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J.B. Mitton · B.R. Kreiser · G.E. Rehfeldt

Primers designed to amplify a mitochondrial *nad1* intron in ponderosa pine, *Pinus ponderosa*, limber pine, *P. flexilis*, and Scots pine, *P. sylvestris*

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Abstract The b/c intron of the mitochondrial *nad1* gene, was sequenced to characterize the indel region of ponderosa pine, *Pinus ponderosa*. The sequence in ponderosa pine was aligned with the sequence in Scots pine, *Pinus sylvestris*, to design seven primers that are useful for sequencing and for revealing size variation in amplified fragments in ponderosa pine, Scots pine, and limber pine, *Pinus flexilis*. These primers reveal variability in all three species, and the pattern of variability within ponderosa pine is described by a preliminary survey. The indel region of ponderosa pine contains three distinct elements with lengths of 31, 32, and 34 bp.

Keywords mtDNA · Polymorphism · *Pinus ponderosa* · *Pinus flexilis* · *Pinus sylvestris*

Introduction

The organellar genomes of many conifers are ideally suited for studies of gene flow and population structure, fas they have contrasting patterns of inheritance. For the majority of species within the genus *Pinus*, the mitochondrial DNA (mtDNA) shows maternal inheritance, and the chloroplast DNA (cpDNA) is paternally inherited (Neale et al. 1986, 1989; Wagner et al. 1987; Neale and Sederoff 1989; Strauss et al. 1989, 1993; Wagner 1992; Strauss et al. 1993; Dong and Wagner 1993, 1994; Hong et al. 1993). Exceptions to the general pattern of maternal inheritance of mtDNA have been reported in *Pinus monticola* and *Pinus banksiana* (Bruns and Owens 1989; Wagner et al. 1991). Because the pollen of pines is dispersed by wind, cpDNA has the potential for high levels of gene flow. In contrast, the mtDNA of pines has a low potential for dispersal, for seeds are typically blown by the wind for less than 100 m. Pines with seeds dispersed by birds have a greater potential for mtDNA gene flow (Tomback and Linhart 1990), but even in these species, the potential for gene flow of mtDNA is much lower than for cpDNA.

The disparate potentials for gene flow in mtDNA and cpDNA will produce contrasting patterns of population structure. For example, within a population of ponderosa pine, mtDNA is spatially structured, revealing open-pollinated maternal families, while the spatial structure of cpDNA is homogenized by the dispersal of pollen (Latta et al. 1998). Within the secondary contact zone between *Pinus ponderosa ponderosa* and *Pinus ponderosa scopulorum*, mtDNA variation reveals a sharp cline, while the cline of cpDNA is attenuated by greater gene flow (Latta and Mitton 1999). Finally, allozymes and cpDNA reveal little population structure in limber pine, *Pinus flexilis* (Latta et al. 1997), but mtDNA reveals that large geographic areas are marked by diagnostic mtDNA haplotypes (Mitton et al. 2000).

Currently, the paucity of polymorphic markers in plant mtDNA restricts the study of the geographic variation of this component of the genome (Schaal et al. 1998). To better understand the variation among mitotypes in ponderosa pine and limber pine, and in the hope of identifying sequence variation that would support phylogeographies, we have sequenced the b/c intron of *nad1*. Size variation in this intron has been used to describe the population structure in both ponderosa pine and limber pine (Latta and Mitton 1997, 1999; Latta et al. 1998; Mitton et al. 2000). These new primers make size variation more apparent, so that surveys can be conducted with agarose gels, and can be used to sequence this region of approximately 2000 bp.

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J.B. Mitton (✉) · B.R. Kreiser
Department of Environmental,
Population, and Organismic Biology,
University of Colorado, Boulder, CO, USA 80309

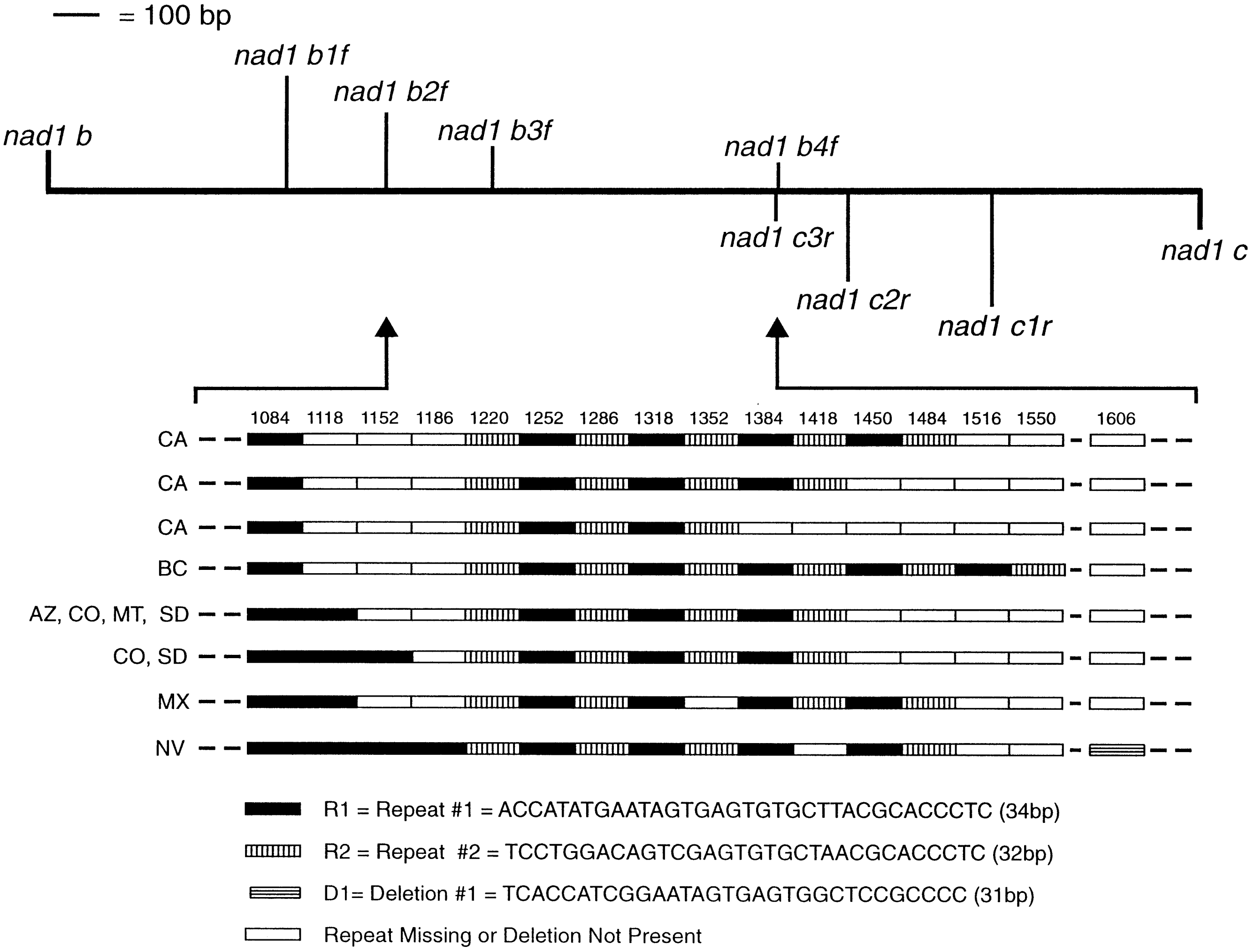
G.E. Rehfeldt
USDA Forest Service,
Intermountain Research Station,
121 South Main Street, Moscow, ID, USA 83843

Materials and methods

Total genomic DNA was extracted from needle tissue with the DNAeasy Plant Mini Kit (QIAGEN Inc.; Santa Clarita, Calif.). Amplifications were conducted in a total volume of 25 µl using: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, 1.25–3.00 mM $MgCl_2$, 200 µM of dNTPs, 1 unit of *Taq* polymerase, 0.3 µM of each primer, approximately 100 ng of template DNA and water to the final volume. PCR-cycling conditions consisted of an initial denaturing step of 94°C for 1 min followed by 30 cycles of 45 s at 95°C, 45 s at 55° to 59.5°C and 2 min at 72°C. A final elongation step of 7 min at 72°C ended the cycle. The PCR product was cleaned using the QIAquick PCR purification kit (QIAGEN Inc.; Santa Clarita, Calif.). Both strands of the fragment were cycle-sequenced with the original primers using ABI Big Dye terminator chemistry and visualized on an ABI Prism model 377 automated DNA sequencer (MCDB Sequencing Facility; University of Colorado). The *nad1b2f-nad1c3r* region was sequenced using the methods described above, but the template for the reaction was the fragment amplified with the *nad1b1f* and *nad1c1r* primers.

Results

We began with the *nad1b* and *nad1c* primers (Demesure et al. 1995), which are anchored in the *b* and *c* exons. In ponderosa pine, these primers amplify a fragment of approximately 2000 bp. We sequenced inward with both of these primers, and designed an internal set of primers (*nad1b1f*, *nad1c1r*) from sequences that were identical in ponderosa pine and *Pinus sylvestris* (GenBank accession # AJ223312). We repeated the process to design a second pair of internal primers, *nad1b2f* and *nad1c3r*. The sequence for the intron in *P. ponderosa* has been deposited in GenBank (accession # AF231325). Additional primers (*nad1c2r*, *nad1b3f*, *nad1b4f*) were designed to amplify fragments of appropriate size for sequencing, or to detect size differences among amplified fragments. The sequences of these primers are listed in Table 1 and their positions in the intron are presented in Fig. 1.



▬ R1 = Repeat #1 = ACCATATGAATAGTGAGTGTGCTTACGCACCCTC (34bp)

▥ R2 = Repeat #2 = TCCTGGACAGTCGAGTGTGCTAACGCACCCTC (32bp)

▤ D1= Deletion #1 = TCACCATCGGAATAGTGAGTGGCTCCGCCCC (31bp)

▭ Repeat Missing or Deletion Not Present

Fig. 1 The positions of nested primers within and beside the intron between exons b and c in the mitochondrial Nad1 of ponderosa pine and Scots pine, and the diversity of haplotypes found in a preliminary survey of ponderosa pine. *Numbers* above the repeat blocks correspond to the 5′ position of the particular repeat block in the consensus strand of the ponderosa pine sequences (the alignment is available from the authors upon request). Sequences are identified by the geographic region in which they were observed. Abbreviations are as follows: *AZ* = Arizona, *BC* = British Columbia, *CA* = California, *CO* = Colorado, *MT* = Montana, *MX* = Mexico, *NV* = Nevada and *SD* = South Dakota

Table 1 Nested primers for the intron between exons b and c of the mitochondrial NAD1 in ponderosa pine, *P. ponderosa*, limber pine, *P. flexilis*, and Scots pine, *P. sylvestris*

Primer (f)	Sequence	Primer (r)	Sequence
NAD1B[a]	5′ - GCATTACGATCTGCAGCTCA - 3′	NAD1C[a]	5′ – GGAGCTCGATTAGTTTCTGCC - 3′
NAD1B1f	5′ – ATGCCGCCCGTTTCCATTTC – 3′	NAD1C1r	5′ – TGCTGCAAAAGGGTTAGGGGG – 3′
NAD1B 2f	5′ – CGAGGGGTAGGTATCGGTCCGA – 3′	NAD1C2r	5′ – GCATGCTTACTCACCCTCTCCCG – 3′
NAD1B 3f	5′ – CTTTTTGGTTTGCTTATTGGGTGGGGGG – 3′	NAD1C3r	5′ – TTTTAAGTGACTCGCCCGACC – 3′
NAD1B 4f	5′ – CGGGCGAGTCACTTAAAAGTCAC – 3′		

[a] From Demesure et al. (1995)

The indel region of ponderosa pine is between the primers *nad1b2f* and *nad1c3r*, which produces fragments ranging in size from 751 to 922 bp. The diversity of genotypes revealed in our preliminary survey demonstrates the utility of these primers for revealing genetic variability. Although sequence variation is rare (three nucleotide substitutions), variation in repeated elements is common. The various haplotypes are represented schematically (Fig. 1), with R1 and R2 representing repeats of 34 and 32 bp, respectively, and D1 representing a 31-bp fragment. In these preliminary data, all trees sampled in California and British Columbia are distinguished by deletion of the R1 repeat at base 1118.

In limber pine, the *nad1b2f* and *nad1c3r* primers produce a fragment of approximately 300 bp, and this region does not contain the indel region detected as RFLPs in surveys of the population structure of limber pine (Latta and Mitton 1997; Mitton et al. 2000). Instead, the indel region is between the primers *nad1b4f* and *nad1c1r*, which amplify fragments of 500 to 700 bp, depending on the mitotype. We have not yet explored the structure of this region through sequencing.

Discussion

These primers work in *P. ponderosa*, *P. flexilis* and *P. sylvestris*, and some of them work in *Pinus edulis*, *Picea engelmanii* and *Pinus glauca*. Three of the primers (*nad1b1f*, *nad1b2f*, and *nad1c2r*) align perfectly with the sequences in *Picea abies* (Parducci and Szmidt 1999; GenBank Accession #AF142641–2).

The *nad1 b/c* intron should serve as a useful molecular marker in population genetic surveys, for it has size variants that reveal population structure across the range of both *P. ponderosa* and *P. flexilis*. Previously, size variation in the *nad1b/c* intron was detected by amplifying the entire intron, cutting the fragment with *RsaI*, sorting the fragments on a polyacrylamide sequencing gel, and revealing fragments by silver staining (Latta and Mitton 1997, 1999; Latta et al. 1998; Mitton et al. 2000). The new primers amplify smaller fragments, which allow the size variants to be distinguished on agarose gels.

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