Thirty Polymorphic Nuclear Microsatellite Loci From Black Walnut

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Black walnut (Juglans nigra L.) is a large tree, native to the eastern United States, that is prized for its high-quality timber and edible nut. Thirty (GA/CT)n nuclear microsatellite markers were identified from black walnut for use in population genetic studies, genome mapping, DNA genotyping of important clones, studies of gene flow, and tree breeding. The markers were polymorphic based on a diversity panel of 10 black walnut individuals from eight Midwestern U.S. states.

Black walnut (Juglans nigra L.) is a large tree that is native throughout the eastern United States from New England to Texas (Fowells 1965). Black walnut is prized as a multipurpose species: it provides valuable timber, produces a high-quality edible nut, and is attractive to wildlife (USDA Forest Service Fire Effects Information System website). More than 15 million acres of timberland in 30 states contain black walnut (Schmidt and Kingsley 1997). The vast majority of this species exists in natural stands, with a small percentage grown in plantations. An estimated 15 million cubic feet of black walnut are harvested annually in the United States (USDA Forest Service Forest Inventory and Analysis website). In 1997, 29 million pounds of in-shell black walnut nuts were purchased for processing and about 2 million pounds of nutmeat were sold (Hammonds 1998). Nearly all processed nuts came from uncultivated trees growing in wild populations (Reid 1990).

There have been successful genetic improvement efforts in black walnut for both timber and nut traits (Beineke 1989; Funk 1979; Tourjee 1998). There is now a need for DNA-based genetic markers to investigate critical problems in black walnut breeding and conservation. For example, breeders need a method for genotype typifying important cultivars to verify their identity (Bish C, personal communication). Efforts to understand the wild black walnut germplasm have been largely limited to provenance tests (Brennan et al. 1992; Rink 1997). Provenance tests, and the associated morphological and phenological data, have provided important tools for breeders and foresters where they are available, well designed, and well maintained (Guries et al. 1981), but provenance tests of black walnut are expensive and time consuming because the species has a long juvenility and mature trees are large. Genetic markers such as restriction fragment length polymorphisms (RFLPs; Fjellstrom and Parfitt 1994; Fjellstrom et al. 1994), random amplified polymorphic DNA (RAPDs; Woeste et al. 1996), internal transcribed spacer (ITS) sequence polymorphisms (Potter D, personal communication), and allozymes (Arulsekar et al. 1985) have been identified for several members of the Juglandaceae, and they permit a rapid parsing of the genetic differentiation within J. nigra. While these markers are more informative than phenotypes in terms of their ability to identify species substructure and diversity, microsatellite DNA markers [simple sequence length polymorphisms (SSLPs)] can provide greater levels of resolution in a cost-effective manner. Microsatellites overcome some of the limitations of other marker systems (Goldstein and Pollock 1997), and a large number of methods for statistical analysis of microsatellite data are available (Luikart and England 1999).

We intend to use the microsatellites published here as part of a larger effort to understand the genetics of black walnut. While the commercial value of this species both for nuts and timber is based almost entirely on exploitation of the wild resource, there are large gaps in our understanding of the genetic structure of wild populations of black walnut, and there is no published information on the effects of timber and nut harvests on the long-term health of the species. In addition, the markers will be used for genetic mapping, DNA genotyping (fingerprinting) of important clones, and studies of gene flow in seed orchards as part of a breeding effort.

Materials and Methods

DNA was isolated from the leaves of three black walnut selections using Nucleon Phytopure DNA extraction columns (Amersham, Buckinghamshire, UK). The trees were part of a walnut breeding and genetics program in the Department of Forestry and Natural Resources at Purdue University. A pooled DNA sample from these trees was used by Genetic Identification Services (San Diego, CA) to create an enriched (GA/CT)n microsatellite library. The library was plated on a selective medium, and 1500 colonies were robotically picked (Genetix, Hampshire, UK) into 96-well plates, miniprepped (Qiagen REAL 96 Prep, Valencia, CA), and sequenced using an ABI 3700 (Perkin-Elmer, Foster City, CA). We analyzed the resulting sequences using Sequencher software (version 3.1.1; Gene Codes, Ann Arbor, MI) and discarded candidate sequences if they contained no discernible microsatellite repeat, or if there was insufficient flanking sequence to construct suitable polymerase chain reaction (PCR) primers. The sequences that remained were assigned to contigs whenever possible. When sequence contigs were available, we derived a consensus sequence for the regions flanking the microsatellites and used it for primer design. Primers (18–20 bp) for amplification of microsatellite-containing sequences (100–400 bp) were designed using Primer 0.5 (Whitehead Institute for Biomedical Research, Cambridge, MA).

To develop a preliminary screening panel, DNA from 10 J. nigra individuals representing populations in eight Midwestern U.S. states was isolated from mature leaves using an automated nucleic acid extractor (Autogen, Framingham, MA) and a CTAB extraction buffer modified with 2× PVP and 2× CTAB. PCR amplification of primer pairs was performed with an MJ Research thermal cycler (Waltham, MA) using 20 μl reactions. The PCR reaction mixture contained 20 ng of DNA template, 1.5 mM MgCl2, 0.4 U AmpliTaq Gold (Perkin-Elmer), and 0.8 μM (each) primer. All other components of the PCR mixture were as recommended by the manufacturer (Perkin-Elmer). PCR amplification was for 50 cycles of 92°C for 30 s, 45°C for 1 min, and 72°C for 1 min. All primers were annealed at 45°C. The reaction products were then held at 0°C until aliquots could be loaded into 1.5% Trevigels (Trevigen, Gaithersburg, MD) containing ethidium bromide. Electrophoresis was in 1× TAE buffer, and gels were photographed using...
were combined in a separate tube, denatured (Promega, Fitchburg Center, WI) for 200 s, 51°C (BMA, Rockland, ME) at 3000 V, 60 mA, Ranger (polyacrylamide) denaturing gels (Promega, Fitchburg Center, WI). Electrophoresis was in 6% Long Quick-Comb 96-well comb (Sigma, St. Louis, MO). Sequences from unique microsatellite-containing sequences were generated from an enriched (GA/CT)n library, yielding 450 sequences. From the screening population, to confirm that microsatellites were polymorphic, PCR was performed with ABI fluorescent dCTP according to manufacturer’s instructions (Perkin Elmer), and 1 μl of the PCR product and 2 μl of CXR 350 bp Ladder Standard (Promega, Fitchburg Center, WI) were combined in a separate tube, denatured for 2 min at 95°C, and loaded onto a Quick-Comb 96-well comb (Sigma, St. Louis, MO). Electrophoresis was in 6% Long Ranger (polyacrylamide) denaturing gels (BMA, Rockland, ME) at 3000 V, 60 mA, 200 W, 51°C for 2.5 h using an ABI 377 (Perkin Elmer) with 36 cm plates and 0.2 mm spacers.

Results and Discussion

Our initial screening of 1500 colonies from an enriched (GA/CT)n library yielded 450 unique microsatellite-containing sequences from *J. nigra*. The remaining colonies produced sequences containing no discernible microsatellite repeats or insufficient flanking DNA to construct PCR primers. Of the 450 positive sequences, 141 (30%) were grouped into contigs of two or more sequences (presumably derived from the same locus). Because our microsatellite-containing sequences were derived from the pooled DNA of three *J. nigra* individuals, 67% (95/141) of these contigs contained sequences that differed in the number of (GA/CT)n repeats. This indicates that the three arbitrarily chosen *J. nigra* individuals that we used to construct our microsatellite library were polymorphic at about two-thirds of the microsatellite loci we identified.

Thirty of the microsatellites showed clear polymorphism in the screening population (Table 1). Alleles ranged from 150 to 242 bp, a range that should facilitate multiplexing of samples. About 66% of the microsatellite sequences contained perfect (GA/CT)n repeats. The average number of (GA/CT)n repeats was 18.2, and the range was between 8 and 30 repeats. The remaining 34% of the microsatellites contained repeats that were interrupted, and five microsatellites contained repeats other than (GA/CT)n, including WGA33, which contained the tetranucleotide repeat (GAGT)n.

An average of 8.6 individuals was analyzed at each locus, and we observed an average of more than seven alleles per locus. This high level of polymorphism may have been the result of how the microsatellite library was developed and screened. Sequence contigs polymorphic among the three individuals that were used to make the library were selected for analysis first. We will not know if the allelic richness found in these 30 microsatellites is characteristic of all walnut microsatellite loci in the species until more of the library has been screened with larger populations. By comparison, Fjellstrom and Parfitt (1994) found an average of 1.47 alleles per RFLP locus in five *J. nigra* populations containing 11 individuals each.

The black walnut microsatellite loci we describe here may be useful for population-level studies, genetic mapping, plant breeding, and cultivar identification. The ordering of these microsatellites into a genetic map will be especially useful for development of cultivars in the black walnut.
Inheritance and Linkage of Allozymes in a Balkan Endemic, Pinus peuce Griseb.

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This article presents a study of isozyme variation in Pinus peuce Griseb., a Balkan endemic. Among the enzyme systems studied, five were monomorphic and eight were polymorphic in at least one locus. The segregation analysis of the polymorphic loci was consistent with a Mendelian mode of inheritance. No significant deviation from the expected ratio was observed both at the individual and pooled segregation data levels. Segregation patterns were homogeneous across individuals. Two significant linkage groups were found in P. peuce: FEST-2: LAF-2 and 6PG-1: 6PG-2, which correspond to the results obtained for other pine species.

Pines (Pinus spp.) are among the most extensively studied forest tree species, due to their widespread distribution in temperate and subtropical zones and their economic importance as timber-producing species (Critchfield and Little 1966; Mirov 1967; Vidakovic 1991). Genetic studies on the genus encompass a wide range of approaches and methods, ranging from provenance and progeny testing to the application of biochemical markers, such as terpenes (Hanower 1992), polyphenols (Lebreton 1995), isozymes, DNA, etc. (Müller-Starck et al. 1992). There are some species, however, which have been studied to a lesser extent, mainly due to their relatively limited distribution. Among these species is the Balkan endemic, Pinus peuce Griseb.

Pinus peuce, referred to as Macedonian pine or Rumelian pine, grows in the mountains of the Balkan peninsula at altitudes of between roughly 800 and 2300 m (Vidakovic 1991). This species possesses numerous remarkable characteristics, such as ecological tolerance and frost resistance, that make it very valuable for forest tree breeding (Mitchell 1996). Because of its limited area of natural distribution, this species requires special attention and implementation of measures for its conservation.

To date, the genetic studies on this species have been limited to monoterpene composition and provenance/progeny performance (Dobrev 1992, 1998). No isozyme study on the species currently exists, with two insignificant exceptions, dealing with single individuals in a botanical garden and bulk seed lot (Bergmann and Gillet 1997; Shukhal et al. 1992). The objective of the present study is to investigate the genetic control of isozyme variants and possible linkage relationships among loci. This may produce a better understanding of the genetics of this species and could be useful for future studies based on allozyme markers.

Materials and Methods

Material was collected from 42 individuals. Eighteen originated from a natural stand situated in the Vitosha Mountains (42°27′N, 23°12′E) at approximately 1900 m altitude, while the remaining 24 trees were clones originating from a seed orchard in the Yun- dola Experimental Forest (Rhodopes) at 1600 m altitude (42°04′N, 23°51′E). Cones were harvested in October 1997 in the seed orchard and in September 1998 in the natural stand. Seeds were extracted from the cones of each individual and kept separately at 2–4°C before the analysis.

Seeds were soaked on moist filter paper for several days before the analysis. Since the seeds of this species are characterized by long-term dormancy, no germination