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Differentiation of Isolates of *Discula umbrinella* (Teleomorph: *Apiognomonia errabunda*) from Beech, Chestnut, and Oak Using Randomly Amplified Polymorphic DNA Markers

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Genetic variation in 30 isolates of *Discula umbrinella* derived from beech, chestnut, and oak was assessed using randomly amplified polymorphic DNA (RAPD) and restriction fragment length polymorphic markers. Polymerase chain reaction amplifications with 17 primers produced 134 different DNA fragments. Three RAPD fragments were subsequently used for Southern hybridization. By these techniques up to four different individuals could be detected in the same leaf. The presence of several individuals within a single leaf indicates a finely tuned balance between the endophyte and its host. Cluster analysis of all arbitrary primed amplified DNA fragments showed that the isolates could be placed into four groups corresponding to their host origin. The high percentage of private RAPD variants within groups is consistent with low gene flow.

Additional keywords: Castanea sativa, Fagus sylvatica, Quercus spp.

Discula umbrinella (Berk. et Br.) Morelet, the anamorph of the ascomycete *Apiognomonia errabunda* (Rob.) von Höhnel is frequently found in the leaves of a number of trees, including *Fagus sylvatica* L. and *Quercus* spp. (von Arx 1970; Monod 1983). *D. umbrinella* is reportedly the causal agent of leaf anthracnose of beech and other trees, and at regular intervals it has caused severe epidemics (Butin 1989; Morelet 1989). However, Sieber and Hugentobler (1987) have isolated endophytes from leaves of beech collected at several sites in Switzerland, recovering *D. umbrinella* as a symptomless endophyte from virtually all beech leaves investigated. In a survey carried out over more than 4 yr, colonization rates by this fungus in July reached almost 100% of the leaves investigated (Sieber and Hugentobler 1987; L. Toti and O. Petrini, unpublished). Virtually nothing is known of the mechanisms of infection and colonization of *D. umbrinella*. Sieber and Hugentobler (1987) have postulated that infection may occur by airborne inoculum and by progressive colonization of the leaf tissues by individuals located in the twigs and entering the leaf through the petiole. If this is correct, it can be assumed that from some leaves more than one individual can be isolated.

Recently, Stone (1987) has provided histological evidence for the presence of more than 15 infection loci per square millimeter within one needle of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) for the endophyte *Rhabdocline parkeri* M. A. Sherwood, J. K. Stone & Carroll, but he could not conclusively demonstrate the presence of more than one individual in each needle. Preliminary investigations with *D. umbrinella* using classical vegetative compatibility group testing have suggested that more than one fungal individual is likely to be present within the same leaf (U. A. Haemmerli and O. Petrini, unpublished). This points either to the lack of a response by the host after the first infection or to a localized colonization with subsequent limited local defense by the host tissue.

This study has been undertaken to test whether several genetically distinct individuals of *D. umbrinella* can be detected within the same beech leaf. The polymerase chain reaction (PCR) technique using arbitrary primers (Martin *et al.* 1991; Williams *et al.* 1990) has been applied to distinguish isolates by randomly amplified polymorphic DNA (RAPD) markers (Williams *et al.* 1990). In addition to investigating multiple infections, RAPD markers and restriction fragment length polymorphism (RFLP) analysis have been used to study genetic variation within beech isolates collected from separate geographic regions and among isolates from different hosts.

MATERIALS AND METHODS

Fungal isolates. Isolates of *D. umbrinella* used and their origin are listed in Table 1. All strains were isolated from symptomless leaf tissues as described by Sieber and Hugentobler (1987) and were all mycelial isolates. Cultures were maintained on 2% malt agar slants (2% malt extract, 2% agar, both Difco) at 4 C.

DNA isolation. Isolates were grown for 10 days at 20 C in shake cultures containing 50 ml of V8 medium (Benhamou *et al.* 1984). The mycelium was removed by centrifugation (10 min at 3,000 × *g*), lyophilized, and ground to a fine powder. From 30 to 40 mg of mycelium DNA was extracted and purified by a total DNA CTAB mini-prep extraction method (Zolan and Pukkila 1986).

PCR amplification conditions. Amplification reactions were carried out in volumes of 25 μl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ (primers

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P1, P2, P14, OPE2, OPE4, OPE7, OPE12, OPE15), 2.5 mM $MgCl_2$ (primers P11, OPE3, OPE5, OPE6, OPE11, OPE16, OPE20), 3 mM $MgCl_2$ (P4, OPE1), 100 μM each of dATP, dCTP, dGTP, and TTP (Boehringer Mannheim), 0.2 M primer, 25 ng of DNA, and 1 U of *Taq* DNA polymerase (Boehringer Mannheim). Amplification was performed in a Perkin Elmer Cetus Gene Amp PCR system 9600 programmed for two cycles of 30 sec at 94 C, 30 sec at 36 C, 120 sec at 72 C, 36 cycles of 20 sec at 94 C, 15 sec at 36 C, 15 sec at 45 C, 90 sec at 72 C, followed by 10 min at 72 C. Reaction products were resolved by electrophoresis (4 V/cm) in a 1.5% agarose gel, run in 1× TPE for 4 hr and stained with ethidium bromide.

Table 1. Isolates of *Discula umbrinella* used in this study

Isolate no.	Host	Origin	Tree no.	Branch no.	Leaf no.
1	*Fagus sylvatica*	Switzerland, Benglen, 10 km from Zurich	1	4	1
2	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	1	4	1
3	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	1	4	1
4	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	1	4	1
5	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	2	6	3
6	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	1
7	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	1
8	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	1
9	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	2
10	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	2
11	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	2
12	*F. sylvatica*	Switzerland, Benglen, 10 km from Zurich	3	5	2
13	*F. sylvatica*	Switzerland, Zürichberg, 2 km from Zurich	1	4	1
14	*F. sylvatica*	Switzerland, Zürichberg, 2 km from Zurich	1	4	1
15	*F. sylvatica*	Switzerland, Zürichberg, 2 km from Zurich	1	4	1
16	*F. sylvatica*	Switzerland, Zürichberg, 2 km from Zurich	1	4	1
17	*F. sylvatica*	Switzerland, Zürichberg, 2 km from Zurich	1	4	1
18	*F. sylvatica*	Switzerland, Zürichberg, 2 km from Zurich	2	5	3
19	*F. sylvatica*	Switzerland, Adlisberg, 3 km from Zurich	1	7	1
20	*F. sylvatica*	France, Alsace, Strassburg	1	4	2
21	*F. sylvatica*	Switzerland, Geeren, 5 km from Zurich	—	—	—
22	*Quercus petraea*	France, Alsace, Ferrette	—	—	—
23	*Q. petraea*	Austria, Vienna	—	—	—
24	*Q. petraea*	Austrai, Vienna	—	—	—
25	*Q. petraea*	France, Alsace, Hesingue	—	—	—
26	*Q. robur*	England, Devon, Exeter	—	—	—
27	*Q. robur*	England, Devon, Exeter	—	—	—
28	*Castanea sativa*	Switzerland, Zarei, 8 km from Lugano	—	—	—
29	*C. sativa*	Switzerland, Zarei, 8 km from Lugano	—	—	—
30	*C. sativa*	Switzerland, Cureglia, 5 km from Lugano	—	—	—

A total of 17 random primers with 10 nucleotides length and a minimum of 50% GC content were used. The primers, supplied by Operon Technologies Inc., Alameda, CA, were OPE1 (CCCAAGGTCC), OPE2 (GGTGCGGGAAP), OPE3 (CCAGCGGGAA), OPE4 (GTGACATGCC), OPE5 (TCAGGGAGGT), OPE6 (AAGACCCCTC), OPE7 (AGATGCAGCC), OPE11 (GAGTCTCAGG), OPE12 (TTATCGCCCC), OPE15 (ACGCACAACC), OPE16 (GGTGACTGTG), OPE20 (AACGGTGACC). Primers P1 (AGGAGGACCC), P2 (ACGAGGGACT), P4 (GGTTCCAGCA), P11 (GCGCACGTCT), and P14 (CCACAGCACG) were synthesized by MicroSynth AG, Windisch, Switzerland.

Hybridization and autoradiography. Three RAPD marker products—no. 211: 2,100-bp product of isolate 11 by primer P2; no. 214: 1,170-bp product of isolate 2 by primer P14; no. 1814: 1,000-bp product of isolate 18 by primer P14—were transferred from the agarose gel to reaction tubes using autoclaved toothpicks and amplified under the conditions described above. Twenty-five nanograms of reamplified DNA was labeled with 50 μCi (α-^{32}P)dCTP (3,000 Ci/mmole, Amersham International; random primed DNA labeling kit, Boehringer Mannheim) and used as hybridization probes on Southern blots. *Eco*RI digested total genomic DNA was separated by horizontal agarose electrophoresis for 15 hr and blotted to Hybond N+ membranes (Amersham) (Southern 1975). Prehybridization and hybridization reactions were carried out in plastic bags at 65 C with 5× SSPE, 5× Denhardt's solution, 1% SDS 0.5 mg of herring sperm DNA. Hybridizations were incubated at 65 C for 14 hr.

Data analysis. Presence or absence of each amplification product in the gel was scored as 1 and 0, respectively. The resulting matrix was used to compute indices of between-isolates distance using the PCT option offered by the software package SYSTAT 5.1 (Wilkinson 1989). This metric computes the percentage of comparisons of values resulting in disagreements in two column or row profiles and is particularly useful for categorical or nominal scales, as it does not apply any weighting to the matrix. The dendrogram was constructed by UPGMA-clustering (Sneath and Sokal 1973).

RESULTS AND DISCUSSION

A total of 134 different, arbitrary primed DNA fragments, summing over all isolates tested, were amplified by the 17 primers, ranging from 500 to 2,500 kb. Only bands that amplified regularly over several DNA extractions and PCR experiments with the isolates were considered as reproducible and used for the analysis (Fig. 1). On the average, three DNA fragments per isolate per oligonucleotide decamer were detected. The distribution of all polymorphic RAPD markers among beech isolates is listed in Table 2. With RAPD analysis inference can only be made on the degree of difference between two given isolates, as isolates similar for all markers need not necessarily be identical. Of the 55 DNA fragments amplified in isolates derived from beech, thirty-four (62%) were fixed in all isolates and 21 (38%) markers were polymorphic. Of these 21 variable bands, 12 were unique to a single

isolate, two were observed in two isolates, two in three isolates, and one band was present in each of five, six, eight, nine, and 10 isolates. These RAPD markers were useful in unequivocally distinguishing *D. umbrinella* individuals isolated from the same leaf and to determine the degree of genetic diversity among the variable isolates. In some cases, isolates (nos. 1, 2 and 4; nos. 13 and 17; Table 2) appeared to be genetically identical, at least based on sharing all of their 40 arbitrary primed DNA bands. In other cases, isolates from the same leaf can be identified as genetically distinct individuals (nos. 6,7,8; 9,10,11,12; 14,15,16; Table 2), indicating that multiple colonization by *D. umbrinella* is a common occurrence in beech leaves.

The Southern hybridization experiments confirmed the results of the RAPD marker analysis. Figure 2 shows one of these probes. Isolates 1, 2, and 4 have identical RFLP pattern with probes 211, 214, and 1814 and are clearly distinct from isolate 3. Isolates 6, 7, 8, 9, 11, 12, 15, and 20 are all distinguishable based on the three RFLP probes. Hybridization of the probes derived from beech isolates to DNA extracted from oak and chestnut isolates yielded no signals except for one case (lane 22, Fig. 2).

The RAPD markers described here are useful in determining genetic similarities within and among groups of isolates from different geographic origin or host provenance. The correlation of RAPD marker patterns and of the corresponding RFLPs with other physiological traits such as pectinase production (L. Toti and O. Petrini, unpublished), host, and geographic origin suggests close genetic relationships and can thus be analyzed in a manner similar to that used for classical RFLP work.

The 30 isolates cluster into four groups corresponding mainly to their host origin (Fig. 3). Three isolates are characterized by RAPD polymorphisms that are atypical considering their host origin. Isolate 21, derived from *Fagus*, shows characters typical for the *Quercus* isolates; isolate 22, from *Quercus*, has polymorphisms otherwise found only in *Fagus*; and 24, from *Q. petraea*, presents the typical *Q. robur* polymorphisms. This may be indicative of an ongoing process of host specialization within *A. errabunda*, with a limited number of individuals showing a particular set of characters but still able to infect different

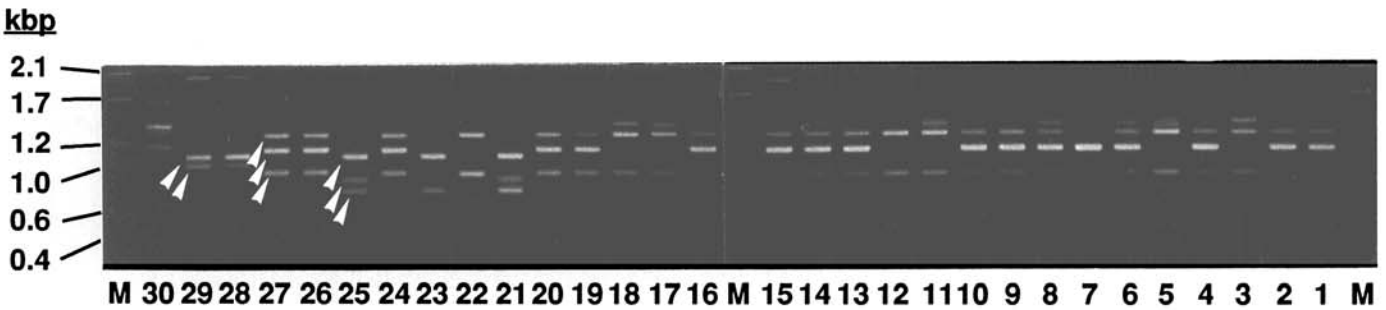

Fig. 1. Amplified DNA polymorphisms of 30 isolates of *Discula umbrinella* with primer P14. Size markers are in first, middle, and last lanes. Lanes labeled 1–4, 5, 6–8, 9–12, 13–17, 18, 19, 20, and 21 are all isolated from *Fagus sylvatica* with the groupings representing separate leaves. Lanes 22–25 are isolates from *Quercus petraea*. Lanes 26 and 27 are isolates from *Quercus robur* and lanes 28–30 are isolates from *Castanea sativa*. Bands scored for the analysis are marked by an arrow. Lane numbers refer to the isolate numbers listed in Table 1. Further details see text.

Table 2. Number of bands not shared by isolates of *Discula umbrinella* derived from the same *Fagus sylvatica* leaf and their haplotypes, based on 21 polymorphic RAPD markers[a]

	Isolate no.[b]						Haplotype[c]
Leaf no. 1, tree no. 1, Benglen		1	2	3	4		
	1	...					10110000110100000000
	2	0	...				10110000110100000000
	3	6	6	...			11110000000110001010 0
	4	0	0	6	...		10110000110100000000
Leaf no. 1, tree no. 3, Benglen		6	7	8			
	6	...					10110000100100100000
	7	3	...				10110000100101000010 0
	8	4	2	...			10110000100101000100 0
Leaf no. 2, tree no. 3, Benglen		9	10	11	12		
	9	...					10110000100101100100 0
	10	3	...				10110000110101000000 0
	11	8	5	...			10110000010000000010 1
	12	6	5	8	...		01110000010101001100 0
Leaf no. 1, tree no. 1, Zürichberg		13	14	15	16	17	
	13	...					10110000110100000100 0
	14	1	...				10110000110100010100 0
	15	3	4	...			00110000100100000101 0
	16	0	1	3	...		10110000110100000100 0
	17	4	5	5	4	...	10110000000101000100 1

[a] Isolate, leaf, and tree numbers according to Table 1.
[b] For groups of isolates derived from the same leaf, the number of different polymorphisms are given.
[c] The presence or absence of a RAPD marker in a given isolate is identified by a 1 or a 0, respectively.

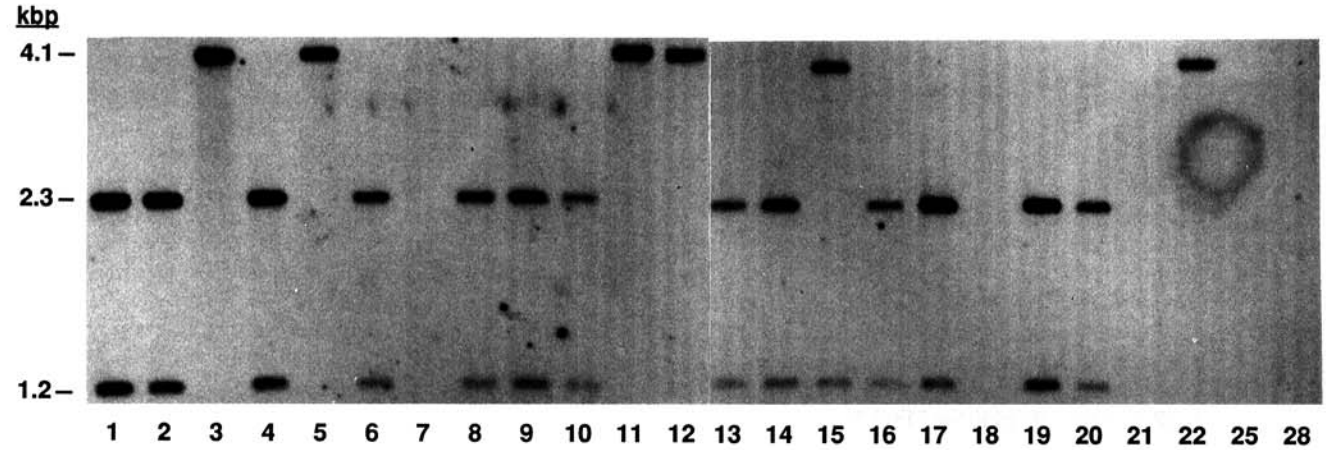

Fig. 2. Southern autoradiograph of 24 isolates of *Discula umbrinella* hybridized with RAPD fragment no. 213, 1,170 bp. Lane numbers refer to the isolate numbers listed in Table 1. 1–21: Isolates from *Fagus sylvatica*. 22 and 25: Isolates from *Quercus petraea*. 28 is an isolate from *Castanea sativa*.

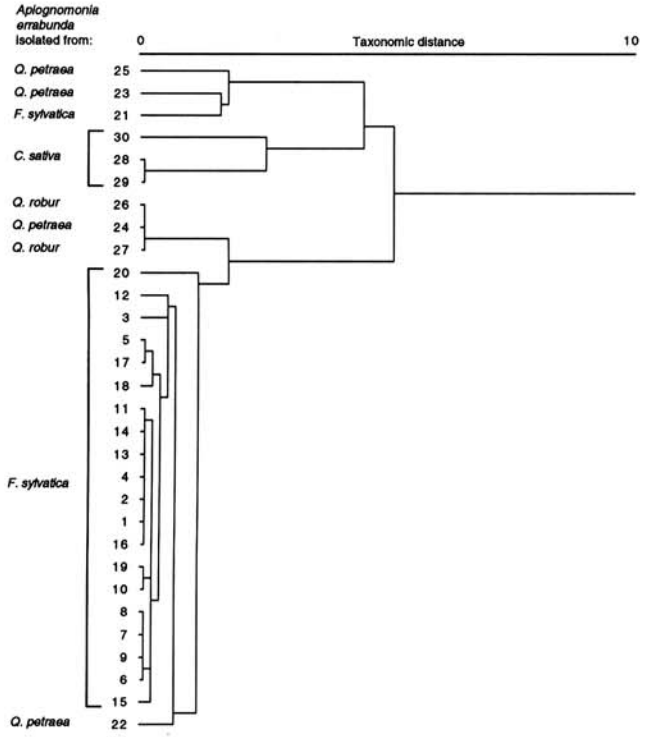

Fig. 3. UPGMA clustering of 30 isolates of *Discula umbrinella*. Further details see text.

Table 3. Distribution of shared arbitrary amplified bands, using 17 different decamer primers, among the different isolates collected from the four host species

Origin of the isolates				Number of DNA fragments (%)
F. sylvatica	*Q. petraea*	*Q. robur*	*C. sativa*	
x				22 (16.4)
	x			39 (29.1)
		x		31 (23.1)
			x	2 (1.5)
x	x			10 (7.5)
x		x		10 (7.5)
x			x	1 (0.7)
	x	x		0 (0)
	x		x	6 (4.5)
		x	x	1 (0.7)
x	x	x		2 (1.5)
x	x		x	7 (1.5)
x		x	x	2 (1.5)
	x	x	x	0 (0)
x	x	x	x	1 (0.7)
Total				134 (100)

hosts. On the other hand, the number of isolates from *Quercus* spp. studied here is too small to allow any definitive conclusion. The results of the RAPD marker analysis reveal that 94 (70.1%) of the DNA fragments were amplified specifically in only one host group, 28 (21%) were common to two, only 12 (8.2%) to three, and only one fragment (0.8%) was found in all isolates (Table 3).

The considerable variability within the 20 isolates of *D. umbrinella* from beech is striking. However, similar patterns have been observed in other fungi. Metzenberg (1991) has found similar levels of polymorphism at the molecular level in isolates of *Neurospora crassa* collected in a small geographic area. McDermott *et al.* (1989) have demonstrated considerable variation in both isozyme and rDNA variation from isolates of *Rhynchosporium secalis* (Oudem.) J. J. Davis collected from a single barley plot. Goodwin and Annis (1991) have detected significant levels of variation using the RAPD assay on only 15 isolates of the ascomycete *Leptosphaeria maculans* (Desm.) Ces., the causal agent of the blackleg disease of crucifers. It could be argued that some of the RAPD polymorphisms may arise from mutations occurring during the storage of the isolates under laboratory conditions. Although this cannot be excluded *a priori*, tests of freshly isolated strains of *D. umbrinella* yielded an identical picture provided by the isolates used in this study (data not shown). Moreover, classical vegetative compatibility testing by crossing carried out with the same isolates used in this study has given compatible results (U. A. Haemmerli and O. Petrini, unpublished).

Colonization of beech leaf tissues by *D. umbrinella* may occur by infection through air inoculum and/or by systemic spreading from the twigs through the leaf petiole. Sieber and Hugentobler (1987) have postulated that air inoculum

is the most important colonization factor for *D. umbrinella*. In our study, the genetic variability observed in the *D. umbrinella* isolates from the same leaf supports this hypothesis. The identification by RAPD marker analysis of up to four distinct individuals in a single leaf indicates that infection of a small tissue area by more than one individual may occur without elicitation of a generalized defense response. The host-endophyte relationship between *F. sylvatica* and *D. umbrinella* is apparently well balanced; the endophyte infects the tissue either without provoking any defense responses or inducing only a mild local host reaction that limits the fungal growth. This would lead to the symptomless infection described for a number of plant pathogens (Cerkauskas 1988; Cerkauskas and Sinclair 1980; Huang 1989; Kulik 1984; Leslie *et al.* 1990) and also explain the frequency of many symptomless endophytic fungi (Petrini 1991).

Morphological, physiological, and biochemical investigations have pointed to host-specific differences among *D. umbrinella* isolates derived from different hosts. Toti *et al.* (1992) have observed morphometric differences in the conidial sizes of isolates derived from beech, chestnut, and oak. Ongoing investigations in our laboratories have shown host-specific patterns of pectinase isozymes among *D. umbrinella* isolates (Toti *et al.*, unpublished). In this study, two-thirds of the markers have shown host-specific patterns and only a single marker was found in all isolates. The large number of private RAPD variants is consistent, with extremely limited or no gene flow among the groups defined by host species origin. Strong selection may have generated postreproductive isolation and maintained the genetic divergence among the ecological groups. Alternatively, the groups may be reproductively isolated through sexual incompatibility, thus precluding gene flow. Thirdly, geographic isolation by distance may have contributed to some of the differences. However, the sample used in this preliminary investigation is not ideal for distinguishing among possible hypotheses leading to restricted gene flow.

The present study demonstrates conclusively the occurrence of multiple infection in a host-endophyte system and points to a strong host specificity of the endophytic symbiont. The characterization of fungal isolates by PCR techniques using arbitrary primed amplified DNA fragments leads to results comparable to those obtained by using RFLP analysis (Brown *et al.* 1990; Förster *et al.* 1989; Manicom *et al.* 1987) and the DNA fingerprinting with synthetic oligodeoxynucleotides (Weising *et al.* 1991; Meyer *et al.* 1991). It also provides a quick and reliable method of population analysis in fungal ecology and pathology.

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