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Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*

U. G. MUELLER, S. E. LIPARI* and M. G. MILGROOM*
*Section of Neurobiology & Behaviour, Cornell University, Ithaca, NY 14853 and *Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA*

Abstract

A PCR-based fingerprinting technique based on amplified fragment length polymorphisms (AFLP) is used to screen symbiotic fungi of the fungus-growing ant *Cyphomyrmex minutus* for genetic differences. AFLP fingerprints reveal several fungal 'types' that (a) represent distinct clones propagated vegetatively by the ant, or (b) correspond to free-living fungi that may be acquired by the ant. Fungal types identified by AFLP fingerprints correspond to vegetative-compatibility groups established previously, suggesting that vegetative compatibility can be used as a crude indicator of genetic differences between fungi of *C. minutus*.

Keywords: AFLP fingerprinting, attine fungi, *Cyphomyrmex minutus*, fungus-growing ants, symbiosis

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Introduction

Molecular techniques allowing differentiation between strains, genotypes, or individuals have become indispensable tools in population genetic surveys. An impressive number of such techniques are available, but the various techniques differ in their ease of use and their power to resolve genetic differences. Techniques such as minisatellite multilocus fingerprinting (Jeffreys *et al.* 1985; Kirby 1990) or microsatellite single-locus typing (Queller *et al.* 1993) yield maximal resolution, but they are time consuming. More time-efficient techniques such as protein electrophoresis (Hames & Rickwood 1990) are frequently plagued by insufficient resolution because of low variance in allozyme markers. One technique combining time-efficiency with adequate resolution uses randomly amplified polymorphic DNA (RAPD-PCR) (Williams *et al.* 1990; Hadrys *et al.* 1992), but this technique has proven flawed because of potential PCR artefacts (Riedy *et al.* 1992; Weeden *et al.* 1992; Ellsworth *et al.* 1993; but see Micheli *et al.* 1994; Bielawski *et al.* 1995; Tommerup *et al.* 1995).

Zabeau & Vos (1993; Vos *et al.*, in preparation) recently developed a novel PCR-based fingerprinting technique using Amplified Fragment Length Polymorphisms (AFLP) that promises to be free of PCR artefacts, while retaining time-efficiency. This technique is similar to RAPD-PCR in that randomly chosen segments (specifically, restriction fragments) are amplified by the polymerase chain reaction, but differs fundamentally from RAPD-PCR in that these random sequences are amplified at high stringency; high stringency eliminates the problem of spurious PCR products created when amplifying under low stringency as in typical RAPD-PCR.

The basic protocol of generating AFLP fingerprints involves two key steps: (1) Small amounts of DNA (≤ 2.0 μg) are restricted with an endonuclease producing staggered (sticky) ends, which are then ligated to specifically designed adapters (10–30 nucleotides, double-stranded). Restriction and ligation are performed simultaneously. To ensure that fragment-fragment ligations are recleaved and that all fragments are ligated to adapters, the adapters are designed such that ligation to an adapter does not reconstitute the restriction site; in contrast, fragment to fragment ligation does reconstitute the site and reopens the possibility of cleavage. Reactions are performed with an excess of endonuclease to avoid partial digestion. (2) Tagged fragments are then amplified using a primer sequence incorporating the adapter sequence, the part of the restriction site remaining on the fragment, and several (2–5) randomly chosen nucleotides extended into

Correspondence: Ulrich G. Mueller Smithsonian Tropical Research Institute, Unit 0948, APO Miami, FL 34002–0948, USA. Fax: +1 507 32 6274.

the unknown portion of the fragment. Extension of the primer by several nucleotides into the fragment ensures that only a subset of the fragments is amplified, thus creating a series of discrete bands when electrophoresing the products. Longer extensions yield fewer bands (amplify a smaller subset of fragments), and primers with different nucleotide sequences in the extension generate different banding patterns. High stringency of the PCR conditions ensures that primers differing by only one nucleotide in the extension produce distinct banding patterns. The number of distinct banding patterns that can be generated is large, because the number of different primers that can be used increases exponentially with the length of the extension (16 different primers with an extension of 2, 64 with an extension of 3, etc.). It is likely therefore that one or several of the banding patterns reveal fragment length or presence/absence polymorphisms, making this procedure ideal for rapid fingerprinting.

Materials and methods

Fungal samples

We used AFLP fingerprints to screen 14 isolates of fungi cultivated by the fungus-growing ant *Cyphomyrmex minutus* (Attini: Formicidae). Fungi had been isolated from 13 nests collected at four sites (all within a 2-km × 2-km area) at the Archbold Biological Station (ABS), Lake Placid, Florida, and from one nest collected 150 km north of the ABS in Orlando (Chapela *et al.*, in preparation). At ABS site 1, nine colonies of *C. minutus* were collected within a 20-m × 20-m area; two colonies collected at ABS site 2 were separated by 16 m; one colony each was collected at ABS sites 3 and 4. Sites were separated by at least 500 meters. Fungal isolates were grown in liquid culture (Potato Dextrose Broth, 0.5% Tryptone; Difco) for two weeks, and total DNA was extracted from lyophilized mycelium following the methods of Milgroom *et al.* (1992).

Restriction-ligation

Fungal DNA was restricted with the endonuclease Pst1 and ligated to double-stranded Pst1 adapters. Restriction and ligation were performed simultaneously in a volume of 20 μL for 4 h at 37 °C, using the following conditions: 2 μg of DNA; 0.2 μg of adapters; 20 units of Pst1; 1 unit of T4 DNA-ligase; 10-mM Tris.HAc (pH 7.5), 10-mM MgAc, 50-mM KAc; 2-mM dithiotreitol; 0.5-mM ATP. As suggested by Zabeau & Vos (1992), the adapter sequence and its staggered end were as follows:

5-C T C G T A G A C T G C G T A C A T G C A-3
3-G A G C A T C T G A C G C A T G T-5

Note that the recognition sequence of Pst1, 5-CTGCAG-3, is not restored when ligating this adapter to a restriction fragment, because a 5′ A (see underlining in adapter) replaces the 5′ C in the original recognition sequence. This substitution prevents recleavage of the adapter-fragment product and allows ligation in the presence of Pst1. After ligation, volumes were increased to 100 μL, 50 μL of 7.5-M NH_4Ac was added, and the DNA was precipitated with two volumes of cold 100% ethanol to remove unbound adapters. DNA pellets were rinsed with 70% ethanol, dried, and resuspended in ddH_2O. About 50–70% of the original DNA was recovered after precipitation.

PCR amplification

Adapted Pst1 fragments were amplified under standard conditions (1 ng of DNA; 150 ng of primer; 0.3 units of Taq polymerase; 200-μM dNTP mix; buffer 10-mM Tris HCl pH 8.5, 1.5-mM $MgCl_2$, 50-mM KCl; ddH_2O to a volume of 25 μL) using the following profile: 1 min at 94 °C, 1 min at 60 °C, ramping from 60 °C to 72 °C at 1 °C per second, and 2.5 min at 72 °C (33 cycles total). A pilot survey showed that primers with a one-base-pair extension yielded too many bands for reliable scoring. Amplifications were therefore performed with primers with the sequence of 5-GACTGCGTACATGCAGXX-3, with the 2-bp extension at the 3′ end (XX in the sequence) varying between the primers. The primer extensions used were GT, GA, GC, AC, AG and CG. PCR products were electrophoresed in 1.4% agarose gels at 80 V for 5 h and stained with ethidium-bromide for visualization.

Results and discussion

Figure 1(a–f) presents AFLP fingerprints of the 14 fungi isolated from nests of the fungus-growing ant *C. minutus*. Each of the six primers generated fingerprint patterns markedly distinct from the other primers, even when primers differed by only one nucleotide in the extension (compare Fig. 1a and 1b; 1c and 1d, etc.). Primers generated between 12 and 26 scorable bands, 4–11 of them variable as presence-absence polymorphisms. Repeated amplifications of the same DNA revealed no artefactual variation like the one found in RAPD (Riedy *et al.* 1992; Weeden *et al.* 1992; Ellsworth *et al.* 1993), except for an occasional slight intensity difference. This demonstrates that AFLP banding patterns are useful for rapid typing and fingerprinting.

Among the 14 symbiotic fungi of *C. minutus*, 4 distinct fingerprint 'types' are evident (Fig. 1a–f). Type 1 includes the first eight isolates from site 1 and both isolates from site 2; Type 2 includes the last isolate from site 1 and the isolate from Orlando; Type 3 and type 4 are represented by a single isolate each from site 3 and site 4, respectively. These distinct fungal 'types' are identifiable in each of the

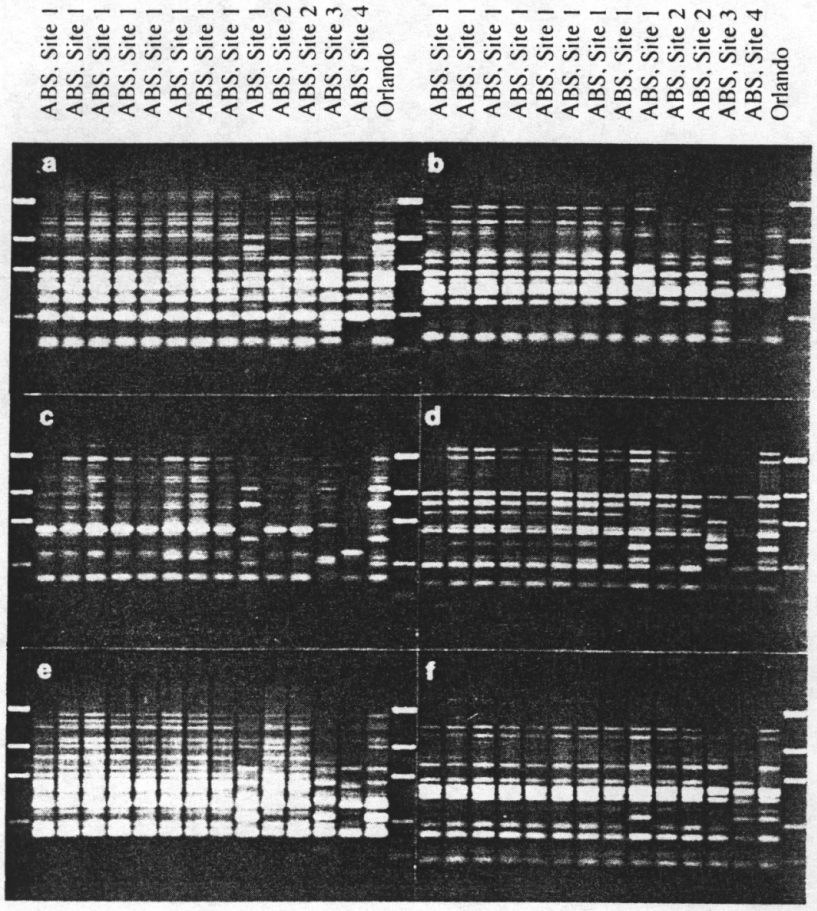

Fig. 1 AFLP fingerprints of symbiotic fungi of the fungus-growing ant *Cyphomyrmex minutus*, generated by electrophoresis of AFLP-PCR products, using primers with two-base-pair extensions of (a) GT, (b) GA, (c) GC, (d) AC, (e) AG and (f) CG. The ladders show fragments of 2000, 1200, 800, 400, 200 and 100 base pairs. Thirteen attine fungi were collected at four sites (Site 1–4) at the Archbold Biological Station (ABS; see Materials and Methods for details) and one isolate from Orlando (150 km north of the ABS). AFLP fingerprints reveal substantial polymorphisms between fungal isolates from the same species of ant. Four fungal types are apparent: type 1 represented by the first eight isolates from site 1 and the two isolates from site 2; type 2 represented by the last isolate from site 1 and the isolate from Orlando; Types 3 and 4 represented each by one isolate from site 3 and 4, respectively. The type 1 fungus therefore was isolated from attine nests at two sites ≈ 500 m apart; the type 2 fungus was isolated from two attine nests collected at 150 km distance. The fungi are grouped into the same types by each of the six AFLP-primer systems. The fungal types therefore are genetically divergèd from each other, suggesting that the fungal types are (a) distinct clones that are vegetatively propagated by the ants, or (b) discrete fungal lineages of acquired fungi that are reproductively isolated from each other.

fingerprints generated by the six different primer systems, indicating considerable divergence among the fungal types. Within each type, different isolates showed identical banding patterns. Each fungal type therefore may represent a distinct clone (the sister species *Cyphomyrmex rimosus* transmits its fungi clonally from parent to offspring nest; personal observation). Alternatively, if the ants occasionally acquire fungi from free-living stocks, as has been suggested for the basal lineages of fungus-growing ants (Chapela *et al.* 1994), each fungal type could correspond to a free-living species of fungus. It is therefore possible that (a) a more comprehensive survey of symbiotic fungi of *C. minutus* at the ABS could reveal a substantially larger number of fungal types propagated by this species of ant; and that (b) comparisons of AFLP fingerprints of symbiotic fungi with those of sympatric, free-living lepiotaceous fungi could reveal genetic identity between symbiotic and free-living fungi, as predicted by fungal acquisition.

The genetic differences among the fungal types are congruent with findings from vegetative-compatibility (VC) assays predicting genetic differences among the 14 isolates (Chapela *et al.*, in preparation). VC incompatibility reactions are assumed to reflect genetic differences among isolates (Hansen *et al.* 1993), because (a) vegetative compatibility in dikaryotic fungi is regulated by multiple, polymorphic loci (b) only strains with identical alleles at each of these loci are compatible, and (c) the intensity of incompatibility increases with the number of alleles differing between interacting strains. For the 14 fungi of *C. minutus*, VC pairings in all combination had revealed incompatibilities that varied on a microgeographic scale and that were a function of the distance between collection sites. With one exception, only fungi from nests collected within a site (within about 20 m) were compatible. The absence of incompatibilities among fungal isolates from the same site therefore predicted genetic similarity or identity between fungi from the same site. AFLP fingerprints confirm these predicted pattern. With only one exception, isolates collected in close proximity were genetically similar; the only exception was the last isolate from site 1 that belonged to a different 'type' from all other isolates from that site (see Fig. 1); this distinct isolate was also vegetatively incompatible with the other isolates from site 1. This study therefore support the findings of others (Hansen *et al.* 1993) that

vegetative compatibility can be used as a crude indicator of genetic differences between dikaryotic fungi.

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UGM is an evolutionary biologist working on the ecology, population genetics, and phylogenetic history of the symbiosis between fungus-growing ants and their fungi. SEL and MGM are plant pathologists working on the population genetics of plant pathogenic fungi and fungal viruses, primarily with the chestnut blight fungus.
