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A cryptic microbial community persists within micropropagated *Bouteloua eriopoda* (Torr.) Torr. cultures

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ABSTRACT

Higher plants are ubiquitously colonized with fungal endophytes that often lack readily detectable structures. This study examines the diverse endophyte population within a single line of micropropagated *Bouteloua eriopoda* (Torr.) Torr., using microscopy and comparison of internal spacer (ITS) gene sequences obtained from both plant and isolated fungal tissues. Microscopy revealed fungal hyphae and lipid bodies, the majority of which lacked distinguishing characters. Internal transcribed spacer (ITS) sequences amplified from fungal isolates and micropropagated plant tissues were subjected to Bayesian analysis, which clearly distinguished six endophyte taxa.

Results confirm a diverse, cryptic endophyte consortium is retained within this micropropagated plant line. The probability of similar complexity in other plant species is discussed. The development of controlled systems in which to study single plant-fungal interactions within such consortia presents significant technical challenges. However, potential for such systems to reveal species interactions that influence plant growth and development is high.

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1. Introduction

Identification of cryptic endophytes[1] in plants presents a perplexing task. Yet in recent years, molecular techniques have provided overwhelming evidence that fungal communities residing in plants are complex, dynamic, and diverse [1–3]. These findings, based largely on molecular methods, have contributed to the general acceptance that fungi inhabit all natural ecosystems, survive in extreme environments [4], and are present in tissues of all examined plants [5,6].

The role of fungi as symbionts is now accepted as a universal phenomenon and a fundamental condition of plants and animals [7]. Specific benefits fungi confer to plants include enhanced tolerance to disease, drought, herbivory, heavy metals, temperature extremes, increased photosynthetic efficiency, enhanced nutrient uptake, and increased plant biomass [8–13]. These characteristics make fungi appealing cohorts for plant systems. Yet despite overwhelming evidence that fungi confer multiple benefits to the host, and despite the generally accepted knowledge that as many as 99% of the microorganisms existing in natural environments cannot be cultured in the laboratory, mainstream plant biologists continue to perceive healthy, micropropagated plants as axenic organisms [14,15].

Fungal endophytes associated with the forage grass species, *Bouteloua eriopoda*, and other plants native to the Chihuahuan Desert are perceived as mutualists in arid ecosystems [16,17]. Micropropagated and regenerated lines established from excised embryos to reduce the microbial complexity of these plant systems have failed to eliminate cryptic fungal associations [18–20]. This systemic, cellular level association and cryptic behavior in healthy, rapidly growing, micropropagated plants lends support to hypotheses of symbiotic interactions, while micropropagated lines offer powerful contained systems in which plant–microbe interactions may be explored.

Although routine culturing of micropropagated *B. eriopoda* produces asymptomatic plants, hyphae have occasionally emerged from roots and shoots, permitting isolation. Factors regulating the emergence of previously cryptic hyphae are unclear, and reported isolations have proven difficult to reproduce [9]. Moreover, isolates collected from a single micropropagated line on different occasions represent diverse species, suggesting the line is colonized by complex consortia. This investigation explores the complexity of

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[1] In this manuscript, the term "endophyte" will refer to fungi that are present, "in" plants, with no readily detectable pathogenic effects.

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the *B. eriopoda* endophyte community using universal and specific PCR primers to amplify internal spacer region (ITS) rDNA from a single plate of *B. eriopoda* callus tissue. Sequences cloned from these amplicons were compared to the host grass, isolated endophytes, and known fungal sequences to classify source organisms and to evaluate the diversity of both cultured and uncultured fungal endophytes present.

2. Methods

2.1. Micropropagation of *B. eriopoda*

The regenerated plant lines described in Osuna and Barrow [21] provided the source material for this study.

2.2. Tissue staining and light microscopy

Established methods for trypan blue and sudan IV [18,22] staining were used to detect fungal presence in regenerated plant tissues. Slides were mounted and examined with a Zeiss Axiophot microscope with conventional and differential interference contrast optics at 1000×. Digital images were captured and processed using Auto-Montage 3D software by Syncroscopy.

2.3. Electron microscopy

For scanning electron microscopy, fresh tissues were harvested and analyzed within 1 h by placing samples in the vacuum chamber of a Hitachi S3200N scanning electron microscope under variable pressure mode.

2.4. DNA extraction, amplification, cloning, and sequencing of isolated endophytes

Fungal hyphae observed on the root surface of nutrient depleted, regenerated *B. eriopoda* plantlets were physically scraped from the root and harvested hyphae were divided into two aliquots. One aliquot was freeze dried, the second was plated on PDA. Four fungal species were isolated in this manner, each from a different plantlet originating from the same callus line. One species, putatively identified as "unknown," did not proliferate on PDA. The others included a putative *Aspergillus ustus* and two species which only exhibited vegetative growth, a putative *Engyodontium album* and a putative *Moniliophthora*. Rooted *B. eriopoda* plantlets with protruding hyphae and fungal isolates thought to be *Moniliophthora* were donated to M. Catherine Aime, who confirmed identification as *Moniliophthora* to the genus level [23]. DNA from living or freeze-dried fungal tissues of the remaining isolates was extracted using the UltraClean Plant DNA Isolation Kit as per the manufacturer's instructions (MoBio Laboratories, Inc., Solana Beach, CA). The internal transcribed spacer (ITS) region consisting of ITS-1, the 5.8S ribosomal RNA gene, and ITS-2 was amplified from each isolate using the primers and annealing temperatures described in Table 1. The majority of primers used were among the universal and fungal specific primers described by White [24] or by Gardes and Bruns [25]. Amplicons were cloned and sequenced as described in the following section.

2.5. DNA extraction, amplification, cloning, and sequencing of plant and unisolated endophytes

Total genomic DNA was sampled from *B. eriopoda* callus tissue produced from regenerated plants. Callus was frozen in liquid nitrogen, ground to a fine powder, and stored at −80 °C prior to DNA extraction. Extraction was performed as described above. DNA was amplified using primer pairs and annealing temperatures indicated in Table 1. PCR products were cloned into a pCR2.1 cloning vector using the TA cloning® kit (Invitrogen product number K2040-40) according to the manufacturer's protocol. Clones were sequenced using M13 forward and reverse primers, Applied Biosystems Big Dye Terminator v3.1 Cycle Sequencing Kit, and an applied Biosystems 3100 Genetic Analyzer. To minimize potential for contamination of PCR reactions, all amplifications were carried out in a PCR hood which was UV treated for 6 h prior to each use. Dedicated, UV resistant pipettors were used with factory sterilized barrier tips to avoid introduction of extraneous DNA. The pipettors were stored in the PCR hood and UV treated prior to each use.

DNA from callus and fungal isolates was also amplified using the species-specific primers AustsF2-1 and AustsR1 and annealing temperatures described by Haugland [26]. Products were amplified using Taq PCR Master Mix (Qiagen, Inc.) according to the manufacturer's instructions.

2.6. Phylogenetic analysis of ITS sequences

DNA extracted from whole plant, leaf, or callus tissues of the *B. eripoda* line used in this study was believed to contain a mixture of plant and fungal genomes. Although the universal primers developed by White et al. [24] were designed to target fungal rDNA, plant sequences are frequently amplified with these primers. Seven unique clone sequences obtained from the micropropagated plants are listed in Table 1, along with sequences obtained from fungal isolates. These sequences were included in a multiple alignment with 5 previously characterized GenBank accessions (AB106650 from *Engyodontium album* [27], AY373873 from *Aspergillus ustus* [26], DQ303008 from *Mycosphaerella pseudafricana* [28], AF126816 from *Phoma glomerata* [29], and U05195 from *Alternaria alternata*

Table 1
Sequences amplified from *B. eriopoda* plant tissues or fungal isolates

Forward primer ID	Reverse primer ID	Annealing temperature (°C)	DNA source	Taxonomic grouping	GenBank accession
ITS1 [23]	ITS4 [23]	53	Micropropagated *B. eriopoda*	Grass	DQ497245
ITS1 [23]	ITS4 [23]	53	Micropropagated *B. eriopoda*	Grass	DQ497244
18S [40]	28S [40]	55	Micropropagated *B. eriopoda*	Grass	EU338389
ITS1 [23]	ITS2 [23]	57	Micropropagated *B. eriopoda*	Grass	EU338384
ITS1-F [24]	ITS2 [23]	57	Micropropagated *B. eriopoda*	Basidiomycete	EU338386
ITS1-F [24]	ITS4 [23]	55	Micropropagated *B. eriopoda*	Ascomycete	EU338387
ITS1-F [24]	ITS4 [23]	55	Micropropagated *B. eriopoda*	Ascomycete	EU338388
ITS1-F [24]	ITS4B [24]	55	*Moniliophthora sp.* isolated from micropropagated *B. eriopoda*	Basidiomycete	AY916754
ITS5 [23]	ITS4 [23]	53	Putative *Aspergillus ustus* isolated from micropropagated *B. eriopoda*	Ascomycete	DQ649073
ITS5 [23]	ITS4 [23]	53	Putative *Engyodontium album* isolated from micropropagated *B. eriopoda*	Ascomycete	DQ649066
ITS1-F [24]	ITS4 [23]	53	Unidentified fungal hyphae excised from roots of micropropagated *B. eriopoda*	Ascomycete	DQ520628

Forward and reverse primers and annealing temperatures used in PCR are shown.

[30]) associated with well described source organisms from published phylogenies. Multiple alignment was performed in ClustalX software version 1.81 [31] using a pairwise gap opening of 10 with a gap extension of 0.10. The multiple alignment gap opening was 10, with a gap extension of 0.20. Divergent sequences were delayed 30%. After manual examination of the alignment, alternate parameters were tested. However, they did not improve the alignment quality. The final alignment was trimmed to a 457 base pair consensus sequence which included all of the 5.8S subunit region and partial spans of ITS1 and ITS2. The grass sequence EU338384 was removed from the alignment. The sequence was considered uninformative because it aligned completely with the DQ497244, but lacked ITS2. Another clone, EU338386 also lacked ITS2. However, this clone was left in the alignment because it represented the only putative basidiomycete sequence obtained from direct amplification of total plant DNA. A Bayesian tree (Fig. 3) was constructed Mr. Bayes v. 3.0 [32] using 1,000,000 generations and a sampling frequency of 250.

3. Results

3.1. Microscopy

Fungal endophytes with and without cell walls were observed following staining with trypan blue and sudan IV in photosynthetic, meristematic, and stomatal complex cells (Fig. 1a–c, respectively) of regenerated plantlets. Meristematic tissues integrated with trypan blue stained fungal tissues of a root initial emerging from regenerated *B. eriopoda* are shown (Fig. 1b) in proximity to sudan IV stained lipid bodies.

In undifferentiated suspension cells (Fig. 2a), blue and red stained tissues are observed between the plant cell walls and membranes. Electron microscopy of callus reveals fungal networks associated with the undifferentiated cells (IIb). With the exception of a single *Aspergillus ustus* conidiophore on the root of a regenerated plant (Fig. 2c), which represented <0.001% of examined root sections, and numerous microhair-like teliospores (Fig. 2d) observed on leaf surfaces, fruiting bodies were not detected.

3.2. *Aspergillus ustus*

An *Aspergillus ustus* isolated from the roots of regenerated plants produced an ITS sequence and fruiting bodies consistent with other *Aspergillus ustus* strains (GenBank accession no. DQ649067). A single *A. ustus*-like conidiophore (Fig. 2c) was also observed on single root section taken from a regenerated plantlet. PCR amplification of total DNA extracted from *B. eriopoda* shoots utilizing species-specific primers optimized for detection of *A. ustus* by Haugland et al. [26] produced an 88 base pair fragment (not shown). The same sized fragment was obtained by direct amplification of DNA from *A. ustus* isolates, but was not observed in amplifications of either *Aspergillus niger* or *Monoliophthora* DNA. *A. ustus* has been isolated previously from both native and in vitro populations of *B. eriopoda* [9].

3.3. Putative *Engyodontium album*

A putative *Engyodontium album*, identified only by ITS sequence homology to other isolates of that species [27] was isolated from the roots of regenerated plants (GenBank accession no. DQ649066). This isolate represents the second isolation of *E. album* from the plant line [9].

3.4. Putative Urediniomycete

Light and electron microscopy have revealed a series of bicellular, microhair-like structures in *B. eriopoda* (Fig. 2d) which are connected

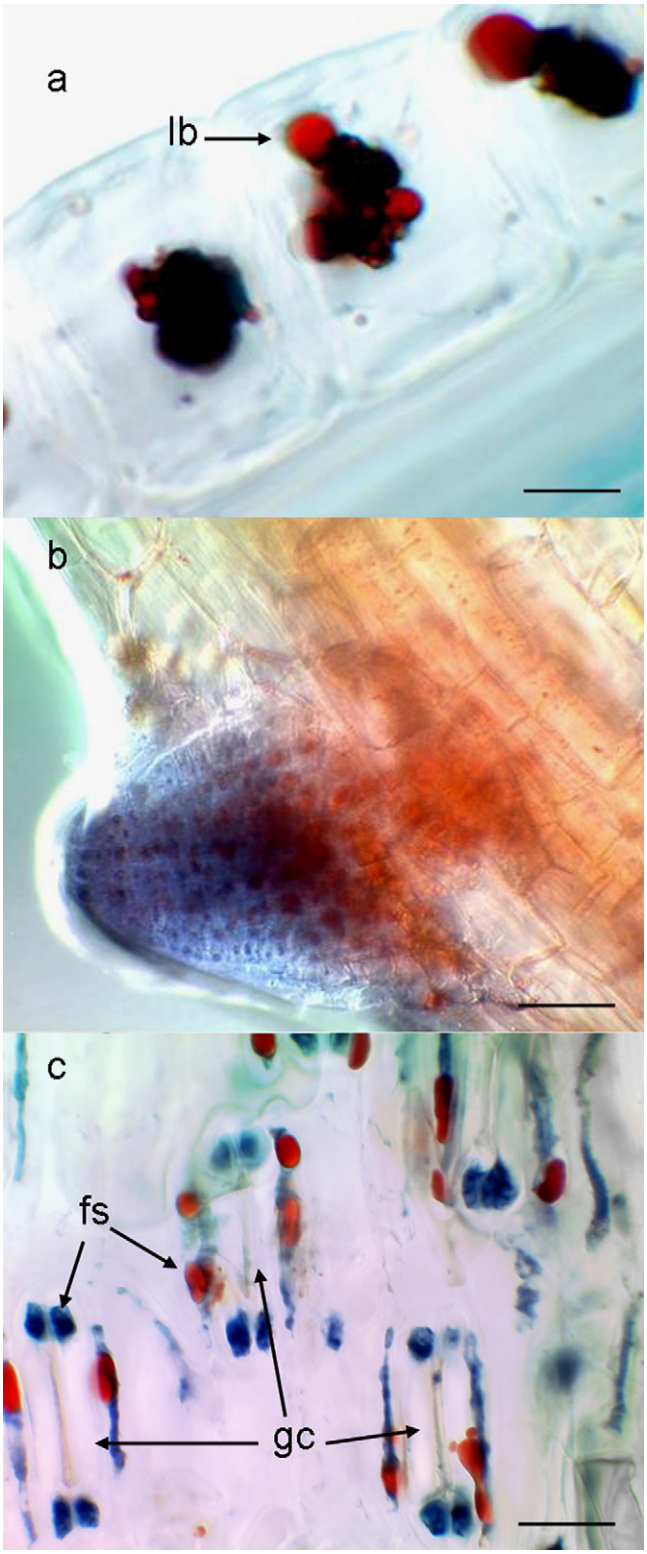

Fig. 1. ***Bouteloua eriopoda*** observed under light microscopy (bars = 10 μm). Trypan blue and sudan IV stained fungal structures with attached lipid bodies (Ia) integrated with photosynthetic bundle sheath cells. A trypan blue and sudan IV stained root initial reveals the association of fungi with meristematic tissue (Ib). Guard cells (gc) of the stomatal complex (Ic) are also associated with stained fungal structures (fs). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

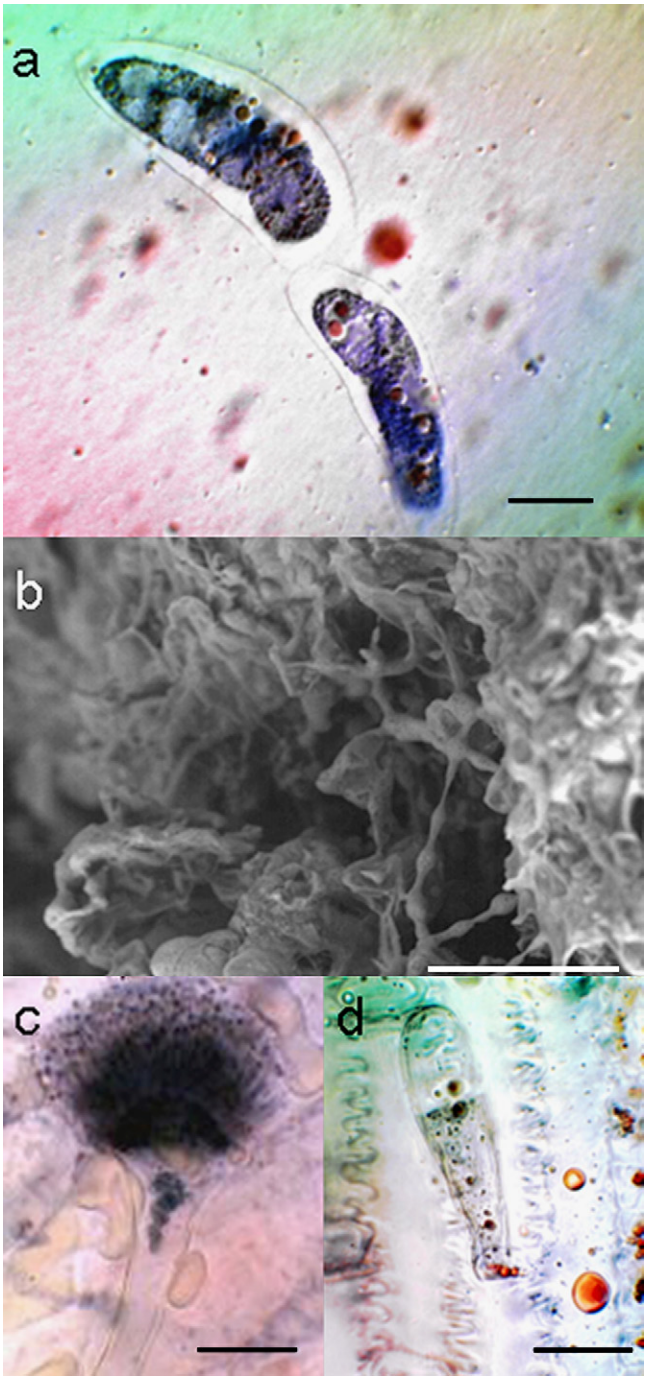

Fig. 2. Stained fungal chitin (blue) and lipid bodies (red) appear to lie between the membrane and cell wall (clear) of individual ***B. eriopoda*** suspension cells (IIa, bar = 15 μm). Scanning electron microscopy reveals branched fungal hyphae (bh) associated with ***B. eriopoda*** callus tissue (IIb, bar = 100 μm). A single ***Aspergillus ustus*** fruiting body was observed on a root section of regenerated, aseptically cultured ***B. eriopoda*** plantlets (IIc, bar = 10 μm). Structures resembling rust teliospores populate the leaf surface of regenerated ***B. eriopoda***. These structures are easily mistaken for plant microhairs, but differ in that they are connected to stained hyphae (h) rather than to leaf short cells (IId, bar = 10 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

to hyphae. These structures are occasionally observed producing spores [19]. The bicellular structures resemble fruiting bodies of *Puccinia* and other Urediniomycetes. Like Urediniomycetes, these fungi appear to be obligately associated with the host plant. Similar fungi are associated with other C4 grasses [19].

3.5. Unidentified ascomycete isolate

DNA extracted from hyphae severed from roots of a regenerated plantlet (GenBank accession no. DQ520628) bore BLASTn homology to several *Mycosphaerella* strains [33]. *Mycosphaerella* have been well described in association with the salt marsh grass *Spartina alterniflora* [34]. *Spartina* and *Bouteloua* both belong to the tribe Cynodonteae. However, *Mycosphaerella* generally grow well on agar based medium, while this isolate did not. Since the root-associated hyphae lacked fruiting bodies or other defining characteristics, only the ITS sequence is available for preliminary identification.

3.6. ***Moniliophthora*** *sp. isolate*

A *Moniliophthora* isolated from roots of regenerated plants from this line was deposited in the U.S. National Fungus Collection under accession MCA2500. This organism did not produce fruiting bodies necessary for species assignment. However, a phylogenetic analysis based on three gene sequences placed it solidly within the genus [23]. A sequence amplified directly from *B. eriopoda* callus DNA (GenBank accession no. EU338386) sequence was homologous to the sequence obtained from the *Moniliophthora* isolate, indicating that this basidiomycete strain persists within the plant line.

3.7. Detection of cryptic, uncultured endophytes

Seven clones generated from amplified ITS DNA extracted directly from *B. eriopoda* are listed in Table 1. Primers used for amplification, the GenBank accession [35] of the clones, and the taxonomic grouping of the source organism are shown. One clone was described with the *Moniliophthora* strain above. Four clones likely represent grass sequences (GenBank accession nos. EU338389, EU338384, DQ497245, and DQ497244). Three of these were utilized for the phylogenetic analysis. The Bayesian analysis illustrated by the tree in Fig. 3 places three of these clones with the grass species, *Bouteloua gracillis* (GenBank accession no. AF019835). Two other clones (GenBank accession nos. EU338387 and EU338388), aligned pairwise with sequences representing *Phoma* (GenBank accession no. AF126816) [29] and *Alternaria* (GenBank accession no. AAU05195) [30] species, respectively. These strains have never been detected as isolates.

The Bayesian cladogram illustrated in Fig. 3 clearly separates the seven clone sequences into distinct "grass" and "ascomycete" and "basidiomycete" clades. Two of the sequences appear to represent taxa from the genera *Phoma* and *Alternaria* which were not previously represented by isolates.

4. Discussion

The four fungal strains isolated from in vitro, regenerated *B. eriopoda* and the obligate, Uredinomycete-like endophyte observed microscopically [19] demonstrate that aseptically propagated, asymptomatic plants are capable of harboring diverse endophyte communities. These isolated strains have all been previously observed in *B. eriopoda* [9]. The ITS sequences obtained from direct amplication of total plant DNA were expected to produce sequences homologous to those obtained from the four isolates, or possibly to an Urediniomycete (Fig. 2d). However, only one ITS sequence (GenBank accession no. EU338386) bore homology to a previously isolated specimen. The two fungal sequences amplified directly from the host, (GenBank accession nos. EU338387 and EU338388) indicate diversity within the micropropagated plant culture exceeds diversity revealed through isolation and microscopy. Failure to detect ITS sequences homologous to each of the isolated strains may indicate that one or more of these strains have been eliminated from the plant line.

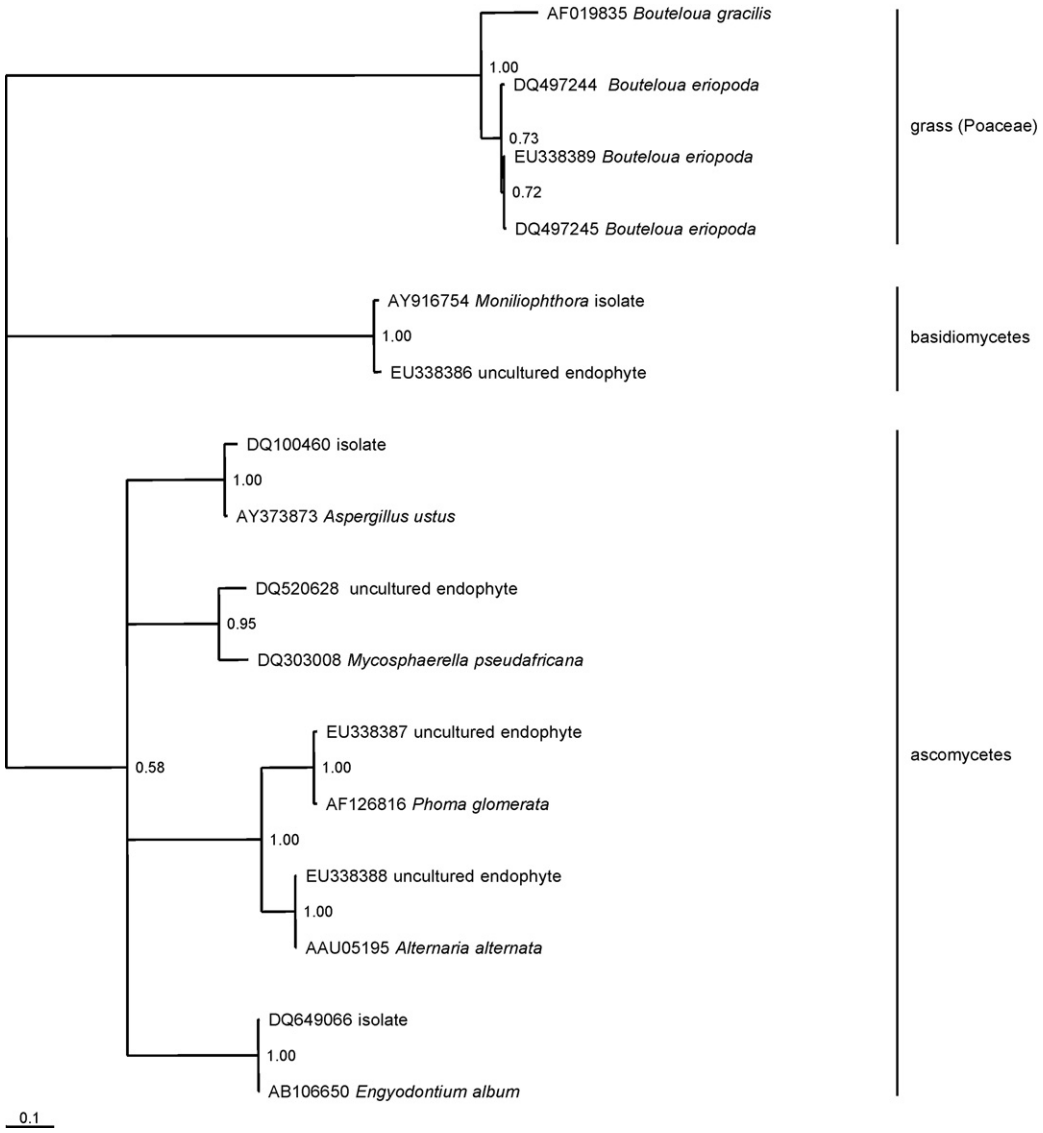

Fig. 3. A Bayesian tree illustrates to diversity of ITS sequences associated with micropropagated ***B. eriopoda*** by comparing sequences to taxa representing the host and putative endophyte genera. Solid clades separate the grass sequences from 5 ascomycete and basidiomycete taxa. Credibility values are shown as probabilities for each clade.

Alternate conclusions are that some species were present below the detection limits of PCR, or that PCR bias resulted in preferential amplication of only a subset of the fungal sequences present in the plant tissue. This last conclusion is supported by the observation that *A. ustus* was detected with specific primers even though universal primers failed to amplify an *A. ustus* sequence. Custom primers designed to target sequences of the *E. album*, *Moniliophthora*, and the *Mycosphaerella*-like fungus all amplified ITS regions from pure isolates, but did not work well in mixed samples (data not shown). Improved specific primers may be necessary in order to detect these species within the mixed consortium. Microdissection may also facilitate amplification of fungal DNA with minimal contamination by plant or other fungal cells. With microdissection, the Urediniomycete-like structures could be severed from leaf tissues, and DNA could be extracted and amplified with universal primers.

The Bayesian tree produced by analyzing ITS regions amplified from host and endophyte DNA illustrates a minimum of 1 plant, 5 ascomycete, and 1 basidiomycete taxa associated with a single micropropagated plant line. While the identified taxa are all associated with pathogenic or saprophytic ecological roles, it must be acknowledged that the callus and regenerated plants obtained from this line are growing asymptomatically under micropropagation. Perhaps more remarkably, even the rapidly growing strains such as *A. ustus*, which readily grows on artificial medium once separated from the plant host, do not readily propagate on medium when the plant is actively growing. Plant and/or fungal processes may be restricting the growth of hyphae to inner plant tissues. This cryptic presence of diverse fungi systemically associated with rapidly growing, regenerated cell lines provides ample opportunity for complex, cross-species interactions [7].

While the diversity of the fungal communities in natural plant habitats is expected to exceed what we report in vitro, [3,36–38] the presence of six or more fungal taxa complicates efforts to demonstrate cause and effect with respect to individual plant–endophyte interactions. Potential for source identification errors is high [39,40], and experiments must proceed with caution. The identification of ITS sequences specific for each endophyte is an important step towards developing molecular tools needed to monitor dynamics of the fungal population that influence establishment, growth, and reproduction of *B. eriopoda* [16].

5. Conclusion

In summary, the continuum of fungal-like structures observed throughout the roots and leaves of regenerated plants, combined with the isolation of associated fungi and the sequencing of ITS regions associated with plant tissues all provide evidence that *B. eriopoda* is inhabited by a diverse community of ascomycete and basidiomycete fungi. This phenomenon is unlikely to be unique, since complex endophyte consortia have already been identified in plants from a range of natural and micropropagated habitats [1,3,9,41].

By demonstrating diverse endophyte associations cryptically maintained in aseptically propagated plants, we highlight a mechanism by which macromolecules and natural products isolated and characterized in micropropagated plants could potentially be of microbial origin. We advise that techniques capable of detecting systemic endophytes be more broadly developed and adopted for studies concerned with identification, isolation, and characterization of plant genes or natural products.

Earlier research has suggested that fungal endophytes associated with *B. eriopoda* contribute to plant hardiness [9,16]. The current study builds on previous efforts by describing previously undetected endophytes present in the consortium and by providing sequence data which can be used to develop specific probes for monitoring changes in endophyte populations that correlate with differences in plant productivity.

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