

Random amplified polymorphic DNA markers reveal genetic variation in the symbiotic fungus of leaf-cutting ants

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Abstract: RAPD markers were used to examine the degree of genetic variation within the putatively asexual basidiomycete fungus (Lepiotaceae: provisionally named *Leucoagaricus gongylophorus*) associated with the leaf-cutting ant species *Atta cephalotes*. We analyzed fungal isolates from ant nests in two geographically distant sites, two isolates from Panama and five isolates from Trinidad. Ten decamer primers were used to amplify total DNA from these seven fungal isolates, and RAPD banding patterns were compared. Genetic similarity among isolates was determined by pair-wise comparisons of the shared number of DNA bands on an agarose gel. There was considerable genetic variation among isolates of the symbiotic fungus even within sites. Pairs of fungal isolates from the two different sites shared an average of only 36% of the bands in their RAPD profiles, while pairs from the within sites shared an average of 72% of the bands. RAPD markers may be useful for further investigation of the genetic structure of the fungal symbiont within species of leaf-cutting ants.

Key Words: *Atta cephalotes*, fungal DNA, genetic variation, leaf-cutting ants, *Leucoagaricus gongylophorus* (Lepiotaceae), population differentiation, RAPD markers, symbiosis

INTRODUCTION

Leaf-cutting ants (Formicidae: Attini: *Acromyrmex* and *Atta*) are considered to be the dominant herbivores of the Neotropics (Hölldobler and Wilson 1990). These ants are obligately dependent on a symbiotic fungus (Weber 1972, Hölldobler and Wilson 1990, Mueller et al 2001), which is cultivated on leaf fragments brought into the nest by foraging workers.

The fungus produces swollen hyphae ('staphylae' or 'gongylidia') that are harvested by the ants for food, particularly for feeding the larvae (Quinlan and Cherrett 1979, Bass and Cherrett 1995).

The symbiotic fungus of leaf-cutting ants generally lacks sexual structures, which has made species identification and nomenclature difficult (reviewed in Pagnocca et al 2001). Progress has been made recently in determining the phylogeny of both the leaf-cutting ants and their symbiotic fungi, based on interspecific comparisons of DNA sequences (Chapela et al 1994, Hinkle et al 1994, Wetterer et al 1998). The fungus is in the basidiomycete family Lepiotaceae (Mueller et al 1998, 2001), and the fungus associated with *Atta cephalotes* provisionally has been named *Leucoagaricus gongylophorus* (Fisher et al 1994).

The symbiotic fungus may have been propagated asexually by leaf-cutting ants for millions of years (Chapela et al 1994, Hinkle et al 1994, Judson and Normark 1996). The usual means of reproduction is clonal; founding queens of leaf-cutting ants initiate gardens in new nests with small bits of fungus carried from their natal nest (Weber 1972). However, there is some evidence that sexual structures can be formed by the fungus on rare occasions (Fisher et al 1994, Pagnocca et al 2001, Mueller et al 2001), which suggests that recombination may be possible.

There has been considerable interest in the genetic structure of clonal lineages (e.g., Jokela et al 1997, Weider et al 1999, Mark Welch and Meselson 2000), though information is available from only a small sample of species. To investigate genetic variation in the symbiotic fungus of the leaf-cutting ant *Atta cephalotes*, we used random amplified polymorphic DNA (RAPD) markers to differentiate fungal clones from different source nests. RAPD markers typically have high overall variability and so can be useful for detecting genetic differences within species (Williams et al 1990, Parker et al 1998, Sunnucks 2000). This technique has been used to investigate intraspecific genetic variation in several fungi (e.g., Fegan et al 1993, Moore et al 2001).

We compared RAPDs of fungal isolates from *Atta cephalotes* nests, both within and between two distant sites (Panama and Trinidad), to test the hypothesis that geographically distant populations will be more genetically distinct than nearby populations.

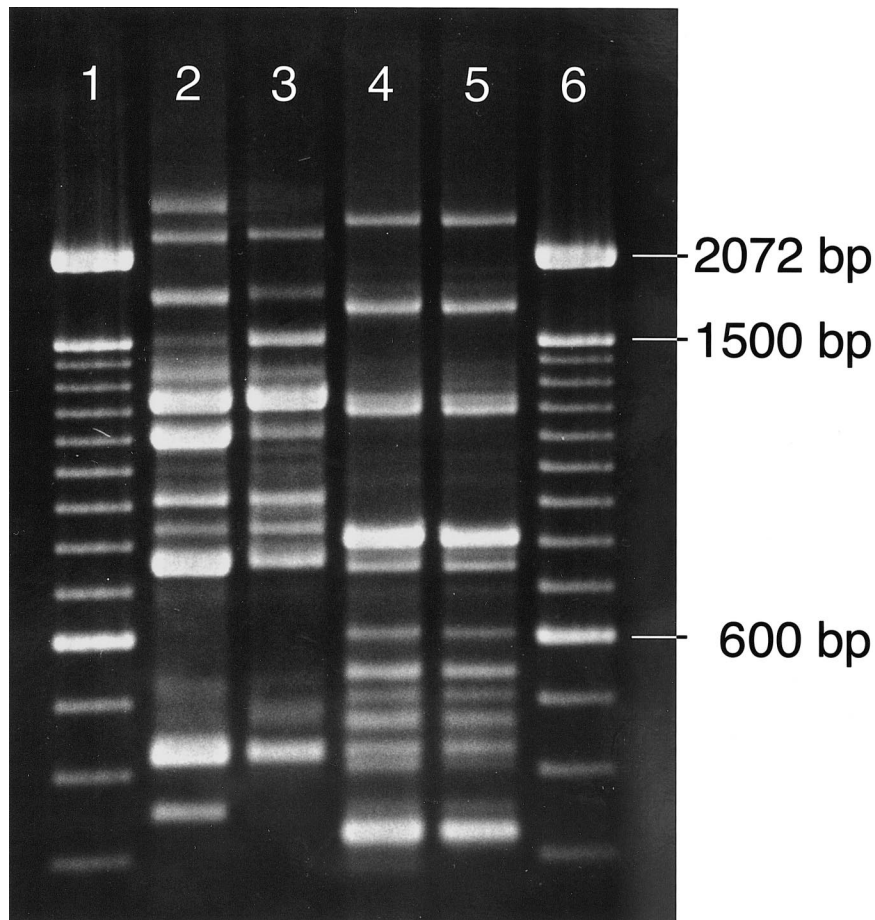


FIG. 1. RAPD profile of the *Atta cephalotes* symbiotic fungus generated using the primer OPS02. Lanes 1 and 6 contain a 100 bp DNA ladder as a size marker; lanes 2 and 3 contain amplified DNA products from the two fungal isolates from Panama; lanes 4 and 5 contain amplified DNA products from two fungal isolates from Trinidad.

MATERIALS AND METHODS

Fungal isolates were obtained from seven different nests of *Atta cephalotes* that were being maintained in either zoos or laboratories in North America. The ants' nests were originally from two sites: two samples from Panama near the city of Gamboa (C. Currie pers comm), and five samples from the Arima Valley in Trinidad (R. Morgan pers comm). We maintained the fungal cultures in the laboratory on slants of potato dextrose agar (PDA) medium, incubated at 25 C. After 3–4 wk of growth on the PDA medium, the fungus was removed by scraping the surface with an inoculating loop and then placed in a sterile mortar (pre-chilled at –80 C). The fungal tissue was ground under liquid nitrogen to a fine powder using a sterile pestle. The tissue powder was then transferred to a microcentrifuge tube, and the liquid nitrogen was allowed to evaporate. Fungal DNA was extracted by using a silica gel membrane-based DNA isolation kit (DNeasy Plant Mini Kit provided by Qiagen Inc., Valencia, California). Yields typically ranged from 0.1–1 µg of genomic fungal DNA.

Each of the seven fungal extracts was amplified with ten different 10-base pair primers (supplied by Operon Technologies, Alameda, California): OPN01–CTCACGTTGG; OPN03–

GGTACTCCCC; OPN14–TCGTGCGGGT; OPN15–CAGCGA-CTGT; OPN19–GTCCGTACTG; OPN20–GGTGCTCCGT; OPS02–CCTCTGACTG; OPS04–CACCCCCTTG; OPS06–GATACCTCGG; OPS07–TCCGATGCTG. PCR amplification was performed using RAPD Analysis Beads (Amersham Pharmacia Biotech, Piscataway, New Jersey). Amplification was conducted in a 25 µL volume containing 20 ng of genomic DNA, 25 pmols of a single RAPD primer, and one Ready-To-Go RAPD analysis bead. PCR conditions were as follows: 1 cycle at 95 C for 5 min followed by 45 cycles at 95 C for 1 min, 36 C for 1 min and 72 C for 2 min, with a final extension of 10 min at 72 C. The PCR products were separated electrophoretically on 1.5% agarose gels in TBE buffer (45.0 mM Tris-borate, 1.0 mM EDTA). DNA bands were visualized by staining with ethidium bromide (0.5 µg/mL), observed under UV light and photographed.

RESULTS

We initially chose over twenty random decamer primers to generate RAPD profiles of the seven fungal isolates. We chose ten of these primers for further study as they produced consistent and reproducible

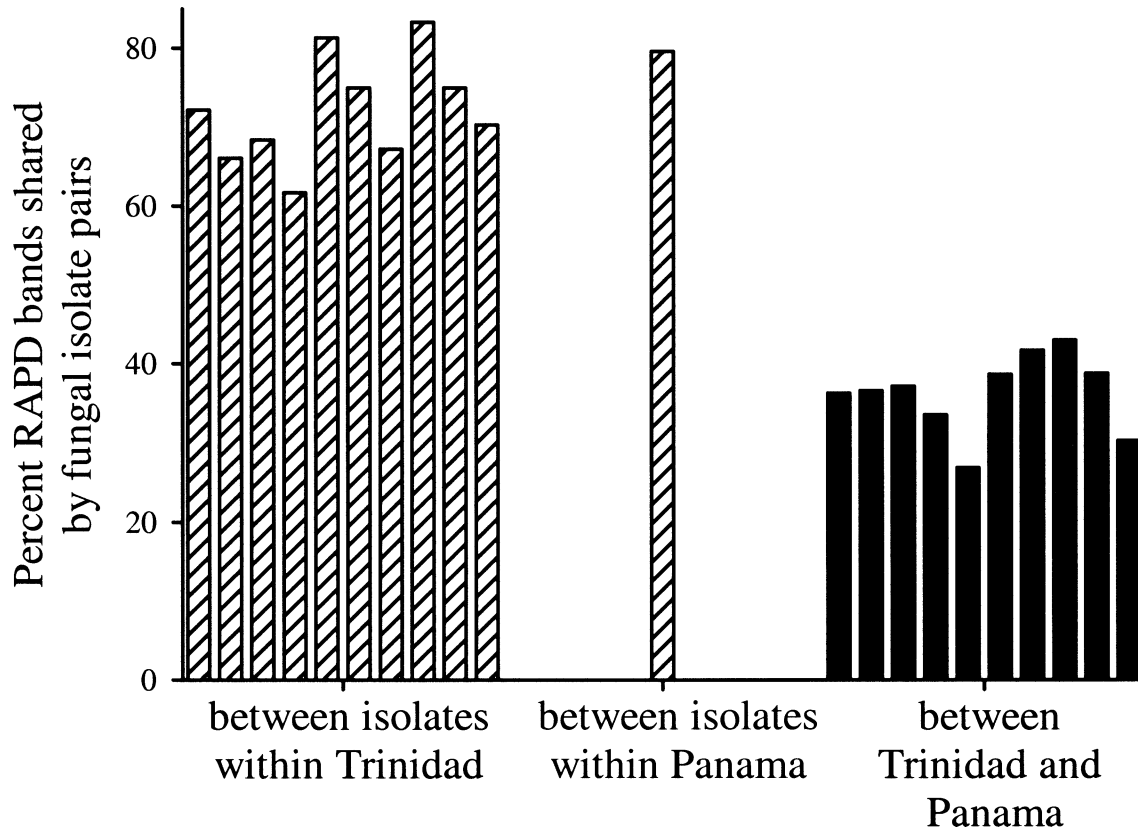


FIG. 2. RAPD band sharing of pairs of isolates of the *Atta cephalotes* symbiotic fungus within and between sites. There were five isolates from Trinidad and two from Panama. All pairwise comparisons between isolates are shown: 10 pairs within Trinidad, 1 pair within Panama, and 10 pairs between Trinidad and Panama. Within-site pairs are shown as striped bars and between-site pairs are shown as filled bars. On average, pairs of isolates had 86.7 informative bands from an average of 7.9 primers.

bands for all of the fungal isolates. The results of one such primer amplification (OPS02) are shown in FIG. 1.

To quantify the similarity between fungal isolates, we compared the number of shared RAPD bands from all primers between each pair of isolates. Band sharing for a pair of isolates was calculated as the number of identical bands shared by both isolates divided by the total number of distinct band locations present in either of the two isolates. The number of distinct bands was different for each pair being compared; the average number of band locations per pair was 86.7 (range = 44–134) from an average of 7.9 primers (range = 5–10).

Pair-wise comparisons of band sharing in the fungal isolates are shown in FIG. 2. The two isolates from Panama shared 79.6% of their RAPD bands. The five isolates from Trinidad shared an average of 72.1% of bands (range = 61.7–83.3%). In contrast, an average of 36.3% of bands were shared between pairs of isolates from the two different sites (range = 26.9–43.0%). Pairs of fungal isolates within sites shared significantly more bands than pairs between sites (t -test, 19 df, $P < 0.001$).

DISCUSSION

We found that RAPD profiles differed markedly between isolates of the symbiotic fungus from different *Atta cephalotes* nests. On average, pairs of fungal isolates from within sites (both Trinidad and Panama) shared more than twice as many RAPD bands as pairs from different sites (FIG. 2). Such a geographically based pattern of genetic differentiation would be expected if there were restricted dispersal among regions, which would allow time for genetic differences to arise by mutation, drift, and selection. The only known method of dispersal of the symbiotic fungus is within the infrabuccal pocket in the head of dispersing queens when they leave the natal nest (Weber 1972). Since Trinidad is an island, the fungus and ant populations there may have been isolated from mainland areas for many generations.

This is the only examination (of which we are aware) of genetic variation of a leaf-cutting ant fungus between distant sites. Pagnocca et al (2001) used RAPD markers on the fungus associated with the leaf-cutting ant *Acromyrmex hispidus* from a single loca-

tion in Brazil, in order to demonstrate that fruiting bodies were identical to sterile mycelium. Bot et al (2001) used AFLP (amplified fragment length polymorphism) markers to compare the symbiotic fungus from nests of *Acromyrmex octospinosus* and *A. echinator* from one location in Panama. The AFLP markers were effective in detecting fungal isolates from different nests, and provided evidence that there is occasional exchange of fungal strains between the two *Acromyrmex* species (Bot et al 2001).

RAPD markers have been useful in other investigations of genetic variation among geographically distant populations of fungi. Li et al (2001) compared RAPD markers in populations of western gall rust fungus (*Endocronartium harknesii*) collected across western and central Canada from two host pine species. Most of the genetic variation in *E. harknesii* occurred between the two host species, but within hosts there was more variation among geographically widespread locations than within locations (Li et al 2001). This is comparable to the pattern we observed. In contrast, Furnier et al (1999) found complete uniformity in RAPD markers in isolates of the butternut canker fungus (*Sirococcus clavignenti-juglandacearum*) collected from sites across the east-central U.S. This lack of genetic variation could be the result of the recent introduction of a single strain of butternut canker into North America (Furnier et al 1999).

Our results demonstrate the utility of RAPD analysis in measuring genetic variation within the fungal symbionts of leaf-cutting ants. RAPD or other molecular markers (Parker et al 1998, Sunnucks 2000) could allow further investigation of the spatial genetic structure of this symbiotic fungus. Samples from a broad range of distant sites would be particularly interesting. RAPD markers may also help address such questions as the extent of recombination in the fungus (Mueller et al 2001, Pagnocca et al 2001) and the importance of horizontal transfer of the fungus between different ant nests (Bot et al 2001).

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