Genetic evidence that butternut canker was recently introduced into North America

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Abstract: Butternut (*Juglans cinerea*) is seriously threatened by a canker disease caused by *Sirococcus clavigignenti-juglandacearum*, a fungus with no known sexual stage. This pathogen was first reported in 1967 and is now found throughout the native range of butternut, suggesting that it was introduced into North America. We used randomly amplified polymorphic DNA (RAPD) markers to examine genetic variation in *S. clavigignenti-juglandacearum* over a large portion of the native range of butternut. The 55 RAPD fragments were all found in all 86 isolates of the fungus. This complete monomorphism is consistent with the fungus having been introduced into North America as a single isolate and suggests that if resistant butternut genotypes are found, the pathogen will not likely be able to rapidly evolve genotypes that can attack them.

Key words: *Sirococcus clavigignenti-juglandacearum*, *Juglans cinerea*, RAPD, randomly amplified polymorphic DNA.

Résumé : Le noyer cendré (*Juglans cinerea*) est sérieusement menacé par un chancre causé par le *Sirococcus clavigignenti-juglandacearum*, un champignon n'ayant aucun stade sexuel connu. Ce campignon pathogène a été rappporté pour la première fois en 1967 et on le trouve maintenant sur toute l'étendue de l'aire indigène de distribution de ce noyer, ce qui suggère qu'il a été introduit en Amérique du Nord. Les auteurs ont utilisé des marqueurs polymorphiques aléatoires de l'ADN amplifié (RAPD) pour examiner la variation génétique chez le *S. clavigignenti-juglandacearum* sur une importante proportion de l'aire indigène de distribution du noyer cendré. Les mêmes 55 fragments RAPD ont tous été retrouvés chez les 86 isolats du champignon. Ce monomorphisme total est congruent avec l'hypothèse d'une introduction en Amérique du Nord sous forme d'un seul isolat et suggère que si des génotypes résistants de noyer sont trouvés, le pathogène ne serait pas susceptible de développer rapidement des génotypes qui pourraient l'attaquer.

Mots clés : *Sirococcus clavigignenti-juglandacearum*, *Juglans cinerea*, RAPD, polymorphisme des fragments de l'ADN amplifié.

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Introduction

Butternut (*Juglans cinerea*) is a species of mixed hardwood forests in northeastern North America (Fig. 1). Its wood is highly valued for furniture, paneling, and other specialty products and its nuts are an important food source for wildlife (Rink 1990). Butternut canker is a lethal disease of butternut that is caused by the fungal pathogen *Sirococcus clavigignenti-juglandacearum* Nair, Kostichka & Kuntz (Nair et al. 1979). It kills trees of all ages and has been found throughout the range of butternut in North America (Ostry 1997). In some states up to 84% of the butternut trees have been killed (Ostry 1997) and over 90% are infected (Cummings-Carlson 1993). As a result, butternut was listed under Category 2 of the Endangered and Threatened Plants list of the U.S. Endangered Species Act of 1973, meaning that there was evidence of vulnerability but not enough data to support full listing (Ostry 1997). This category has since been discontinued and butternut and other species formerly listed in this category can be considered "species at risk."

Three observations point to butternut canker being a disease recently introduced to North America. First, the disease was not reported in North America until 1967 (Wisconsin Conservation Department 1967), despite decades of forest disease surveys in these areas, and in the relatively short period since that time, it has been found throughout the native range of butternut (Ostry 1997). Second, putative resistance to the pathogen is very rare in butternut (Ostry 1997), suggesting that the host and pathogen had not coevolved for a very long period of time prior to the 1960s. Finally, in artificial inoculation studies of various *Juglans* species, Japanese walnut (*J. ailantifolia*) and heartnut (*J. ailantifolia* var. *cordiformis*), both Asian species, showed the highest levels of resistance (Ostry 1997). While the origin of this pathogen

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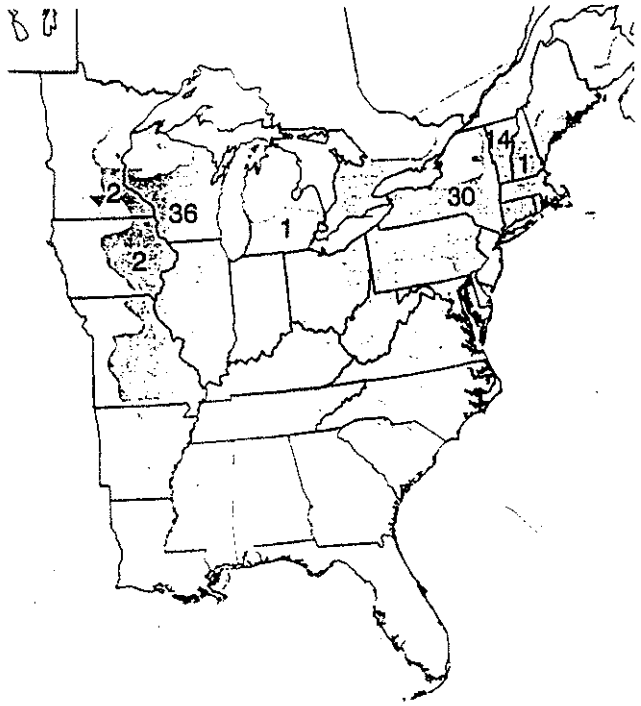
Fig. 1. Map of eastern North America showing the natural range of butternut (*Juglans cinerea*) shaded in gray. *Sirococcus clavigignenti-juglandacearum* is found throughout that range. Numbers represent the number of isolates assayed from each state.



is not known, the latter piece of evidence suggests it may have originated in Asia.

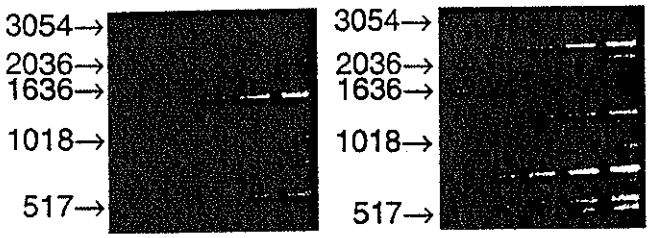
The sexual stage of the fungus is unknown (Nair et al. 1979), so if the fungus was introduced to North America relatively recently as a single isolate or few isolates, one would expect to find relatively little genetic variation throughout its range. The objective of this study was to examine the level of genetic variation among *S. clavigignenti-juglandacearum* isolates collected from butternut in a large portion of its current range in North America.

Materials and methods

A total of 86 isolates of *S. clavigignenti-juglandacearum* were collected in North America (Fig. 1). Butternut branches with 1- to 2-year-old cankers were either collected by us or sent via express mail to our laboratory by our cooperators. The bark was aseptically removed from each canker and 5 mm square pieces of wood were removed from the canker margins. These pieces were soaked in a 0.525% solution of hypochlorite for 1 min, rinsed twice in sterile distilled water, placed onto potato dextrose agar in Petri dishes, and incubated at 20°C in the light. After the fungus grew out from the pieces of wood, subcultures were transferred to fresh plates of potato dextrose agar and again incubated at 20°C in the light. The cultures were then placed into a liquid medium (Griffin et al. 1980) and grow in stationary culture. The mycelia were then collected for DNA extraction.

DNA was extracted (Goodwin and Lee 1993), dissolved in 50 µL of TE (10 mmol/L Trizma base, 1 mmol/L EDTA, pH 8.0), and then cleaned with the Wizard DNA cleanup system (Promega, Madison, Wis.). Prior to polymerase chain reactions (PCR), DNA

Fig. 2. RAPD fragments from isolates of *Sirococcus clavigignenti-juglandacearum*. The left and right photos show fragments generated by primers OPF-01 and OPF-13, respectively. The first lane of each photo is a molecular size marker, with sizes listed in DNA base pairs. The subsequent lanes in each photo represent adjacent pairs of repeated reactions for two isolates.



was diluted to 1 ng/µL in H_2O. PCRs were conducted in a 15 µL volume with final concentrations of 1× Stoffel fragment buffer (Perkin-Elmer, Norwalk, Conn.), 0.25 mmol/L of each dNTP (Promega, Madison, Wis.), 1 µg/µL non-acetylated bovine serum albumin (New England Biolabs, Beverly, Mass.), 4.5 mmol/L $MgCl_2$ (Perkin-Elmer, Norwalk, Conn.), 1.2 µmol/L decamer primer (Operon Technologies, Alameda, Calif.), 1.67 units/µL Stoffel fragment of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.), and 0.33 ng/µL *S. clavigignenti-juglandacearum* DNA. The decamer primers used were OPF-01 (5′-ACGGATCCTG-3′), OPF-03 (5′-CCTGATCACC-3′), OPF-07 (5′-CCGATATCCC-3′), OPF-08 (5′-GGGATATCGG-3′), OPF-09 (5′-CCAAGCTTCC-3′), OPF-11 (5′-TTGGTACCCC-3′), OPF-13 (5′-GGCTGCAGAA-3′), OPF-14 (5′-TGCTGCAGGT-3′), OPF-16 (5′-GGAGTACTGG-3′), and OPF-18 (5′-TTCCCGGGTT-3′). PCRs were conducted in 96-well plates (Hybaid, Ltd., Teddington, Middlesex, U.K.) and each well was overlaid with 25 µL mineral oil. The thermal cycler (Hybaid OmniGene) profile was one cycle of 1.5 min at 94.5°C, followed by 45 cycles of 1 min at 94.5°C, 2 min at 36°C, and 2 min at 72°C, followed by one cycle of 7 min at 72°C. Ramp times between temperatures were the maximum possible, except for 4 s/°C between 36 and 72°C. After amplification, 3 µL of 6× loading buffer was added to each well and samples were subjected to electrophoresis in 1.4% agarose gels in TBE (89 mmol/L Trizma base, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.0) buffer at 140 V (4.7 V/cm) until the bromophenol blue dye front had migrated 10 cm. Gels were stained with ethidium bromide and fragments visualized under ultraviolet light. All isolates were amplified and assayed twice to check for repeatability.

Results and discussion

The 10 primers used generated 55 repeatable amplified DNA fragments (OPF-01 gave fragments of sizes 474, 605, 761, 838, 1071, 1115, 1486, 1789, 1906, and 2206 base pairs (bp); OPF-03 gave fragments of sizes 339, 592, 839, 1052, 1793, and 3474 bp; OPF-07 gave fragments of sizes 705, 890, 1123, and 1424 bp; OPF-08 gave fragments of sizes 252, 331, 370, 803, 1234, and 1298 bp; OPF-09 gave a fragment of size 798 bp; OPF-11 gave fragments of sizes 1159 and 1269 bp; OPF-13 gave fragments of sizes 495, 550, 737, 926, 1238, 1859, 2056, and 2302 bp; OPF-14 gave fragments of sizes 400, 695, 1225, and 1601 bp; OPF-16 gave fragments of sizes 438, 516, 741, 858, 1312, 1685, 2055, and 2309 bp; OPF-18 gave fragments of sizes 349, 627, 1204, 1495, 1691, and 1972 bp; Fig. 2). All of these fragments were present in every individual.

The sexual stage of *S. clavigignenti-juglandacearum* is unknown, but the fact that its conidia are produced in pycnidia strongly suggest that it is an ascomycete (Nair et al. 1979). The vast majority of ascomycetes spend most of their life cycle as haploid cells. If we assume that the isolates we sampled are haploid, the probability of observing this completely monomorphic result is very low. For example, even if there were a low level of variation at these loci, such as five of the loci having alternate alleles present at a frequency of 0.01, the probability of obtaining our results would only be 0.013. Thus, this extreme monomorphism is very strong evidence that all the geographically dispersed isolates we assayed are genetically identical clonal derivatives of one original isolate.

The observed monomorphism fits well with the hypothesis that the presence of *S. clavigignenti-juglandacearum* in North America is the result of a single introduction event. While there may be variation among the sampled isolates at some other loci, the absence of variation in this survey suggests that little genetic variation has been generated since the putative introduction. This is not surprising given that the sexual stage of the fungus is unknown (Nair et al. 1979), suggesting that sexual reproduction and recombination are rare or absent.

Similar results have been found in assays of molecular variation in other forest tree pathogens in North America. While Wang et al. (1997) found high levels of RAPD variation in a single population of *Gremmeniella abietina* in northern Sweden, Hamelin et al. (1998) found very low levels of RAPD variation in a survey of North American populations of the European race of this pathogen. Studies of North American isolates of *Discula destructiva* using RAPDs (Trigiano et al. 1995) and arbitrary signatures from amplification profiles (Caetano-Anolles et al. 1996) showed low levels of variation and suggest separate, recent introductions of this pathogen into eastern and western North America.

These genetic data from *S. clavigignenti-juglandacearum* are of practical value in the design of control strategies. There is an active effort to identify and test resistant butternut germplasm (Ostry 1997). The lack of genetic variation that we observed suggests that one does not need to use a broad geographic base of inoculum when screening butternut germplasm for resistance to *S. clavigignenti-juglandacearum*. Those materials that display resistance to a single isolate in field tests should be resistant to *S. clavigignenti-juglandacearum* throughout North America. Furthermore, once resistance is found, there is a relatively low probability that a fungal isolate will arise that is capable of overcoming the host resistance.

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References

Caetano-Anolles, G., Trigiano, R.N., and Windham, M.T. 1996. Sequence signatures from DNA amplification fingerprints reveal fine population structure of the dogwood pathogen *Discula destructiva*. FEMS Microbiol. Lett. **145**: 377–383.

Cummings-Carlson, J. 1993. Butternut: are there any healthy trees left? Woodland Management, Spring 1993, pp. 11–12.

Goodwin, D.C., and Lee, S.B. 1993. Microwave miniprep of total genomic DNA from fungi, plants, protists and animals for PCR. Biotechniques, **15**: 441–444.

Griffin, D.H., Ehrenshaft, M., and Manion, M. 1980. Host-selective toxins of *Hypoxylon mammatum* for *Populus tremuloides*. Proceedings of the Joint Symposium on Resistance Mechanisms in Poplar Diseases, 1–5 September 1980, Kornik, Poland, pp. 209–214.

Hamelin, R.C., Lecours, N., and Laflamme, G. 1998. Molecular evidence of distinct introductions of the European race of *Gremmeniella abietina* into North America. Phytopathology, **88**: 582–588.

Nair, V.M.G., Kostichka, C.J., and Kuntz, J.E. 1979. *Sirococcus clavigignenti-juglandacearum*: an undescribed species causing canker on butternut. Mycologia, **71**: 641–646.

Ostry, M.E. 1997. Butternut canker: history, biology, impact, and resistance. *In* Knowledge for the Future of Black Walnut. Proceedings of the 5th Black Walnut Symposium, 28–31 July 1996, Springfield, Mo. *Edited by* J.W. Van Sambeek. U.S. Department of Agriculture, Forest Service, North Central Forest Experiment Station, Gen. Tech. Rep. NC-191, pp. 192–199.

Rink, G. 1990. *Juglans cinerea* L., butternut. *In* Silvics of North America, Vol. 2, Hardwoods. *Edited by* R.M. Burns and B.H. Honkala. U.S. Department of Agriculture, Forest Service, Agric. Handb. No. 654.

Trigiano, R.N., Caetano-Anolles, G., Bassam, B.J., and Windham, M.T. 1995. DNA amplification fingerprinting provides evidence that *Discula destructiva*, the cause of dogwood anthracnose in North America, is an introduced pathogen. Mycologia, **87**: 490–500.

Wang, X.-R., Ennos, R.A., Szmidt, A.E., and Hansson, P. 1997. Genetic variability in the canker pathogen fungus, *Gremmeniella abietina*. 2. Fine-scale investigation of the population genetic structure. Can. J. Bot. **75**: 1460–1469.

Wisconsin Conservation Department. 1967. Annual report on forest pest conditions in Wisconsin. Wisconsin Conservation Department, Forest Management Division.