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Detection of leaf-associated fungi based on PCR and nucleotide sequence of the ribosomal internal transcribed spacer (ITS) in *Miscanthus*

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Abstract. PCR amplification of the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA from the genomic DNA isolated from the leaf tissue of *Miscanthus sinensis* varieties and *M. floridulus* resulted in two amplified fragments. Via FASTA search, the shorter length of about 500 base pairs was identified as the ITS region of fungi symbiotic in the grass foliage. Two *Cladosporium* species, one *Fusarium* and one basidiomycete were added to the mycoflora of *Miscanthus* grassland. Using a Southern blotting and hybridization, fungal ITS was also detected from the genomic DNAs isolated from sterilized seedling, a suggestive seed transmission of endophytic fungi. The "contaminated" genomic DNA may be a hindrance to the phylogenetic analysis of *Miscanthus* itself, but may provide other insights into the biodiversity of grassland ecosystems. The endophytic foliage fungi were not detected in *M. sinensis* var. *condensatus*, probably a result of its apomictic reproduction.

Keywords: Contamination; Foliage fungi; *Miscanthus sinensis*; Mycoflora; Phylogeny; rDNA ITS.

Introduction

The association between fungi (both endophytic and epiphytic) and higher plants is thought to be ubiquitous (Clay, 1988; Legault et al., 1989; Petrini et al., 1992). Nevertheless, the prevalence of symptomless fungi on healthy plants has become clear only lately (cf. Camacho et al., 1997). Bills (1996) even suggested that endophytic fungi are basic components of microbiota and are distinct from pathogens ecologically. In grasslands, where Poaceae are dominant, symbiotic fungi, both endophytic and epiphytic, may play critical roles in plant-microbe interactions. Diverse taxa of fungi have been isolated and identified from the leaf tissue of many grasses (Harada, 1987; Nakase et al., 1987; Watson and Dallwitz, 1992).

Molecular techniques have been applied widely in ecological studies (e.g., Wirgin et al., 1997) and wildlife conservation (Avise and Hamrick, 1996; O'Corry-Crowe et al., 1997) in the past several years. In many studies the polymerase chain reaction (PCR) has proved powerful in detecting cryptic genetic variation, e.g., the DNA of symbionts that can not be cultured and separated from their cosymbionts (Haddad et al., 1995), and the microbial floristic composition (cf. Hansen and Hanson, 1996). For systematic and ecological studies, many universal primers have been designed, which may be used across a large range of taxa (Dumolin-Lapegue et al., 1997; Chiang et al., 1998; Chow and Hazama, 1998). The primer sequences of the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA have been widely used for resolving phylogenetic relationships at the species or generic levels (White et al., 1990; Baldwin, 1992). Two recent reports documented the amplification of fungal sequences from plant foliage (Liston et al., 1996; Klein and Smith, 1996). Employing phylogenetic analysis, Camacho et al. (1997) was able to identify the endophytic fungi in *Picea* based on the ITS nucleotide sequences. In addition, sequences of the nuclear ribosomal internal transcribed spacer region are known providing species-specific genotypes in many fungi (Egger, 1996; Norman and Egger, 1996; Holst-Jensen et al., 1997; Zhang et al., 1997; Vralstad et al., 1998).

A species complex of *Miscanthus sinensis* Anders. consists of several intraspecific taxa that occur in different elevations on Taiwan (Chou et al., 1999a). The history of adaptive radiation and secondary contacts in the species complex has been reconstructed based on genetic variation of both nrITS and cpDNA *atp*B-*rbc*L spacer sequences (Chou et al., 1999b). Most taxa play a dominant role in the floristic composition of grasslands at different elevations. Examples include *M. sinensis* var. *transmorrisonensis* (Hayata) Lee in high mountains, *M. sinensis* var. *formosanus* Hack. at middle elevations, and *M. sinensis* var. *glaber* (Nakai) Lee in lowlands. In contrast to these taxa with a wide range in distribution, *M.*

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sinensis var. *condensatus* (Hack.) Makino is restricted to the coasts of southeast Taiwan and two offshore islands (Hsu, 1978; Koyama, 1987). In addition, apomixis with sterile pollen has been documented in *M. sinensis* var. *condensatus* (Adati, 1958), while most other taxa of the complex were found to have outcrossing (Chou et al., 1999a).

In this study we detected the presence of four symbiotic fungal species in leaf tissue of the taxa of *Miscanthus sinensis* complex and another related species, *M. floridulus*. We also found evidence, when the template DNA was extracted from seedlings after seed sterilization, for systemic infection in most *Miscanthus* taxa.

Materials and Methods

Plant Samples and DNA Extraction

Young and healthy shoots of taxa of the *Miscanthus sinensis* complex, i.e., *M. sinensis* var. *transmorrisonensis*, *M. sinensis* var. *formosanus*, *M. sinensis* var. *glaber*, and *M. sinensis* var. *condensatus* (cf. Lee, 1995), as well as a closely related species, *M. floridulus*, were collected in the field (Table 1). Seedlings were also cultivated using the seeds of the same individuals used in leaf collections and were harvested for DNA isolation. One seed from each individual was planted in the experimental greenhouse of the Academia Sinica, Taipei. Both leaves and seeds were surface-sterilized with 0.5% sodium hypochlorite for 2 min and rinsed three times with sterile water (Holme and Petersen, 1996). Leaf tissue was powdered in liquid nitrogen and stored at -70°C until use. Genomic DNA was isolated following the CTAB protocol of Murray and Thompson (1980) and gel-quantified.

PCR, Sequencing and FASTA Search, and Hybridization

PCR for amplifying the internal transcribed spacer region (ITS) of nuclear ribosomal DNA was performed in a volume of 100 µl using 10 ng of template DNA, 10 µl of 10X reaction buffer (Promega, Madison, USA), 10 µl $MgCl_2$ (25 mM), 10 µl dNTP mix (8 mM), 10 pmole of each primer, 10 µl of 10% NP-40, and 2 U of *Taq* polymerase (Promega, Madison, USA). The reaction was programmed on an MJ Thermal Cycler (PTC 100) as one cycle of denaturation at 95°C for 4 min, 30 cycles of 45s denaturation at 92°C, 1 min 15s annealing at 52°C, and 1 min 30s extension at 72°C, followed by 10 min 72°C extension. Template DNA was denatured with the reaction buffer, $MgCl_2$, NP-40 and ddH_2O for 4 mins (first cycle), and cooled on ice immediately. A pair of universal primers, ITS1 and ITS4, for amplifying the nrDNA ITS (White et al., 1990), dNTP and *Taq* polymerase were added to the above ice-cold mixture. Reaction was restarted at the first annealing at 52°C.

PCR fragments were eluted using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals, Basel, Switz.), ligated in a p-GEM-T easy Vector (Promega, Madison, USA), and cloned in competent *Escherichia coli* DH5α. Plasmid DNA was purified using Wizard Plus SV Minipreps DNA Purification Systems (Promega, Madison,

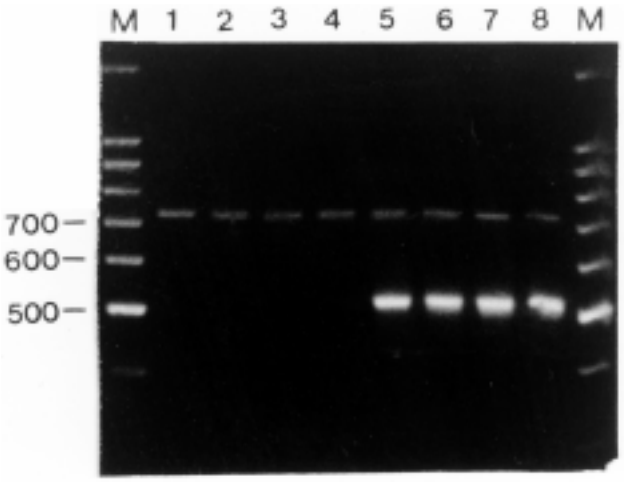
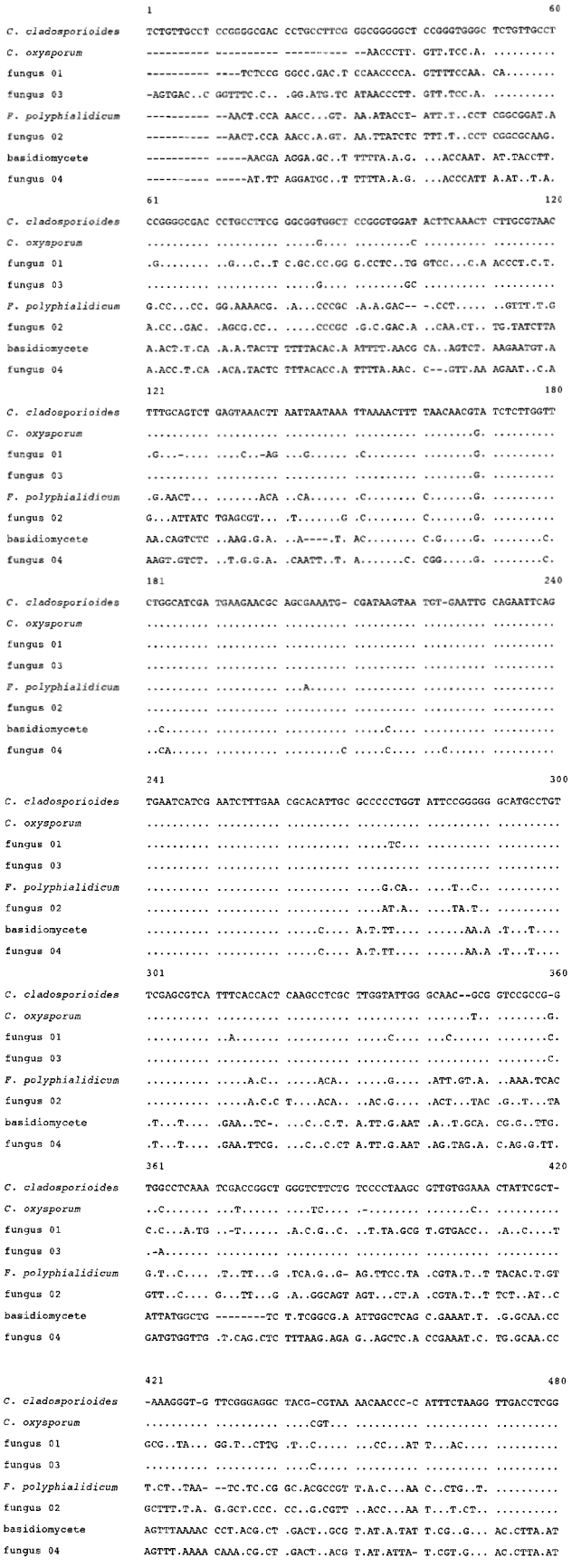


Figure 1. Agarose electrophoresis of the PCR products of nrDNA ITS amplified from genomic DNA isolated from foliage (1, 2-7) or seedlings (2, 8, representative sample) of *Miscanthus.* 1-4, *M. sinensis* var. *condensatus*; 5, *M. sinensis* var. *glaber*; 6, *M. sinensis* var. *formosanus*; 7-8, *M. sinensis* var. *transmorrisonensis*; M, 100 bp ladder marker.

Table 1. Materials collected for amplification of the nrDNA ITS region.

Taxon	Locality	Source	Isolate	Fungal contamination
M. sinensis var. *glaber*	Taipei (121°40'E, 25°09'N)	Leaf tissue	*Chiang 1588*	+
	Taipei (121°40'E, 25°09'N)	Seedling	*Chiang 1588*	+
M. sinensis var. *formosanus*	Alishan (120°40'E, 23°29'N)	Leaf tissue	*Chiang 1673*	+
	Alishan (120°40'E, 23°29'N)	Seedling	*Chiang 1673*	+
M. sinensis var. *transmorrisonensis*	Mt. Yushan (120°48'E, 23°28'N)	Leaf tissue	*Chiang 2499*	+
	Mt. Yushan (120°48'E, 23°28'N)	Seedling	*Chiang 2499*	+
M. sinensis var. *condensatus*	Taitung (121°00'E, 22°42'N)	Leaf tissue	*Chiang 2534*	–
	Taitung (121°00'E, 22°42'N)	Seedling	*Chiang 2534*	–
	Islet Orchid (121°30'E, 22°05'N)	Leaf tissue	*Chiang 2544*	–
	Islet Green (121°30'E, 22°40'N)	Leaf tissue	*Chiang 2545*	–
M. floridulus	Wushe (121°10'E, 24°00'N)	Leaf tissue	*Chiang 2573*	+
	Wushe (121°10'E, 24°00'N)	Seedling	*Chiang 2573*	+



USA). Purified plasmid DNAs were sequenced in both directions by standard methods of the *Taq* dye deoxy terminator cycle sequencing kit (Perkin Elmer) on an Applied Biosystems Model 377A automated sequencer (Applied Biosystems). Primers for sequence determination were the SP6 and T7 promoters located on the p-GEM-T easy Vector termination site. In this study, we sequenced the nrDNA ITS amplified from the leaf-tissue DNAs. DNA sequences were compared to other homologous sequences registered in GenBank using the FASTA search, a function implemented in the GCG software package (Devereux et al., 1991).

In order to identify the PCR amplification of the fungal ITS from the seedling DNAs, a conserved region between sites 120 and 317 was amplified from the leaf-tissue DNAs of the isolate *Chiang 1673* with two additional primers (5'-CTTTGCAGTCTGAGTAAACT-3' and 5'-GGTGAAATGACGCTCGAACAG-3') and was labeled using the PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals, Basel, Switz). The synthesized probe was used to hybridize with the PCR products of the nrDNA ITS, which were agarose-gel electrophoresed and amplified from both leaf-tissue and seedling DNAs of *Miscanthus*. Southern blotting and hybridization were carried out and modified as described in Tregear et al. (1996).

Results

Two DNA fragments, 725 bp and 500 bp, of the internal transcribed spacer (ITS) region, consisting of ITS1, ITS2, and 5.8S rRNA gene, were PCR amplified from most *Miscanthus* except for a single PCR band in *M. sinensis* var. *condensatus* (Table 1; Figure 1). In the taxa with two amplified DNA bands, genomic DNAs obtained from both foliage and seedlings all yielded two ITS bands (Table 1).

DNA fragments of both sizes were eluted, ligated in a T-vector, and sequenced. Via FASTA searches the larger fragments were identified as the nrDNA ITS sequences of angiosperms, i.e. *Miscanthus* in this study (cf. Chou et al., 1999b); and the sequences of the smaller fragments appeared to be closely related to fungal ITS sequences. Clone fungus01 (AJ289867), isolated from the foliage of an individual of *M. sinensis* var. *glaber* (isolate *Chiang 1588*), and clone fungus03 (AJ289869), isolated from the foliage of an individual of *M. sinensis* var. *formosanus*

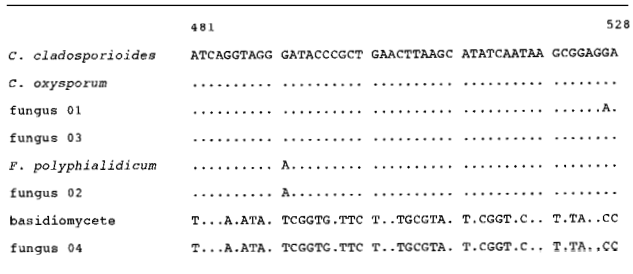


Figure 2. Consensus alignment of nucleotide sequences of nrDNA ITS of fungal clones (Fungus01-04) and the related taxa.

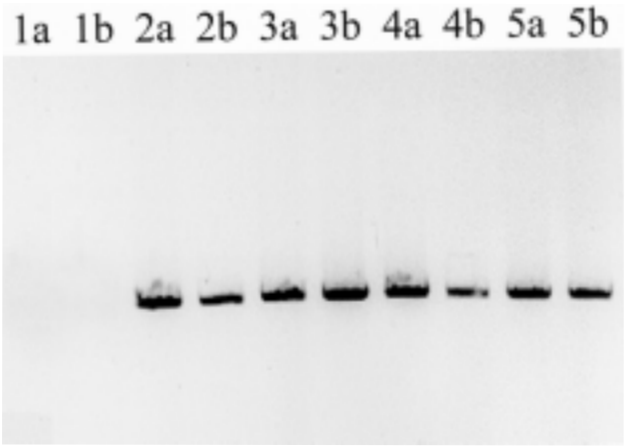


Figure 3. Southern blotting of the PCR products of the ITS amplified with both foliage (a) and seedling DNAs (b), using the probe of the shorter fragment of fungal ITS. 1, *Miscanthus sinensis* var. *condensatus*; 2, *M. sinensis* var. *glaber*; 3, *M. sinensis* var. *formosanus*; 4, *M. sinensis* var. *transmorisonensis*; 5, *M. floridulus*.

(isolate *Chiang 1673*), shared 86.6% and 98.1% similarity with *Cladosporium oxysporum* (L25432) and *Cladosporium cladosporioides* (L25429) (Loculoascomycetes), respectively. Likewise, clone fungus02 (AJ289870), isolated from the foliage of an individual of *M. floridulus* (isolate *Chiang 2573*), shared 79.3% similarity with *Fusarium polyphialidicum* (X94172) (Pyrenomycetes); and the clone fungus04 (AJ289868), isolated from *M. sinensis* var. *transmorrisonensis* (isolate *Chiang 2499*), shared 84.6% identity with a basidiomycete (U65599), which, however, was not indicated with species name.

The probe based on the sequence (sites 120-317, Figure 2) of the clone fungus03 from isolate *Chiang 1673*) hybridized with the shorter ITS fragments only. Both leaf-tissue and seedling DNAs of all *Miscanthus*, except for *M. sinensis* var. *condensatus*, yielded fungal nrDNA ITS (Figure 3).

Discussion

Contaminant fungal DNA has been reported previously from gymnosperms (Klein and Smith, 1996; Camacho et al., 1997) and grasses (Zhang et al., 1997). Outside of this study, the first to focus on *Miscanthus*, the problem of DNA contamination has been frequently encountered when doing PCR of nrITS in various plants, including mosses (Chiang and Schaal, 1999), *Begonia* (Peng and Chiang, 1999), and ferns (cf. Hsu et al., 2000).

Genomic contamination will cause noise in PCR-based fingerprinting, such as RAPD amplification. Genomic loci, annealing to the oligo primers, of both green plants and parasitic fungi will be amplified with no discrimination. Under such circumstances, an overestimate of the genetic variation based on PCR-based fingerprints becomes inevitable (cf. Hudson, 1995; Camacho et al., 1997). Using cpDNA, which is absent in the fungal genome, or more specific genes of the nuclear DNA provides markers independent from the contaminant fungal genome for systematic and ecological studies. Otherwise, precautions ought to be taken when contaminated DNA is manipulated. According to this and many other studies, PCR amplification of the internal transcribed spacer (ITS) using universal primers could possibly identify the hidden contamination, even in healthy-looking leaf tissue.

In addition to the documented mycoflora in foliage of *Miscanthus*, including *Puccinia, Sphacelotheca, Ustilago* (Watson and Dallwitz, 1992) and *Sporobolomyces* (Nakase et al., 1987), two *Cladosporium* species, one *Fusarium*, and one basidiomycete were recorded for the first time. Among them, basidiomycetes seem widespread in the Poaceae (cf. Zhang et al., 1997). Interestingly, according to the ITS amplification carried out with the sterilized seedlings and Southern blotting, the infection of fungi in *Miscanthus* seems to be systemic and may indicate seed-borne transmission. Accumulated evidence has suggested that microbial interactions are critical determinants of plant biodiversity in successional fields (cf. Clay, 1992; Clay et al., 1993; Tsai et al., 1994). Clay and Holah's (1999) experiments indicated that fungal endophytes altered plant community structure and reduced the species richness significantly.

Fungal infection was not detected in *M. sinensis* var. *condensatus*, a taxon distributed in saline habitats. According to previous cytological (Adati, 1958) and RAPD fingerprinting (Chou et al., 1999a) investigations, the reproductive mode in *M. sinensis* var. *condensatus* is obligate apomixis. That is, the formation of seeds does not accompany the regular gametic fusion, but rather proceeds from a diploid ovule. Some recent investigations have revealed that apomixis may provide an opportunity for avoiding the transmission of systematic pathogens in many crops (Nassar et al., 1998). On the other hand, the absence of genomic contamination in *M. sinensis* var. *condensatus* might be associated with environmental selection as well.

Although contaminated DNA causes difficulties in detecting the genetic variability of host plants, it may provide another opportunity for detecting the presence of cryptic parasitic or symbiotic organisms (Camacho et al., 1997). The merit of sensitivity of polymerase chain reaction and the universal presence and high identity of the ribosomal RNA genes (both 18S and 28S) across phyla can be used for the detection of mycoflora. Via this screening, more understanding of the ecological association between fungi and host plants could be obtained, as a supplement to standard-culturing and morphological identification of fungal species.

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利用 PCR 擴充及分子序列核核糖體 DNA ITS 片段偵測芒屬植物葉共生的菌類

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山中國芒各變種及五節芒葉組織萃取的基因體 DNA，利用 PCR 擴充核核糖體 DNA ITS 發現兩個大小不同的片段，以 FASTA 搜尋，其中較小約 500 bp 的片段，鑑定其較可能來自此禾草葉片中共生的菌類，其中包含二種 *Cladosporium*，一種 *Fusarium* 及一個 basidiomycete。利用南方黑點雜合反應，此菌類 ITS 片段亦存在於經消毒處理的小苗，指示此菌類可經由種子傳遞。此一〝污染的〞基因體 DNA 對於芒屬植物的親緣重建可能造成障礙，另一方面卻提供我們了解草原生態系多樣性的訊息。內生菌並未在八丈芒中被偵測到，可能與其無配生殖有極大相關。

關鍵詞：污染；葉組織共生菌；中國芒；菌類誌；親緣；核糖體 DNAITS。