanceolate blade that is pinnate-pinnatifid to tripinnate and a densely hairy lower surface. *C. feei* is common in central and western North America (Mickel, 1979; Figure 3).
*Cheilanthes lanosa*, the Hairy Lip Fern, is a close relative to *Cheilanthes feei* (Gray, 1973; Figure 4). *Cheilanthes lanosa* grows in the shaded substrate on top of cliffs and rocky slopes. It has a slender, short creeping rhizome. The blade is pinnate-pinnatifid to tripinnate, and the fronds reach 50 cm tall. *C. lanosa* is frequent in the eastern and southern United States (Mickel, 1979; Figure 5).

These particular fern species were chosen for several reasons. First, they are closely related. They both grow on sedimentary rocks, which frequently provide water. These species are neither rare nor endangered. In addition, they are found locally, whereas most North American species of *Cheilanthes* are found in the southwestern United States and in Mexico. Aside from their similarities, the two ferns were also chosen because of the differences between them. *C. feei* inhabits a more xeric environment than that of *C. lanosa*, and it possesses xerophytic adaptations that *C. lanosa* lacks. In this study, I examined *C. feei* and *C. lanosa* in southeastern Missouri and southern Illinois for the presence of mycorrhizae.
Figure 2. *Cheilanthes feei.*

*Cheilanthes feei* sporophyte growing out of limestone formation at Reis Biological Station (Crawford County, MO). Note microphylly in the sporophylls, the recurved nature of the margins and the gray-green appearance.

Figure 3. **North American distribution of* Cheilanthes feei.*

The fern inhabits the Western and Midwestern regions of the United States, from California north to Canada and east to Illinois and south to Texas.
Figure 4. Cheilanthes lanosa.

Cheilanthes lanosa sporophyte in Makanda, IL. Note the recurved margins visible on the abaxial portion of the pinnae and the bright green coloration. The ferns are growing in the shaded substrate at the base of trees on top of a sandstone bluff.

Figure 5. North American distribution of Cheilanthes lanosa.

Cheilanthes lanosa inhabits the Midwestern and Eastern regions of the United States, from Florida north to New York and west to Kansas and south to eastern Texas.
MATERIALS AND METHODS

Collection of Plant Materials

*Cheilanthes lanosa* was collected from: (1) Frey’s Bluff (n=5) at Mingo Wildlife Refuge in southeast Missouri in November 2000, (2) La Rue/Park Hills (n=5) from rocky outcrops near the Big Muddy River in Lincoln County, Illinois in January 2001, and (3) the top of a sandstone bluff approximately 0.25 miles north of Makanda, Illinois (n=3) in January 2001.

*Cheilanthes feei* sporophytes were collected from three sites in Southeast Missouri: (1) Reis Biological Station (n=12)-from an Eminence–Potosi Limestone Dolomite Formation in Crawford County in November 2000, (2) Cedar Hill (n=7)-limestone cliffs 1.3 mi east of Cedar Hill in Jefferson County on Hwy BB in February and May 2001 [Leo and Mary Bequette estate], and (3) Mammoth Road (n=5)-limestone bluffs 2.5 mi south of County Hwy H on Mammoth Road along Big River in Jefferson County in February 2001.

Clearing and Staining of Roots

Roots from specimens at each site were excised and rinsed with tap water. These were fixed overnight in 2% (v / v) glutaraldehyde in commercial bottled drinking water (Aberfoyle, CA). Roots were cleared with 10% (w / v) KOH at 100°C for 5 minutes. After clearing, roots were lightened with 30% (v / v) H$_2$O$_2$ for 4 minutes. Roots were acidified with 1% (v / v) HCl for 20 minutes, then placed in a second change of HCl overnight.
To view vesicles, arbuscules, and to obtain fungal spore diameters, a portion of the cleared specimens was stained with Trypan Blue in acidic glycerol [50% (v/v) glycerol, 45% (v/v) ddH$_2$O, 0.05% (v/v) HCl, 0.05% (w/v) Trypan Blue]. Roots were heated in stain at 90°C for 60 minutes and destained at room temperature in acidic glycerol without Trypan Blue (modified from Brundrett et al. 1996).

Stained specimens were examined with light microscopy using an Olympus BH microscope. Qualitative data (i.e. structures that indicate the presence or absence of mycorrhizal associations) were documented as brightfield images. These were captured digitally with a Spot Insight color camera 3.2.0 and Spot Advanced version 3.2 for Windows.

Roots that were too dark to visualize following clearing and staining were dehydrated in graded ethanol baths (70% ethanol for 10 minutes, 95% ethanol for 10 minutes, 95% ethanol for 10 minutes), followed by two changes of 100% Hemo D for 10 minutes each. Roots were then placed in two changes of paraffin wax with 1% (w/w) beeswax for 1 hour at 60°C, and a third change overnight. The whole mounts were then sectioned (35 µm sections) using an 820 Spencer Microtome. Paraffin sections were floated on ddH$_2$O and dried at 40°C. Paraffin was cleared with two baths of 100% Hemo D for 5 minutes each. Root sections were rehydrated with two changes of 95% ethanol for 3 minutes each and one change of 70% ethanol for 3 minutes. Specimens were stained with Lactophenol Cotton Blue (Larone, 1993) for 20 minutes and destained in three successive ddH$_2$O baths. Roots were then mounted in ddH$_2$O and examined with light microscopy using an Olympus BH microscope. Brightfield images were captured
digitally with a Spot Insight color camera 3.2.0 and Spot Advanced version 3.2 for Windows. All measurements were processed within this software.

**Spore Identification**

**Light Microscopy**

For spore identification, fungal spores were removed from the soil matrix using a stereomicroscope and tweezers. Spores were placed in a solution containing equal parts of Melzer’s Reagent [4.5% (w/v) chloral hydrate, 45.0% (v/v) ddH₂O, 0.7% (w/v) iodine, and 2.3% KI] and polyvinyl-lacto-glycerol [PVLG; 45.5% (v/v) ddH₂O, 45.5% (v/v) lactic acid, 4.5% (v/v) glycerol, and 4.5% (w/v) polyvinyl alcohol] (INVAM, 2001). The spores were allowed to stain for a minimum of 48 hours, then viewed with either differential interference contrast optics (DIC optics) on an Olympus BH2 microscope or with brightfield microscopy on an Olympus BH microscope.

DIC micrographs were captured on Ektachrome 160T film. Brightfield images were captured digitally with a Spot Insight color camera 3.2.0 and Spot Advanced software version 3.2 for Windows.

**Color Assay**

Spore color was assessed with an INVAM Color Chart (INVAM, 2001; generously provided by Dr. Joseph Morton, West Virginia University, WV). Digitized images of the spores were used to compare spore color-to-color swatches on the chart. Color ranges were determined using a color picker (Adobe Photoshop 5.0) and haphazardly chosen pixels (n = 6). The color range was recorded as a series of four numbers representing the percent blue (cyan), red (magenta), yellow, and black, respectively.
RESULTS

Vesicular arbuscular mycorrhizae (VAM) were present in both *Cheilanthes lanosa* and *Cheilanthes feei* sporophyte roots at each sight. Diagnostic features (i.e. vesicles, arbuscules, spores) were documented in specimens at every site. Although all features were not identifiable in every sporophyte, specimens at each site contained the appropriate structures or spores for identification and for documentation of the fern / fungal relationship (Table 1).

*Colonization of Cheilanthes lanosa by endophytic fungi*

**Site 1: Makanda, IL**

Golden hyphae were associated with whole root specimens of *Cheilanthes lanosa* sporophytes and were visible under a dissecting scope (not shown). Stained roots examined with light microscopy revealed extraradical hyphae associated with the roots and rhizomes of *C. lanosa* sporophytes (Figure 6). Hyphae were aseptate and ranged from 2 - 8 µm in diameter. The hyphae contained characteristic swellings along the length of the hyphae (Figure 7). The hyphae entered the roots through root hairs (Figure 7) or through direct penetration of the cortical layer (not shown). Penetrating hyphae entered the sporophyte roots via appressoria on the root surface (Figure 8). Hyphae also bifurcated upon entry into the cortical region of the root (Figure 9).

Vesicles were identified in the extraradical matrix. These were borne at the terminal ends of hyphae, either singly (Figure 10) or in clusters on intercalary axes (Figure 11). The vesicles measured 10–25 µm in diameter.

Straw-colored globose spores were also present in the soil and were borne either singly or grouped in sporocarps (Figure 12). The spore walls measured *ca.* 15 µm in
diameter. The spores were associated with subtending hyphae (Figure 12) that measured ca. 50 µm in diameter at the widest part and then tapered to 36 µm.

Intraradical fungal structures were present. Intraradical fungal hyphae measured ≤4 µm. These hyphae colonized the cortical parenchyma and did not penetrate the vascular cylinder (Figure 13). The hyphae ran intercellularly along the longitudinal root axis (Figure 13). Intraradical arbuscules (Figure 13) and vesicles (Figure 14) were also present.
Table 1. Fungal structures present in association with *Cheilanthes lanosa* and *Cheilanthes feei* sporophytes.

Arbuscules, hyphae, vesicles, and spores were documented in sporophytes at every site with the exception of arbuscules at site #3 (*Cheilanthes lanosa*, Mingo Wildlife Refuge). Identifying spores were also documented at every site.
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<th>CHEILANTHES LANOSA</th>
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<td>Site 1</td>
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<td>Spores</td>
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Figure 6. Extramatrical fungal mycelium in association with *Cheilanthes lanosa* sporophyte rhizome and roots.

Extraradical hyphae (EH) are aseptate and measure *ca.* 8 μm in diameter. Rhizome is brown and sporophyte roots are stained blue (two shown). Fungal structures are stained with Trypan Blue and viewed with brightfield microscopy. IH = Intraradical hyphae. A = arbuscule. * = Y junction. Bar = 50 μm.

Figure 7. Fungal hypha inside *Cheilanthes lanosa* sporophyte root hair.

Hypha is aseptate and contains characteristic small protuberances. Intraradical fungal hypha (IH) is stained with Trypan Blue and viewed with brightfield microscopy. Hyphal diameter is *ca.* 4 μm, to *ca.* 8 μm with protuberances. Arrow = protuberance. RH = root hair. Bar = 10 μm.

Figure 8. Fungal hypha forms an appressorium and penetrates a *Cheilanthes lanosa* sporophyte root.

The hypha constricts then regains its previous diameter following penetration. Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. Arrow = hyphal constriction. A = appressorium. EH = extraradical hypha. IH = intraradical hypha. Bar = 10 μm.

Figure 9. Fungal hypha bifurcates in *Cheilanthes lanosa* sporophyte root.

The hypha (IH) bifurcates into four hyphae in the sporophyte root. Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. Arrows = bifurcation. Bar = 10 μm.
Figure 10. **Extraradical vesicle in the soil matrix of a *Cheilanthes lanosa* sporophyte.**

Vesicle is borne at the terminal end of an extramatrical hypha (EH). Hyphal diameter is *ca.* 5 µm. Vesicle (EV) diameter is *ca.* 40 µm. Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. Bar = 50 µm.

Figure 11. **Cluster of vesicles in the soil matrix of a *Cheilanthes lanosa* sporophyte.**

Vesicles (EV) are borne at the end of intercalary axes of fungal hyphae (EH). Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. Bar = 50 µm.

Figure 12. **Fungal spores from the soil matrix of a *Cheilanthes lanosa* sporophyte viewed with differential interference contrast optics (DIC).**

Spores are stained with Melzer's reagent and mounted in Melzer's Reagent and polyvinyl-lacto-glycerol (PVLG). Spores measure *ca.* 150 µm in diameter. Spore wall (SW) measures *ca.* 15 µm. Septum is V-shaped (arrow = septum). Subtending hypha (EH) measures *ca.* 50 µm at the widest part then tapers to *ca.* 36 µm. Hyaline sporocarp is visible. SP (white) = fungal spore. SP (black) = sporocarp. Bar = 50 µm.

Figure 13. ***Cheilanthes lanosa* sporophyte root containing arbuscules.**

Arbuscules (A) are present within cortical root cells (CRC). Intercellular hyphae (IH) that produce the arbuscules are clearly visible. Note that the vascular tissue (VT) is not compromised. Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. Intraradical hyphae measured ≤4 µm. Bar = 10 µm.
Figure 14. *Cheilanthes lanosa* sporophyte root containing intercellular hyphae and vesicle.

Vesicle (IV) measures ca. 10 µm wide by ca. 25 µm long. Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy.

IH = intercellular hyphae. Bar = 10 µm.
Site 2: La Rue / Park Hills

Stained roots mounted on microscope slides and examined with light microscopy revealed extramatrical hyphae associated with the roots of *Cheilanthes lanosa* sporophytes (Figure 15). The hyphae were aseptate and ranged from 2 - 6 µm in diameter. Occasional bifurcations produced Y-shapes (Figure 15).

Intraradical hyphae were present and measured ≤4 µm in diameter (Figure 15). These hyphae ran parallel to the longitudinal axis of the root. They formed arbuscules and intraradical vesicles *ca.* 30 µm in diameter (Figure 15).

Extraradical hyphae formed vesicles in the soil matrix (Figure 16). These vesicles were globose and measured *ca.* 30 µm in diameter. Fungal spores were present in the soil (Figure 17). These were golden and measured *ca.* 50 µm in diameter. Globose swellings were formed in groups at the terminal ends of extramatrical hyphae (Figure 18). These organs averaged 19 µm in diameter (n = 15) and may have been extraradical vesicles or immature spores.
Figure 15. Fungal hyphae in association with *Cheilanthes lanosa* sporophyte roots.

Extraradical hyphae (EH) are visible in association with sporophyte roots (SR). The hyphae penetrate the root and give rise to intraradical hyphae (IH) and arbuscules (A). Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. Bar = 50 µm.

Figure 16. Fungal vesicle in the soil matrix of *Cheilanthes lanosa* sporophyte.

Extramatrical fungal hyphae (EH) produce globose vesicles (EV). Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy. Bar = 10 µm.

Figure 17. Fungal spore from the soil matrix of *Cheilanthes lanosa* sporophyte viewed with differential interference contrast optics (DIC).

Spore (SP) is stained with Melzer's reagent and mounted in Melzer's Reagent and polyvinyl lacto-glycerol (PVLG). Spore measures *ca.* 50 µm in diameter. Spore wall (SW) measures *ca.* 5 µm. Bar = 25 µm.

Figure 18. Globose fungal structures from rhizosphere of *Cheilanthes lanosa* sporophyte.

Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy. Globose structures measure *ca.* 20 µm in diameter and may be extramatrical vesicles or immature spores. EH = subtending hyphae. FS = fungal structures. Bar = 10 µm.
Site 3: Mingo Wildlife Refuge

A low level of fungal colonization was observed in specimens of *Cheilanthes lanosa* obtained from Mingo Wildlife Refuge. Virtually every sporophyte root examined was free of fungal structures (Figure 19). A few hyphae were found in the extraradical matrix of the specimens (Figure 20). These hyphae were aseptate and measured 2 - 6 µm in diameter. No extramatrical vesicles were observed. The soil matrix contained light brown fungal spores (Figures 21-24). The spores ranged from *ca.* 60 µm to *ca.* 250 µm in diameter and possessed V-shaped septa (Figure 22). The spores were keyed out to the genus *Glomus* and specifically to *Glomus mosseae* using the keys provided by Hall (1984). This identification was supported by information and pictures on the INVAM website (INVAM, 2001).

In addition to fungal spores, intraradical fungal structures were present in specimens from Mingo Wildlife Refuge. Hyphae ranged from 1–4 µm in diameter and ran parallel to the root longitudinal axis (Figure 25). Intracellular vesicles were present. These were ovoid and measured *ca.* 15 µm by 50 µm (Figure 26).
Figure 19. Sporophyte roots of *Cheilanthes lanosa* with no fungal colonization.

No fungal structures are present in association with this sporophyte root (SR). Roots measure *ca.* 40 µm in diameter. Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy. Bar = 50 µm.

Figure 20. Fungal hyphae in the soil matrix of a *Cheilanthes lanosa* sporophyte.

Extramatrical hypha (EH) measures *ca.* 4 µm in diameter. Specimen is stained with Lactophenol Cotton Blue, mounted in ddH_2_0 and viewed with brightfield microscopy. 35-µm section. Bar = 10 µm.

Figure 21. Fungal spore in soil matrix of *Cheilanthes lanosa* sporophyte.

Spore wall (SW) is clearly visible in this specimen. Fungal spore (SP) is mounted in Melzer's Reagent and polyvinyl-lacto-glycerol (PVLG) and viewed with brightfield microscopy. Spore diameter measures *ca.* 70 µm. Spore wall measures *ca.* 7 µm. Bar = 10 µm.

Figure 22. Extramatrical fungal spore with V-shaped septum.

Subtending hypha (SH) is visible and septum (arrow) is clearly V-shaped. Spore wall (SW) measures *ca.* 7 µm. Spore is mounted in Melzer's Reagent and polyvinyl-lacto-glycerol (PVLG) and viewed with brightfield microscopy. Bar = 10 µm.
Figure 23. Fungal spore from rhizosphere of *Cheilanthes lanosa* sporophyte.

Specimen is mounted in Melzer's Reagent and polyvinyl-lacto-glycerol (PVLG) and viewed with brightfield microscopy. Spore (SP) diameter is *ca.* 50 µm and spore wall (SW) measures *ca.* 4 µm in diameter. EH = Subtending hypha. Bar = 10 µm.

Figure 24. Fungal spore from rhizosphere of *Cheilanthes lanosa* sporophyte.

Fungal spore (SP) diameter is *ca.* 230 µm. The subtending hypha (EH) is still attached. Specimen is mounted in Melzer's Reagent and polyvinyl-lacto-glycerol (PVLG). Bar = 50 µm.

Figure 25. Intraradical hyphae in root of *Cheilanthes lanosa* sporophyte.

Hypha (IH) bifurcates (arrow) and proceeds parallel to the longitudinal axis of the root. Specimen is stained with Lactophenol Cotton Blue and mounted in ddH₂O. 35-µm section. CRC = cortical root cell. Bar = 10 µm.

Figure 26. Vesicle in root of *Cheilanthes lanosa* sporophyte.

Vesicle (IV) measures *ca.* 15 µm by *ca.* 40 µm. Specimen is stained with Lactophenol Cotton Blue and mounted in ddH₂O. 35-µm section. Bar = 10 µm.
Colonization of *Cheilanthes feei* by endophytic fungi

**Site 1: Mammoth Road**

Golden hyphae were associated with whole root specimens of *Cheilanthes feei* sporophytes and were visible under a dissecting scope (not shown). Stained and sectioned roots, mounted on microscope slides and examined with light microscopy, revealed extraradical hyphae associated with the roots of *C. feei* sporophytes (Figure 27). These hyphae ranged from 2-4 µm in diameter. Golden fungal spores were also identified in the soil matrix. These spores measured *ca.* 60 µm in diameter and possessed V-shaped septa (Figure 28). The unstained spores ranged from (0-10-40-0) to (0-30-60-10) according to the INVAM color chart. This is within the range for *G. mosseae*, which has spores that range from a straw-color (0-5-20-0) to dark orange-brown (0-30-100-10). Most *G. mosseae* spores are yellow-brown (0-10-60-0) (INVAM).

Intraradical fungal structures were also identified in association with *C. feei* sporophyte roots from Mammoth Road. A few ovoid vesicles were identified. These measured *ca.* 10 µm by *ca.* 15 µm in diameter (Figure 29). Degraded arbuscules were present in the form of fungal clumps (Figure 30).
Figure 27. Fungal hypha penetrates root of *Cheilanthes feei* sporophyte.

Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy. Hypha penetrates the sporophyte root (SR) and proceeds parallel to the longitudinal axis of the root. EH = extraradical hypha. IH = intraradical hyphae. A = appressorium. Bar = 10 µm.

Figure 28. Fungal spore from soil matrix of *Cheilanthes feei* sporophyte.

Spore measures *ca.* 60 µm in diameter. Specimen is stained with Trypan Blue, mounted in glycerol and viewed with brightfield microscopy. V-shaped septum (arrow) is visible. SP = fungal spore. Bar = 10 µm.

Figure 29. Vesicle inside *Cheilanthes feei* rhizome.

Vesicles (IV) are present in this specimen and measure ca. 10 µm by *ca.* 15 µm. Specimen is stained with Trypan Blue, mounted in ddH₂O and viewed with brightfield microscopy. SR = sporophyte root. IH = subtending hypha. Bar = 25 µm.

Figure 30. *Cheilanthes feei* sporophyte root.

Arbuscules (A) are visible within a cortical root cell (CRC). Specimen is stained with Trypan Blue, mounted in ddH₂O, and viewed with brightfield microscopy. Bar = 10 µm.
Site 2. Reis Biological Station

Fungal hyphae were associated with the sporophyte roots of *Cheilanthes feei* (Figure 31). These measured *ca.* 4 µm in diameter. An extraradical vesicle was found in the soil matrix (Figure 32). This measured *ca.* 10 µm in diameter. Golden brown spores were also present (Figure 33) and measured from *ca.* 40 µm to *ca.* 160 µm in diameter.

Intraradical hyphae were present and measured ≤4 µm in diameter and ran parallel to the longitudinal axis of the root (Figure 34). Also present were arbuscules (Figure 35). No intraradical vesicles were observed.
Figure 31. Extraradical hypha in association with *Cheilanthes feei* sporophyte root.

Extraradical hypha (EH) measures ca. 4 μm in diameter. Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy. SR = sporophyte root. Bar = 10 μm.

Figure 32. Extramatrical fungal vesicle in association with *Cheilanthes feei* rhizome.

Extramatrical vesicle (EV) and subtending hypha (EH) are visible in association with sporophyte rhizome (RH). Specimen is stained with Trypan Blue and mounted in glycerol, and viewed with brightfield microscopy. Bar = 10 μm.

Figure 33. Fungal spore from the rhizosphere soil of *Cheilanthes feei* sporophyte.

Specimen is unstained, mounted in ddH₂O and viewed with brightfield microscopy. The spore (SP) measures ca. 155 μm in diameter. Bar = 50 μm.

Figure 34. Extraradical hypha penetrates *Cheilanthes feei* sporophyte rhizome.

An extraradical fungal hyphae (EH) forms an appressorium (A) and penetrates the sporophyte rhizome (RH). Specimen is stained with Trypan Blue, mounted in glycerol, and viewed with brightfield microscopy. Hypha measures ca. 4 μm in diameter. Bar = 10 μm.

Figure 35. Arbuscules inside cortical cells of *Cheilanthes feei* sporophyte roots.

Arbuscules are visible inside sectioned root cells. Specimen is stained with Lactophenol Cotton Blue, mounted in ddH₂O, and viewed with brightfield microscopy. 10-µm section. Arrow = arbuscules. Bar = 10 μm.
Site 3: Cedar Hill

Fungal hyphae were associated with the roots and rhizomes of *Cheilanthes feei* sporophytes (Figure 36). The hyphae measured 4-14 µm in diameter. Extramatrical vesicles were found in the soil (Figure 37). These measured *ca.* 20 µm by 40 µm. Fungal spores were identified in the soil matrix (Figure 38). These spores were golden brown and measured *ca.* 70 µm in diameter. The spore walls measured *ca.* 5 µm in diameter.

Intraradical fungal structures were present in the roots of *C. feei* sporophytes. Fungal hyphae measured ≤4 µm in diameter and ran parallel to the longitudinal axis of the root (Figure 39). Arbuscules were present (Figure 40). Intraradical vesicles were also present. These were ovoid and measured *ca.* 25 µm by 35 µm (Figure 41, 42).
Figure 36. Extraradical fungal hyphae in association with *Cheilanthes feei* sporophyte root.

Specimen is stained with Trypan Blue, mounted in ddH₂O, and viewed with brightfield microscopy. Hypha (EH) measures *ca.* 16 µm in diameter before the bifurcation and *ca.* 7 µm each afterwards. Arrow = bifurcation. SR = sporophyte root. Bar = 10 µm.

Figure 37. Brightfield micrograph of extramatrical fungal vesicle in association with *Cheilanthes feei* sporophyte root.

Specimen is stained with Trypan Blue, mounted in ddH₂O, and viewed with brightfield microscopy. Vesicle (EV) measures *ca.* 20 µm by 40 µm in diameter. Bar = 10 µm.

Figure 38. Fungal spore viewed with differential interference contrast optics (DIC).

Spore (SP) is stained with Melzer's Reagent and mounted in Melzer's Reagent and polyvinyl-lacto-glycerol (PVLG). Spore measures *ca.* 70 µm in diameter. Spore wall (SW) measures *ca.* 5 µm. Bar = 25 µm.
**Figure 39.** Fungal hypha inside *Cheilanthes feei* sporophyte root.

Specimen is stained with Trypan Blue, mounted in ddH$_2$O, and viewed with light microscopy. Hypha (IH) measures *ca.* 5 µm in diameter without protuberances. Hypha measures up to *ca.* 8 µm in diameter with protuberances (arrow). SR = sporophyte root. Bar = 10 µm.

**Figure 40.** Arbuscule in *Cheilanthes feei* sporophyte root.

Specimen is stained with Trypan Blue, mounted in ddH$_2$O, and viewed with light microscopy. Hyphal "trunk" measures *ca.* 6 µm prior to first bifurcation. A = arbuscule. Arrow = bifurcation. Bar = 10 µm.

**Figure 41.** Intraradical vesicle inside *Cheilanthes feei* sporophyte root.

Specimen is stained with Trypan Blue, mounted in ddH$_2$O, and viewed with light microscopy. Vesicle (IV) measures *ca.* 25 µm by *ca.* 35 µm. SR = sporophyte root. Bar = 10 µm.

**Figure 42.** Intraradical vesicle inside *Cheilanthes feei* sporophyte root.

Specimen is stained with Trypan Blue, mounted in ddH$_2$O, and viewed with brightfield microscopy. Vesicle (IV) measures *ca.* 15 µm by *ca.* 45 µm. Bar = 10 µm.
DISCUSSION

Based on evidence from light microscope examination, both *Cheilanthes lanosa* and *Cheilanthes feei* support mycorrhizal associations. Without variation, fungal endophytes in these ferns bore fungal structures characteristic of mycorrhizae.

First, fungal mycelia were present in every sporophyte at every site (Table 1), as was evidenced by the presence of hyphal structures that stained with Trypan Blue and Lactophenol Cotton Blue. A positive reaction with these stains indicates the presence of chitin (Larone, 1993), the primary polysaccharide polymer of which fungal cell walls are comprised (Griffin, 1994).

Secondly, the fungus can be identified as a member of the Zygomycota based on the mycelial structure (Webster, 1993). Mycelial hyphae are flat, ribbon-like, and aseptate (Figure 7-9, 20, 25, 31, 36). As with most Zygomycetes, occasional septa can be observed at the hyphal branches (data not shown).

In addition, the presence and/or absence of other diagnostic structures allows the fungus to be identified as an endomycorrhiza, and specifically as a vesicular-arbuscular mycorrhiza (VAM). For example, no Hartig Net was formed. This is a diagnostic characteristic of ectomycorrhizae and ectendomycorrhizae (Harley and Smith, 1983). This feature alone narrows the classification to endomycorrhiza.

The presence of other fungal structures allows the fungi in this study to be further classified as VA mycorrhizae. For example, the presence of vesicles and arbuscules defines the fungus as a VAM (Vesicular-Arbuscular Mycorrhizae). "The presence of arbuscules is a *sine qua non* to identify a VAM infection in a root" (Bonfante-Fasolo, 1984). Arbuscules were present (Figure 6, 13, 15, 30, 35) at every site with the exception
of *Cheilanthes lanosa* site # 3 (Mingo Wildlife Refuge). However, the lack of an arbuscule at this site does not preclude VAM infection. Older, established VAM colonies may consist predominantly of unbranched intracellular hyphae (INVAM, 2001). Since *Cheilanthes* ferns are evergreen, and the fungal presence was documented in the winter, the fungal colonies were probably older, more established colonies. In addition, since VAM spores can be identified to species, and some spores at this site were tentatively identified as *Glomus mosseae* (Figure 24), arbuscule production is implied.

In this study, I hypothesized that *Cheilanthes lanosa* and *Cheilanthes feei* survive in part with the help of mycorrhizal associations. Although mycorrhizal associations exist at every site for both *C. lanosa* and *C. feei*, these relationships are not obligatory. The relationships between these ferns and their endophytes appear to be facultative. For example, specimens collected in the winter (2000 A.D.) from *Cheilanthes feei* site #1 (Cedar Hill) exhibited low-level, unidentifiable infection (data not shown). Alternatively, specimens collected in the spring (2001 A.D.) were colonized to a much greater extent (Figure 36-37, 39-42).

Although the extent of fungal infection was not quantitatively determined in this study, it was apparent that *Cheilanthes feei*, in general, was colonized to a lesser extent than was *Cheilanthes lanosa*. This is interesting in light of the fact that *C. feei* occupies a drier niche than *C. lanosa* and is therefore expected to undergo comparatively more drought stress. Further studies are necessary to determine the cost/benefit relationship, and water-use efficiency of mycorrhizal *C. feei* sporophytes under drought stress conditions.
In conclusion, *Cheilanthes lanosa* and *Cheilanthes feei* are mycorrhizal. However, since the associations appear to be facultative, these data suggest that mycorrhizal associations are not the sole basis for the ferns' ability to survive in arid environments. Clearly, there are other factors (i.e. physiological, biochemical, morphological) that, in combination with mycorrhizal associations, enable these ferns to survive.
LITERATURE CITED


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