Mycologia, 85(2), 1993, pp. 180–186.

GENOTYPIC DIVERSITY IN POPULATIONS OF A FUNGAL ENDOPHYTE FROM DOUGLAS FIR

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ABSTRACT

Rhabdocline parkeri, an endophyte of Douglas fir, forms symptomless infections within single epidermal cells of healthy needles. *Rhabdocline parkeri* strains were isolated from trees growing in various habitats ranging from virgin old growth forests to a single young tree growing isolated in a meadow. Different genotypes of *R. parkeri* were distinguished by using the Random Amplified Polymorphic DNA (RAPD) technique. A significantly lower number of *R. parkeri* genotypes per unit foliage were isolated from trees within a 20-year-old managed stand and from an isolated tree than from old growth trees. Foliage from young trees (20 years old) in a stand directly adjacent to the sampled old growth trees showed levels of genotypic endophyte diversity comparable to that in the old growth trees. Genotypic diversity is estimated to be at least three orders of magnitude greater in foliage of the old growth trees and adjacent young trees than in foliage from the managed stand and from an isolated tree. This difference is ascribed to differences in tree age and access to inoculum.

Key Words: Douglas fir, fungal endophyte, genotypic diversity, population biology, RAPD

The term endophyte refers to fungi which reside completely within plant tissues. Endophytic infections are often quiescent; they inhabit the host without causing obvious damage, but colonize other areas quickly when host tissue becomes damaged or senescent. Endophytic fungi are common; latent fungal infections have been reported in grasses (Bacon et al., **1977**; Clay, **1988**; Wallner et al., **1983**), shrubs (Petrini et al., **1982**), and evergreen trees (Carroll and Carroll, **1978**; Katz and Lieth, **1980**) and are likely to be found within any host plant investigated.

Rhabdocline parkeri Sherwood-Pike, Stone, & Carroll is a fungal endophyte which causes latent infections in the needles of Douglas fir, *Pseudotsuga menziezii* (Mirb.) Franco, (Sherwood-Pike et al., **1986**) and has been found inhabiting nearly every tree in which it has been sought in western Oregon and Washington (Carroll and Carroll, **1978**). Individual infections in healthy needles are limited to single epidermal cells, where they remain dormant until the onset of needle senescence, typically after 5–9 yr. The fungus produces slimy conidia which are dispersed by rain to new needles from abscised needles lodged in the canopy (Sherwood-Pike et al., **1986**; Stone, **1987**). The role of the sexual *Rhabdocline* state in the infection cycle is still unknown (Carroll, **1986**).

Endophytes are often mutualistic, producing toxins antagonistic to herbivores (Calhoun et al., **1992**; Clay, **1986**; Lyons et al., **1986**; Miller, **1986**). Carroll (**1986**, **1988**) has shown *R. parkeri* to be antagonistic toward gall midges (*Contarinia* spp.) in Douglas fir needles. Gall midge larvae showed greatly increased mortality in galls infected by the fungus compared to an uninfected control group. Because the fungus does not invade the bodies of living larvae, fungal toxins were thought to be responsible for larval death. Independent study revealed that *R. parkeri* produced substances in culture that were toxic to both HeLa cells and spruce budworm larvae (*Choristoneura fumiferana* Clem.) (Miller, **1986**).

Carroll (**1991**) has argued that mutualistic association with fungal endophytes could serve to defend long-lived plants against short-cycle herbivores such as insects. This theory proposes that rapid adaptation by pests to host chemical defense is offset by the possession of multiple ge-

netic strains of an endophyte, each of which produces a unique suite of mycotoxins.

If this hypothesis is correct, one would expect the *R. parkeri* population within a Douglas fir stand to consist of a large number of different genotypes. Further, genotypic diversity should vary among individual trees such that older trees contain a higher number of endophyte strains than younger trees simply because they contain more foliage. However, because spores are easily transferred from tree to tree in wind-blown rainfall, one would predict the number of strains per unit area of foliage to be constant in localized areas regardless of tree size. It might also be expected that trees distant from an inoculum source would have few fungal strains while young trees growing close to a diverse inoculum source would harbor a higher diversity of strains. This work addresses these questions by using the RAPD technique to distinguish genotypes of *R. parkeri* collected from Douglas fir of different ages and at different locations.

MATERIALS AND METHODS

Sample collection.—Old growth needles were collected from trees located in the H. J. Andrews Experimental Forest (site 1) near Blue River, Oregon (44°15'N, 22°15'W, elevation approximately 460 m). One branch was collected from the lower canopy from each of three trees using a 12 gauge shotgun; 15 needles were randomly selected from each branch for culture. Second growth foliage was collected from three sites. Site 2 was located in the Andrews Experimental Forest in a 19-year-old clear cut stand adjacent to the old growth collection site. Trees in this site were all located within 32 m of old growth Douglas fir trees. One branch was collected at chest height from each of three trees in order to closely imitate the sampling style used in collecting old growth foliage. Fifteen needles were randomly selected from each branch for culturing. In addition, from sites 1 and 2, one needle was also randomly chosen from each tree and cut into nine segments. *Rhabdocline parkeri* strains were then isolated from each segment independently.

Site 3 trees were located at the U.S. Bureau of Land Management Progeny Test Site, in the Oregon coast range near Junction City, Oregon (44°17'N, 123°28'W, elevation approximately 500 m). This site, which is organized into 31 randomized blocks, was established on cleared land in 1972 with seedlings from identified parental trees organized into families. Families are half-sibs with common maternal trees. Two blocks, whose midpoints were approximately 100 m apart, were arbitrarily selected, and within each block three trees from family A and three trees from family B were sampled. One needle from each of six branches was sampled at chest height from each tree.

The final site (site 4) contained a single tree located in Alton Baker Park in Eugene, Oregon, (44°02'N, 123°08'W, elevation approximately 145 m) which was similar in age and size to site 2 and 3 trees. This tree was unique in that it is located in a meadow area where it is isolated from all other trees (nearest neighbor approximately 64 m) and is more than 900 m from the nearest large Douglas fir tree. Three branches were collected from this tree at chest height and five needles per branch were randomly selected for *R. parkeri* isolation.

Four additional strains were tested which had been collected and isolated from site 3 in 1987. These strains were collected from the same trees within the site as those sampled for this study and, following isolation of single spores, were inoculated into autoclaved needles and stored frozen.

Culture methods.—All foliage was stripped and processed within 24 h of collection; only third year needles were used. Arbitrarily selected needles were surface sterilized by soaking for 5 min in 70% ethanol, briefly rinsing in distilled H_2O, soaking for 5 min in 50% commercial Chlorox bleach, followed by a second brief rinse in distilled H_2O. The foliage was immediately planted on petri plates containing 2% malt extract (Sigma) and 2% yeast extract (Sigma) in potato dextrose agar (PDA). The plates were incubated at room temperature until sufficient growth was attained, at which time the plate was transferred to a 14 C incubator to induce sporulation. Plates were kept at this temperature for 2 wk to allow all strains present to sporulate and avoid biasing the sample in favor of fast sporulating strains. Spores were then streaked on 60 mm diam petri dishes containing PDA and allowed to germinate. Single spore isolates were produced by picking germinated spores off the streak plate with a sterile needle and transferring to slants containing 2% malt extract and 2% yeast extract in PDA.

DNA preparation.—Single spore colonies were incubated at 14 C until they had attained approximately 1 g fresh weight. Approximately 0.2 g of fresh mycelium were transferred by sterile technique to a 1.5 ml centrifuge tube and suspended in 1 ml of grinding buffer (0.4 g sodium deoxycholate, 1 g polyethylene cetyl ether, 40 ml 5 M NaCl; H_2O added to a final volume of 100 ml) and lightly ground with a grinding rod. Following grinding the suspension was incubated on ice for 30 min and centrifuged for 10 min at 7000 rpm. The supernatant was combined with 500 μl of TCA/ethanol (1.43 g/ml TCA mixed with equal volume of 100% ethanol), mixed by inversion and incubated on ice for 1 h. The TCA/ethanol mixture was centrifuged at 7000 rpm at 4 C for 20 min and the supernatant discarded. The pellet was rinsed in 5 μl of 70% ethanol, dried at room temperature for 10 min, resuspended in 150 μl of RNAse solution (Sigma) and 120 μl of 5 M NH_4OAc, and incubated at 4 C for 1 h. This mixture was combined with 900 μl of 100% isopropyl alcohol, incubated on ice for 20 min, and centrifuged at 7000 rpm at 4 C for 20 min. The pellet was rinsed with 100% ethanol, dried at room temperature, and resuspended in 100 μl of distilled H_2O.

Amplification.—Strains of *R. parkeri* were distinguished using the Polymerase Chain Reaction (PCR)

TABLE I

NUMBER OF *R. PARKERI* STRAINS FOUND PER NUMBER TESTED WHERE A SINGLE STRAIN WAS ISOLATED AND TESTED FROM EACH NEEDLE

Site	Tree no.	No. of needles collected	No. of needles tested	No. of strains identified
Old growth (site 1)	1	992	15	15
	2	386	15	15
	3	939	15	15
Clear cut (site 2)	1	702	15	14
	2	490	15	15
	3	895	15	14
BLM progeny test site (site 3)	1–6	Not applicable	23	3
	7–12	Not applicable	24	3
Meadow (site 4)	1	606	15	4

in a fashion similar to the Random Amplified Polymorphic DNA (RAPD) technique described by Williams et al. (**1990**). Four μl aliquots from each DNA sample were placed in separate 50 μl reaction mixtures containing 2 μl of a random decamer primer (Operon, Inc), 0.5 units Taq DNA polymerase (Promega), 2.5 μl buffer (500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100), 2 μl dNTPs, and $CaCl_2$ (final Ca^{++} concentration 4 mM) and cycled 40 times (1.5 min at 94 C, 1 min at 36 C, 2.5 min at 77 C) followed by a 7 min soak at 72 C in a Perkins Elmer Cetus DNA Thermal Cycler. Samples were separated by electrophoresis on 1.2% agarose gels. All samples from within an individual tree were run on the same gel for each primer so no comparison from gel to gel was necessary.

This procedure produced clear and reproducible banding patterns for different genotypes. Several tests using control DNA were conducted prior to running experimental DNA to verify reproducibility of results under slightly different reaction conditions. These tests included: amplification of known concentrations of DNA on different days; amplification of DNA prepared on different days; and amplification of highly different concentrations of DNA (McCutcheon, **1992**). In each of these tests, banding patterns were distinct for each of three control genotypes tested.

Each DNA sample was run with two or three different random decamer primers to confirm results. In all cases isolates which were shown to be identical with one primer were also identical when independently run with a second and third primer. All visible bands were scored and in all cases identical strains had all bands in common. The sequence of the five primers used in this work are as follows: TGATCCCTGG; GTCCACACGG; GGACTGGAGT; GTAGACCCGT; CCTTGACGCA. These primers were numbered 1–5, respectively.

RESULTS

All trees from the Andrews Experimental Forest (sites 1 and 2) possessed a different strain of *R. parkeri* for almost every different needle examined regardless of tree age. This diversity of genotypes was markedly higher than the diversity of site 3 and 4 trees, which possessed only 1–4 strains per tree. No genotype was found to be shared between sites. TABLE I summarizes the number of strains found at each site.

Within the Progeny Test Site (site 3) three distinct genotypes were found in each block and no overlap of genotypes between blocks was detected (midpoints of blocks were about 100 m apart). Each tree within a block possessed from one to three of these strains, regardless of tree family, and there were no significant differences between trees within the block. For these reasons, samples from site 3 were pooled into blocks for the purposes of statistical comparison.

The isolated tree at site 4 also had a low diversity of fungal types. This tree possessed four different genotypes which were homogeneously spread over the three branches tested (FIG. 1).

Nested analyses of variance (Sokal and Rohlf, **1969**) of trees nested within sites run pair-wise between sites 1, 2, and 3 confirmed that there were no significant differences in the number of strains between trees within a site but did reveal significant differences between sites 1 and 3 ($p < 0.001$) and between 2 and 3 ($p < 0.001$). There were no significant differences between sites 1 and 2 ($p > 0.25$). Site 4 contained only one tree and was therefore not compared statistically to any other sites.

Sites 1 and 2 possessed a different strain for nearly every needle tested (FIG. 2). One needle was randomly selected from each tree and cut

FIG. 1. Fifteen *R. parkeri* isolates collected from different needles from site 4; four genotypes, denoted by letters, are identified. End lanes contain λ *Hind* III marker. All samples amplified using primer no. 5.

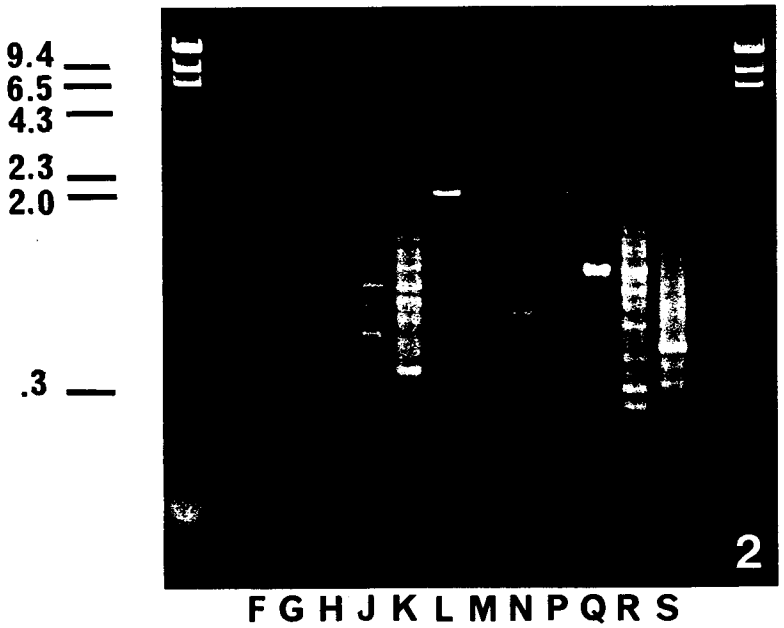

FIG. 2. Twelve isolates from different needles within a single branch of an old growth tree; 12 genotypes, denoted by letters, are identified. End lanes contain λ *Hind* III marker. All samples amplified using primer no. 2.

into nine segments. Single spore isolates obtained from each segment revealed that these trees possess multiple strains within a single needle (FIG. 3, TABLE II). There were no significant differences either within ($p > 0.25$) or between sites 1 and 2 ($p > 0.25$) in the number of strains found per needle.

Five-year-old single spore isolates collected

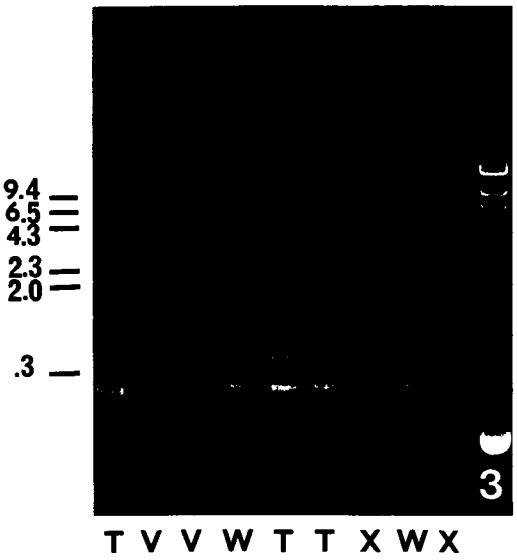

FIG. 3. Nine isolates from within a single needle from a site 2 tree. The isolate from the petiole section is on the left and progress in order down the needle to the tip section on the right. Four different genotypes, denoted by letters, are identified. The right lane contains λ *Hind* III marker. All samples amplified using primer no. 3.

from four of the same trees sampled at site 3 were tested and compared to the six strains found at site 3 during this study. In one case it was shown and confirmed with four different primers that a strain recently collected was identical to a strain collected from the same tree 5 yr earlier, indicating that *R. parkeri* strains are persistent in the field over at least a 5 yr period.

DISCUSSION

These data clearly confirm the hypothesis that genetic diversity of *R. parkeri* is high in large, old trees; they also show that smaller, younger trees can acquire comparable fungal diversity when exposed to inoculum from older trees. Those trees which are isolated from older stands do not acquire a diversity of fungal strains as readily as those trees adjacent to older stands. Young trees located adjacent to old growth trees (site 2) probably contained 10–100 times the number of genotypes found in trees of similar age located in a stand isolated from older trees (site 3).

Comparisons between low and high diversity sites indicate that diversity should be described

TABLE II

THE NUMBER OF DIFFERENT GENOTYPES FOUND PER NINE ISOLATES FROM A SINGLE NEEDLE IN EACH TREE IN SITES 1 AND 2

Site	Tree	No. of strains within a single needle
Old growth (site 1)	1	4
	2	6
	3	7
Clear cut (site 2)	1	6
	2	5
	3	5

at different levels in these areas. In old growth sites, measurements within a single needle revealed as many strains of *R. parkeri* as were found in an entire block of young growth trees at site 3. Within site 3 multiple samples from a few branches within a tree were sufficient to yield an accurate measurement of diversity, while in the old growth site multiple measurements within single needles from many different branches would be required.

To date few investigations have been undertaken to determine diversity of genotypes of an endophyte per unit of foliage. Infection with multiple genotypes of an endophyte within a single leaf has been demonstrated for *Discula umbrinella* (Berk. & Broom) Sutton in beech (Haemmerli et al., 1993). In this work, RAPD markers were used to distinguish as many as four genotypes per leaf.

Data on Douglas fir needle surface area and biomass (Pike et al., **1977**) and counts of *R. parkeri* infections per mm^2 (Stone, **1987**) can be used to approximate the total number of needle infections conservatively at 1×10^{11} infections per old growth tree. Considering the diversity of strains within a single needle revealed here, as many as 1000 different strains may inhabit a single old growth tree. This estimate suggests that *R. parkeri* populations may be among the most diverse ever described for the fungi.

The more general question of how older stands acquire multiple strains probably relates directly to the rate at which *R. parkeri* spreads. Within each block tested at site 3 the trees have readily exchanged genotypes but no overlap of types was observed between blocks. To date this exchange may have been limited by the fact that spores from smaller trees are less likely to be carried

long distances by wind-blown rain. As a stand ages and canopy height increases, exchanges between trees may increase such that diversity within individual trees increases without importation of new strains to the stand as a whole. Although little is known about sexual reproduction in natural *R. parkeri* populations, sexual recombination presumably acts to increase genotypic diversity in all stands.

Carroll has proposed that the main benefit of *R. parkeri* to Douglas fir is shared by the entire population while benefits to any particular individual are minimal (Carroll, **1986**). Evidence presented here which demonstrates the persistence of *R. parkeri* strains within individual trees supports Carroll's ideas. If each strain is capable of perpetuating itself, in the long term trees would be able to accumulate an archive of fungal strains and share that archive with adjacent trees.

There is clear evidence that conifer endophytes, such as *R. parkeri* in Douglas fir (Miller, **1986**), *Phyllosticta* spp. and *Hormonema dematioides* Lagerberg & Melin in balsam fir (Calhoun et al., **1992**), and *Leptostroma* spp. in red spruce (Clark, **1989**) produce metabolites toxic to herbivores. Further reports have demonstrated that fungi may produce mixtures of toxins that act synergistically to increase herbivore mortality (Dowd et al., **1989**). Carroll (**1991**) has suggested that fungal toxins act in long lived plants in a fashion similar to the chemical defenses of short cycle plants. Effective chemical defense in short cycle plants often appears to involve numerous separate compounds which vary in quantity and quality, both in time and space, from one plant to the next (Berenbaum, **1981**, **1985**; Berenbaum and Neal, **1987**) and may act synergistically. It is easy to envision that the highly diverse *R. parkeri* population described here produces a diverse range of toxic metabolites which show synergistic anti-herbivore properties similar to those described in plants.

Some trees in the BLM Progeny Test Site (site 3) have in the past shown high susceptibility to attack from *Contarinia* gall midges. While this susceptibility has been shown to depend, in part, upon the genotype of the host tree (Todd, **1988**), it may also arise as a result of low genotypic diversity of antagonistic endophytes within the stand. Evidence presented here suggests that large clear-cuts that are subsequently planted with nursery trees (e.g., site 3) are effectively isolated from endophyte inoculum, and thus contain an unnaturally low diversity of *R. parkeri* genotypes, a situation which may cause high vulnerability to herbivorous insects.

ACKNOWLEDGMENTS

I would like to thank Bill Bradshaw, Jeff Stone, Kristen McCutcheon, and all other reviewers for their insights. Special thanks to Frank Davis for encouragement when it counted most. This work was supported by a Sigma Xi Scientific Research Society Grant-in-Aid of Research no. 9112.

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Accepted for publication November 30, 1992