

Genetic Characterization of Uncultured Fungal Endophytes from *Bouteloua eriopoda* and *Atriplex canescens*

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Abstract—Obligate fungal endophytes form cryptic communities in vascular plants that can defy detection and isolation by microscopic examination of reproductive structures. Molecular detection by PCR amplification of fungal DNA sequences alone is insufficient, since target endophyte sequences are unknown and difficult to distinguish from sequences already characterized as plant DNA. We have successfully separated fungal and plant ribosomal DNA sequences by amplifying plant-extracted DNA with polymerase chain reaction, and separating sequences with denaturing gradient gel electrophoresis (DGGE). The resulting electrophoregrams theoretically produce specific bands unique for each organism present in a plant-endophyte community. This method has successfully identified endophyte sequences in *B. eriopoda* and *A. canescens*, and has tracked these endophytes as they are transferred to novel hosts.

Introduction

The ability to transfer uncultured fungal endophytes to novel hosts provides us with unprecedented opportunities for developing plant material capable of restoring disturbed landscapes (Barrow and others, this proceedings). The potential for heritable endophytes to dramatically modify the competitive ability of a plant and its progeny also has implications for traditional agriculture and plant biotechnology. However, detection and characterization of endophytes in plant material can be a formidable task. This is attributed to the obligate nature of many endophytes, the diverse nature

of plant-fungal communities (Ganley and others 2004; Lucero and others, in review; Vandenkoornhuyse and others 2002), and the tendency of fungi to propagate *in planta* without forming telltale structures such as fruiting bodies (Barrow 2003). For this reason, only a handful of studies examining the potential long-term ecological impacts of endophyte modified plants are available (Clay and Holah 1999; Clay and others 2005).

In vitro propagation of regenerated plants restricts the complexity of the endophyte community by eliminating those microbes that are strictly associated with the surface layer of plant tissues. In previous reports, light microscopy combined with staining has been utilized to examine fungal colonization of *A. canescens* and *B. eriopoda* (Barrow and Aaltonen 2003). While staining methods reveal the presence of fungi, they fail to distinguish between fungal species. When fruiting bodies and cell walls are not present (as is often the case in *planta*), molecular tools are required to evaluate the nature and complexity of endophyte communities. However, care must be taken when probing plant tissues for endophyte DNA. Because endophytes are often undetected in plant tissues, it is possible that multiple sequences previously characterized as plant genes are actually coming from endophytes (Camacho and others 1997).

To truly distinguish plant from endophyte DNA, it is often necessary to separate endophytes from their plant hosts. However, obligate endophytes do not survive apart from host plants. For this reason, we are using combinations of denaturing gradient gel electrophoresis and an in-vitro system for transferring uncultured endophytes to foreign host plants (Barrow and others, this proceedings) to isolate and identify putative fungal DNA sequences. Efforts reported herein focus on optimization of methods for separation of unique, potentially fungal rDNA sequences extracted from regenerated plants. Efforts to amplify fungal sequences directly from in vitro propagated plants have focused on *B. eriopoda*. Efforts to separate fungi from host plants through endophyte transfer, followed by identification of novel bands in recipient plant fingerprints have utilized both *A. canescens* and *B. eriopoda*.

Methods

DNA Isolation and Amplification

DNA was isolated from shoot or callus of regenerated *B. eriopoda* and *Atriplex canescens* using a MoBio UltraClean™ Plant DNA Kit. PCR was carried out using

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HotStarTaq Master Mix (Qiagen, Inc.). Primer pairs and annealing temperatures are described in table 1. All primers were purchased as custom oligos from Operon Technologies.

Cloning and Sequencing

The amplified PCR products were cloned into pCR 2.1™ plasmids using an Invitrogen TA Cloning Kit™. Individual clones were sequenced using universal M13 forward and reverse primers and a BigDyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in conjunction with an Applied Biosystems 3100 Genetic Analyzer.

Sequence Analysis

Sequences obtained from clones were evaluated for chromatogram purity, then subjected to BLASTn analysis (Altschul and others 1997) against the non-redundant GenBank nucleotide database using the BLASTn interface at the National Center for Biotechnology Information (NCBI).

Endophyte Transfer

Tomato seeds (*Lycopersicon esculentum* var. Bradley) were surface disinfested to remove competing microbes by slowly vortexing in 95% ETOH for 1 min, followed by soaking for 7 min in a 5 percent solution of commercial bleach (Chlorox™) and 0.01 percent Tween 20. Seeds were rinsed thoroughly in sterile water before plating on Murashige and Skoog’s (MS) medium (Murashige and Skoog 1962). Plants were allowed to germinate for 7 days.

Callus cultures of *B. eriopoda* and *A. canescens* were prepared as previously described (Lucero and others 2006), then transferred to hormone free MS media, where they were maintained for 30 days prior to inoculating tomato seedlings. This was done to minimize effects of residual growth regulators utilized to maintain callus.

After this time, callus was transferred to new plates of MS medium, along with germinated tomato seedlings. Care was taken to place seedling radicles in physical contact

with callus. Co-culturing of tomato seedlings with callus continued for 1 week, after which endophytes from callus were presumed to have transferred to the tomato seedlings. Tomato plantlets were transferred to magenta boxes containing MS medium, where they were maintained for 4 weeks. During this time, young leaves were removed and DNA was extracted as described above.

Denaturing Gradient Gel Electrophoresis (DGGE)

DNA extracted from callus cultures of *B. eriopoda* and *A. canescens*, from uninoculated tomato plants, and from tomato plants inoculated with uncultured endophytes from the callus cultures was amplified using the primer sequences 5’CGCCCGGGGCGCGCCCGGGCGGGGCGGGGGC-CCTTMTCATYTAGAGGAAGGAG3’ and 5’CCGCTTATT-KATATGCTTAAA3’. PCR was carried out as described above, except that an initial annealing temperature of 57 °C was used for the first 10 cycles, followed by 52 °C for 20 cycles. Products resulting from this amplification were separated by denaturing gradient gel electrophoresis (DGGE) using a BioRad™ DCode Universal Mutation Detection System. Manufacturer’s protocols were modified by pouring a 40-60 percent gradient. Amplicons were loaded onto the gradient gel and separated at 60 °C, with 75 volts for 16.25 h. Gels were stained with Sybr Green and photographed. Other GC clamped primers shown in table 1 have also been utilized for DGGE; however, less complex banding patterns were obtained with other primers.

Results

DNA Sequence Comparisons

In a recent manuscript, we described four endophytes isolated or identified in *Bouteloua eriopoda* (Lucero and others 2006). None of these isolates were detected using the universal ITS primers shown in table 1. This is not surprising, since PCR is highly biased towards amplification of those sequences most similar to the primers. Sequences

Table 1—GenBank Accessions of putative ITS sequences amplified from aseptically propagated, regenerated *B. eriopoda*. Accession numbers are accompanied by PCR primers utilized for amplification, annealing temperature utilized in PCR, and GenBank Accessions of sequences that produced the closest alignments to amplified sequences when compared against GenBank using BLASTn. Sequences that align with *B. gracilis* most likely represent the *B. eriopoda* ITS rDNA gene.

GenBank Accession	Forward Primer sequence or reference	Reverse Primer sequence or reference	T _z	Closest BLAST match
DQ497245	ITS1/ (White et al., 1990)	ITS4/ (White et al., 1990)	53	AF019835.1 <i>Bouteloua gracilis</i> (grass)
DQ497244	ITS1/ (White et al., 1990)	ITS4/ (White et al., 1990)	53	AF019835.1 <i>Bouteloua gracilis</i> (grass)
DQ645748	CCTTMTCATYTAGAGGAAGGAG	CCGCTTATTKATATGCTTAAA	55	AF019835.1 <i>Bouteloua gracilis</i> (grass)
DQ649069	ITS3/ (White et al., 1990)	CCTGTTTGAGTGTCATGAAA CC	55	AY079521.1 <i>Calectasia cyanea</i> (non-grass monocot)
DQ649074	ITS3/ (White et al., 1990)	CCTGTTTGAGGTCATGAAACC	55	AY753717.1 <i>Begonia alpina</i> (dicot)
DQ649068	ITS5/ (White et al., 1990)	ITS4/ (White et al., 1990)	53	AF019835.1 <i>Bouteloua gracilis</i> (grass)
DQ649075	ITS5/ (White et al., 1990)	ITS4/ (White et al., 1990)	53	AF378603.1 <i>Moringa longituba</i> (dicot)
DQ645749	CCTTMTCATYTAGAGGAAGGAG	CCGCTTATTKATATGCTTAAA	55	AY640053.1 <i>Corydalis saxicola</i> (dicot)

with BLASTn homology to grasses, particularly to *Bouteloua* sp., were expected, since similarities exist between fungal and plant ITS regions. At present, more specific PCR primers designed to uniquely target the isolated species are being used in combination with microscopy (Barrow and others, this proceedings) to validate the persistence of these endophytes.

The fact that more than one ITS sequence resembled *Bouteloua* is easily explained by the highly repetitive nature of ITS DNA. When multiple copies of a gene are present, numerous alleles are no surprise.

Two notable features observed herein were that half of the sequences obtained from PCR exhibited BLAST homology to non-grass monocot and dicot plants, and that sequences of endophytes previously isolated from these cultures (Lucero and others 2006) were not detected.

Sequences resembling plant species that have never been handled in our laboratory were unlikely to represent. We hypothesize that these sequences represent uncultured, obligate fungal endophytes, and that ITS rDNA sequences from related endophytes, present in the plant species *Calectasia cyanea*, *Begonia alpina*, *Moringa longituba*, and *Corydalis saxicola* have been mistaken for ITS sequences from the host. Additional support for this hypothesis is being pursued through DGGE analysis.

Meanwhile, the absence of sequences from endophytes believed to be present in the regenerated plants (Lucero and others, in review) may indicate bias inherent in PCR. Sequence specific probes have since been developed and are being optimized to selectively amplify *Moniliophthora*, *Aspergillus ustus*, *Engyodonium album*, and an unknown endophyte with sequence homology to a *Mycospharella* species, respectively.

DGGE

Figure 1 reveals the potential diversity of PCR products amplified from aseptically cultured plant materials. To identify the source material from which each band is derived, bands may be excised from the gel, reamplified, cloned, and sequenced. In this image, the majority of the bands shown in lanes containing tomato DNA have exhibited sequence homology to tomato ITS sequences and probably originate in the plant genome. However, the arrows reveal two novel bands in tomato inoculated with *B. eriopoda* callus that co-migrate with bands amplified from *B. eriopoda* DNA (Lanes 3 and 5). These bands are expected to reveal the identity of uncultured endophytes. They have been excised from the gels, reamplified, and cloned, but await sequencing.

Many bands that appear on DGGE gels are too faint to pick up in photographed images. Such bands, present in tomato inoculated with *A. canescens*, co-migrated with bands from *A. canescens* callus (not shown). These bands also appeared in the offspring of tomato plants inoculated with *A. canescens*.

Discussion

Barrow and others (this proceedings) have discussed beneficial impacts transferred fungal endophytes may have on host plant survival and fitness. Implications for

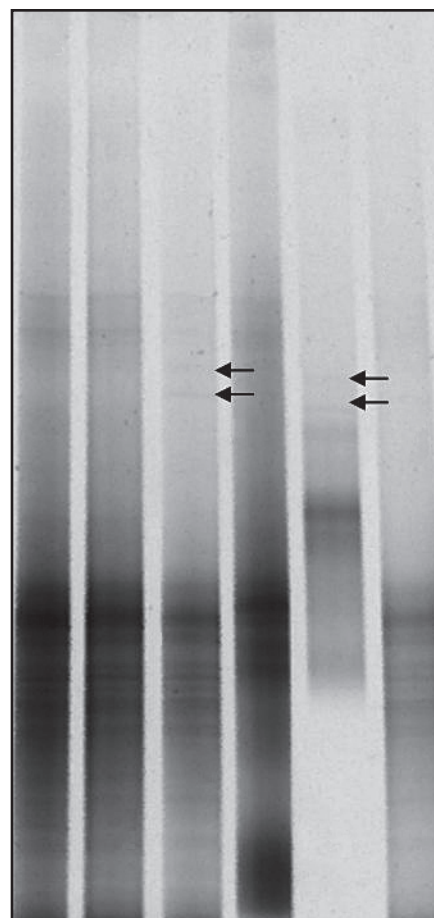


Figure 1—DGGE permits separation of highly similar ITS rDNA amplified from the following plant and endophyte sources (lanes left to right): (1) tomato, (2) tomato inoculated with *A. canescens* callus, (3) tomato inoculated with *B. eriopoda* callus, (4) *A. canescens* callus, (5) *B. eriopoda* callus, and (6) offspring of tomato inoculated with *A. canescens* callus. The high resolution of DGGE permits detection of DNA bands in inoculated tomato plants that appear to have come from the callus source. Dark bands indicate DNA present in tomato plants that appear to have come from *B. eriopoda*. Light bands indicate DNA. Alternate primer sets reveal different bands (not shown).

restoration of shrub and grasslands are profound. Yet direct evidence demonstrating which endophytes have migrated to inoculated plants has been difficult to obtain. The atypical morphology of plant endophytes has made them difficult to observe microscopically, so it is not yet known what species of endophytes have migrated from native plant callus to foreign hosts.

Initially, efforts to identify endophytes present in native plants utilized in vitro propagation and isolation of fungi from internal plant tissues (Barrow and Aaltonen 2001; Osuna and Barrow 2004). More recently, PCR, cloning, and sequencing of ITS rDNA from both the isolated endophytes and the source plant tissues have suggested that both

B. eriopoda and *A. canescens* host not one, but many endophytes (Barrow and others, this proceedings; Lucero and others 2006; Lucero and others, in review). The complexity of these systems impedes identification of individual fungi, particularly when they cannot be reproducibly isolated from the plant tissue. Without sequence data to guide development of selective primers, it has been necessary to use universal primers that amplify both plant and fungal DNA.

The transfer of endophytes to foreign hosts, followed by comparison of DGGE profiles between plants pre-and post-inoculation has provided a powerful, albeit tedious, tool for endophyte isolation. We now have the ability to determine which endophyte(s) are enhancing plant growth, and to isolate DNA specific for such endophytes. As we acquire sequence data describing these endophytes, we will be able to develop PCR assays that can monitor the presence of transferred endophytes across generations. Such assays will be crucial for evaluating the persistence and long term ecological impacts of endophyte-modified plants in field settings.

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