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USE OF DIAGNOSTIC SSR MARKERS FOR IDENTIFICATION OF *LARIX LYALLII* AND *L. OCCIDENTALIS* (*PINACEAE*)

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Two species-specific microsatellite or simple sequence repeat (SSR) markers (*UAKLly10a* and *UAKLla1*) were used to distinguish *Larix lyallii* Parl. (subalpine larch) from *L. occidentalis* Nutt. (western larch) (*Pinaceae*). These markers can easily be used for rapid identification of the two species at any stage in the sporophyte phase of the life cycle. In the future, they should prove to be an invaluable tool for determining the possible occurrence and magnitude of introgressive hybridization in overlap zones between the two species. They are also expected to be useful in validating controlled crosses between *L. lyallii* and *L. occidentalis* and for certification purposes in reforestation and tree improvement programmes aimed at producing genetically improved hybrid stock.

Keywords. Hybridization, *Larix*, microsatellites, PCR, simple sequence repeats.

Introduction

The genus *Larix* Tourn. ex. Adans. comprises 10 commonly recognized species distributed around the Northern Hemisphere, from eastern Siberia westward across Eurasia (except Scandinavia), resuming in eastern North America and westward to Alaska. In many areas they are of considerable economic and ecological importance (Arno, 1990; Gower & Richards, 1990). This has recently been emphasized in a comprehensive examination of *Larix* species of the world (Schmidt & McDonald, 1995). LePage & Basinger (1995) have proposed a phylogeny for *Larix* and divided the genus into two morphologically distinct groups: those with bracts of the female cone not exserted and those with bracts exserted. Another phylogeny based on allozyme markers showed a clear separation between Eurasian and American *Larix* species (Semerikov & Lascoux, 1999). *Larix lyallii* Parl. (subalpine larch) and *L. occidentalis* Nutt. (western larch), having exserted bracts, belong to section *Multiseriales* Patschke (Parker, 1993). They are considered closely related as revealed

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by the restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA (cpDNA; Qian *et al.*, 1995) and have been reported to form natural hybrids where their ranges overlap in the USA (Carlson *et al.*, 1990; Carlson & Theroux, 1993). They could thus be considered as sister species or even subspecies.

In Western Canada and the USA, *L. lyallii* and *L. occidentalis* play an important ecological and economic role. *Larix occidentalis* thrives at lower elevations from 760 to 2300m while *L. lyallii* is best adapted to higher, more climatically rigorous habitats above 2600m. The two species rarely occur sympatrically because they are usually separated by an isolation zone of at least 300m in elevation (Carlson, 1995). This intervening zone is usually occupied by a dense forest of *Abies lasiocarpa* (Hook.) Nutt. (alpine fir) and *Pinus contorta* var. *latifolia* Engelm. (lodgepole pine). Hybridization may occur however, in the only known overlapping populations in two areas of Montana, USA: the Bitterroot Range southwest of Missoula and the Cabinet Range in the northwest of the state (Carlson, 1995). It is reasonably easy to identify mature hybrid *Larix* in the field by observed combinations of the parental characters and an intermediate degree of twig pubescence. Seeds, however, are less useful for hybrid recognition. The high degree of morphological variation reported for the hybrids may be the result of extensive backcrossing and introgression over time, resulting in a heterogeneous population. Many of the morphological characters (e.g. pubescence of current-year twigs, colour and texture of bark of three-year-old branchlets, colour of summer foliage) that distinguish *L. lyallii* from *L. occidentalis* are either found only in mature trees or are not observable on herbarium specimens.

In 2000 about 5.2 million *L. occidentalis* seedlings were raised in British Columbia, of which half were from seed nurseries. In the USA, planting numbers were about 1 million. At present there is virtually no planting of *L. lyallii* in Canada or the USA, other than in small research and land-reclamation plantings. The main reasons for this are slow growth rates and difficulties with seed collection. Because *L. lyallii* is a higher-elevation species while *L. occidentalis* is a low-elevation species not doing so well at high elevations, the question of identity is of obvious concern in genetic resource management, and in ensuring adequate cultural treatments. Foliar terpenes and cone and seed morphology traits have been used to identify individuals of *L. lyallii*, *L. occidentalis*, and their putative natural hybrids (Carlson *et al.*, 1991; Carlson & Theroux, 1993). Molecular markers, which can be applied at any stage in the sporophyte phase of the life cycle, and are little or not affected by ontogenetic and environmental factors, would better address inter- and intra-specific identification. Fins & Seeb (1986) and Jaquish & El-Kassaby (1998) used allozyme markers to study genetic diversity and population structure of *L. occidentalis* in the western USA and Canada, respectively. Genetic relationships among *Larix* species have been studied by analysing RFLP in cpDNA (Qian *et al.*, 1995). In this paper, we report the application of the polymerase chain reaction (PCR) technique to amplify two microsatellite loci, *UAKLly10a* and *UAKLla1*, which allow easy and rapid identification of *L. lyallii* and *L. occidentalis*. Two main advantages of these microsatellite loci are that they are codominant loci and PCR-based, allowing automation and simplicity of use.

Materials and Methods

DNA extraction and PCR protocol

Allopatric populations of *L. occidentalis* and of *L. lyallii* with four individuals each were used in this study (Table 1). DNA was extracted from twigs or germinated seedlings following a modified CTAB method (Khasa *et al*., 2000). The primer sequences for the two diagnostic microsatellite loci are:

For locus *UAKLla1* (GenBank accession X54464):
(Forward) 5′-ATCTCCTTCATCGTCCAC-3′,
(Reverse) 5′-CCCCAACTAATACCTAATCTAC-3′

For locus *UAKLly10a* (GenBank accession AF364543):
(Forward) 5′-TGGTCGGATTGAGTGAAG-3′,
(Reverse) 5′-ACCCATCCCATGATAGGAG-3′

Details of the isolation of these loci are presented elsewhere (Khasa *et al*., 2000). PCRs were carried out on a GeneAmp 9600® thermal cycler (Perkin-Elmer/Cetus) with a touchdown program (Rahman *et al*., 2000): 3 minutes at 94°C followed by two cycles of 30 seconds each at 94°C (denaturation), 60°C (annealing), and 72°C (extension); 11 cycles of 15 seconds each at 94°C, 60°C, and 72°C with stepwise

Table 1. Locations of sampled populations of *L. lyallii* and *L. occidentalis*, all from British Columbia

Population surveyed	Location
L. lyallii	
1. N-1	Baldy Mountain
2. N-2	Burdett Peak–Gray Pass
3. N-3	Mount Kaslo
4. N-4	Fletcher Creek
5. C-1	Inverted Ridge
6. C-2	Sunkist Mountain
7. C-3	Racehorse Pass
8. C-4	Mount Kuleski
9. C-5	Mount Dingley
L. occidentalis	
1. FH	Flathead
2. PL	Plumbob
3. CC	Carrol Creek
4. Sal	Salmo
5. Chr	Christina Lake
6. Kas	Blue Ridge–Kaslo
7. WL	Wilson Lake Rd
8. BL	Becker Lake
9. MT	Merritt

lowering of the annealing temperature from 60°C to 54°C by the 11th cycle; and 27 cycles of 15 seconds each at 94°C, 54°C, and 72°C, followed by incubation at 72°C for 3 minutes as a final extension step.

About 5μl of PCR product was mixed with 10–20μl SSRP loading buffer (10mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol), and 4μl of each mix was electrophoresed on 6% denaturing polyacrylamide gels with 8M urea and 1 × TBE buffer at 75W constant power for 3 hours. Samples were denatured at 95°C for 2 minutes and kept on ice prior to loading. Super low ladder (20bp standard) from Gensura (Bio/Can Scientific) was used as a size standard. After electrophoresis, gels were fixed and stained with silver nitrate using a 'DNA Silver Staining System' kit (Promega Silver Sequence™ Staining protocol) as modified by Echt *et al.* (1996). The allele size was scored with a template made with a 1bp ladder obtained from an M13 mp18 sequencing reaction (Khasa *et al.*, 2000).

Results and Discussion

The two simple sequence repeats (SSRs) (*UAKLla1* and *UAKLly10a*) reported here amplify polymorphic alleles in nine populations of both species (Figs 1 and 2; five representative populations shown). The 176bp allele of the *UAKLla1* locus is present only in *L. lyallii* while absent in *L. occidentalis* (Fig. 1). Conversely, the 179bp allele is present only in *L. occidentalis* while absent in *L. lyallii* (Fig. 1). The allele size range (294–338bp) at locus *UAKLly10a* occurs only in *L. occidentalis* and is absent in *L. lyallii* (Fig. 2). Only the *UAKLla1* locus shows fixed differences, while the *UAKLly10a* locus displays polymorphic alleles with non-overlapping size classes. The development and identification of species-diagnostic genetic markers for closely related species requires the use of individuals typical of each of the two species, that is, individuals from distinctively allopatric populations as sampled in this study, otherwise reliably discriminating alleles are difficult to find. The sample size used in

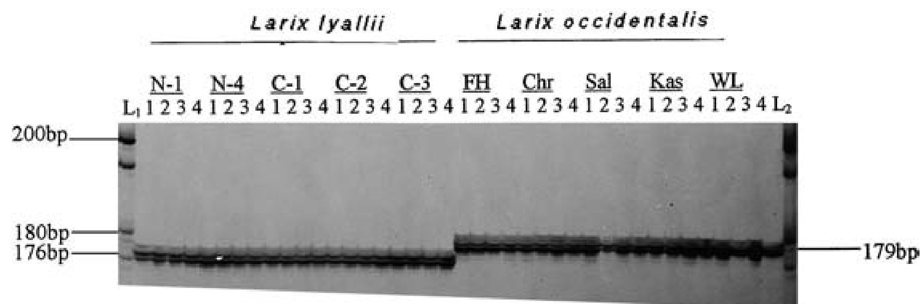

Fig. 1. Pattern of species-specific SSR alleles (*UAKLla1* locus) between *L. lyallii*, shown for five representative populations (N-1, N-4, C-1, C-2, C-3), and *L. occidentalis*, represented by five populations (FH, Chr, Sal, Kas, WL). Population details are given in Table 1. L1 and L2 are 20bp super low ladders.

this study represents a compromise between the need to assess within-population variation and among-population variation (Baverstock & Moritz, 1996).

Biochemical markers such as terpenes, flavonoid and phenolic compounds, total proteins and allozymes have been used to classify species of *Pinaceae* such as *Picea glauca* (Moench) Voss (white spruce) and *Picea engelmannii* Parry (Engelmann spruce), as reviewed by Khasa & Dancik (1996). Molecular markers such as chloroplast (cp) and mitochondrial (mt) DNA RFLP markers (Szmidt *et al*., 1988; Sutton *et al*., 1991a,b), RFLP of the nuclear ribosomal RNA genes (Sutton *et al*., 1994), and *in situ* hybridization probes (Brown *et al*., 1993) have also been used to classify seeds of *Picea* species and for physical mapping purposes.

These methods, however, are time-consuming, labour-intensive, or require relatively large amounts of plant material. Since the development of the PCR in molecular biology, with its advantages over biochemical and RFLP techniques previously used (Mullis *et al*., 1994), the use of PCR-based markers has become an attractive option in molecular taxonomy. Applications of RAPD markers have recently been used to document the origin of the intergeneric hybrid × *Margyraceana skottsbergii* (*Rosaceae*) (Crawford *et al*., 1993), to differentiate closely related *Picea mariana* (black spruce) and *P. rubens* (red spruce) (Perron *et al*., 1995), and to differentiate *Picea glauca* and *Picea engelmannii* (Khasa & Dancik, 1996). One concern about the RAPD technique is its reproducibility and reliability (Khasa & Dancik, 1996; Khasa *et al*., 1997). Also, we noted that the conventional ethidium bromide stain is not sensitive enough for detection on agarose gel of DNA bands, compared with SYBR® stain (Rahman *et al*., 2000).

The discovery of these two microsatellite loci showing patterns of species-specific differences is an important breakthrough in DNA fingerprinting of these closely related species. The conventional methods of identification will still be extremely valuable in the field and will always remain at the forefront of many diagnostic

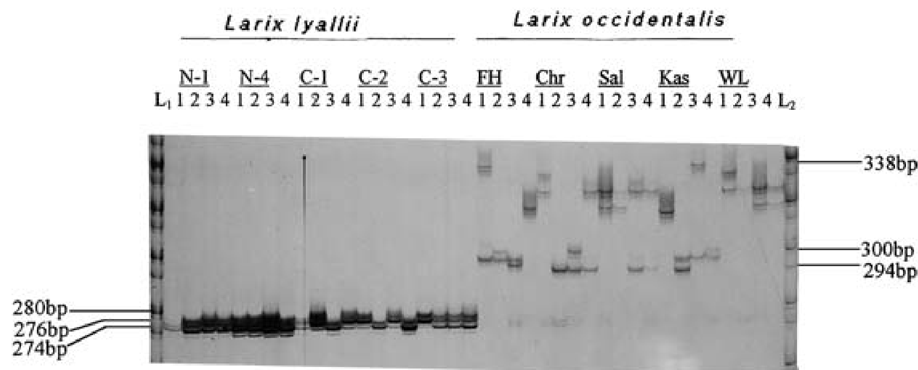

FIG. 2. Pattern of species-specific SSR alleles (*UAKLly10a* locus) between *L. lyallii*, shown for five representative populations (N-1, N-4, C-1, C-2, C-3), and *L. occidentalis*, represented by five populations (FH, Chr, Sal, Kas, WL). Population details are given in Table 1. L1 and L2 are 20bp super low ladders.

procedures. However, molecular tools such as those presented here are extremely useful when there is a requirement for rapid identification of seeds or when material is scarce or immature. The technique appears to offer a quick, easy, reliable and cost-effective method for identification of *L. occidentalis* and *L. lyallii* compared with many of the other methods previously used, especially when morphological characters cannot be observed. Because of the strict altitudinal adaptation of the two species, these diagnostic markers should be useful for rapid identification of seed in operational nursery practices for appropriate seed-to-site matching. They should find application in:

1 defining the species boundaries of *L. occidentalis* and *L. lyallii*;
2 analysing paternity, introgressive hybridization and microevolutionary processes;
3 validating controlled crosses between these two closely related species aimed at producing genetically improved hybrid stock for reforestation.

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