Cross-species Amplification of *Betula pendula* Roth. Simple Sequence Repeat Markers in *Alnus* Species

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Abstract

Alnus glutinosa L. and *Alnus incana* L. Moench are found in natural stands throughout Latvia. *Alnus* species are not commercially grown in Latvia; however there is interest in utilising these species for commercial plantations due to their rapid growth rate and ability to grow in, and to improve poor quality soils. Also of interest are hybrids of these two species, as naturally occurring hybrids have been reported to be faster-growing and larger than the parent species. Molecular markers have not been widely applied or developed in *Alnus* species, particularly for population studies. Our aim was to identify previously developed SSR markers that could be used in *Alnus*, in order to investigate population structure, genetic diversity, and also to discover species-specific markers, to assist in identifying naturally occurring *Alnus* hybrids. We took 15 previously developed birch SSR markers, and tested these on *A. glutinosa* and *A. incana* accessions, as well as potential hybrids identified in natural forest stands. The cross-species PCR amplification success rate is high (8 from 15) however our results show that null alleles are very likely to be a problem when using these markers in *Alnus* species. One of these SSR markers (L3.1) identified clear species-specific allelic separation. When this marker was applied to the morphologically identified species individuals and putative hybrids, the SSR marker results confirmed the species designations, but were not entirely correlated for the putative hybrids. It is possible that the individuals where the morphological designation did not correspond to the DNA marker species designation are not pure hybrids, but backcrosses of hybrids to one of the species. Additional species-specific markers will be required to further investigate the genetic composition of these phenotypically diverse, morphologically identified hybrids.

Key words: *Alnus*, SSRs, cross-species amplification, *Betula*

Introduction

Alnus glutinosa L. and *Alnus incana* L. Moench are found in natural stands throughout Latvia. Currently, over 10% of the forest area in Latvia consists of *A. incana*, while *A. glutinosa* prevalence is approximately 5%. *Alnus* species are not commercially grown; however there is interest in utilising these species for commercial plantations due to their rapid growth rate and ability to grow in, and to improve poor quality soils. To date there have been no studies of the genetic diversity or population structure of these *Alnus* species in Latvia, and selection programs are yet to be established. Also of interest are hybrids of these two species, as naturally occurring hybrids have been reported to be faster-growing and larger than the parent species (Kundzinš and Pīrāgs 1959). *Alnus* species are members of the family *Betulaceae*, where they have been traditionally grouped with the birches (*Betula* spp.) in the subfamily *Betuloideae*. This grouping is supported by more recent molecular evidence (Bousquet *et al.* 1992, Chen *et al.* 1999). Fossil pollen records are extensive in the *Betulaceae*, and show that *Alnus* and *Betula* have been differentiated for over 80 million years (Crane 1989).

The use of molecular markers is becoming more common in applied forestry studies, due to the decrease in the cost of genotyping, and more widespread access to genotyping facilities. Molecular genetic markers are variable regions of DNA that provide valuable genetic tools in genetic linkage mapping, association studies, phylogeographic studies, and for the estimation of many population genetic parameters, such as diversity, gene flow, and inbreeding (Bruford and Wayne 1993). To date, the molecular markers most widely applied to tree species have been isozymes, random fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs). Each marker technique has attributes that offer different advantages (Ritland and Ritland 2000). Isozymes are well studied and established, but are not numerous or highly polymorphic. RFLPs utilize probes derived from either genomic or

coding DNA (cDNA) and are codominant markers, but require a large amount of high quality DNA. RAPDs and AFLPs do not require any sequence knowledge of the genome, and so are easy to apply to uncharacterized genomes. However, they are usually dominant and are often difficult to transfer between different mapping populations or species. Additionally, RAPDs are notoriously difficult to transfer across laboratories (Jones *et al.* 1997). SSR markers exhibit codominance and are usually highly polymorphic, and thus, seem to be the ideal marker (Ritland and Ritland 2000). The major drawback of SSR markers is that the polymerase chain reaction (PCR) primers need to be developed for each species, which requires knowledge of the DNA sequence and involves considerable time and expense. It is possible to use SSR markers developed in one species, in other related species. Some cross-amplification of SSR markers between related species occurs due to DNA sequence conservation. However, the success rate is much lower than for species-specific markers, with SSR markers derived from expressed sequences (EST-SSRs) being more successful at species cross-amplification than SSR markers derived from genomic libraries (Rungis *et al.* 2004).

Molecular markers have not been widely applied or developed in *Alnus* species, particularly for population studies. Ribosomal sequences have been used to infer the phylogeny of *Alnus* species (Navarro *et al*. 2003). To date, there have been no reports of the development of SSR markers in *Alnus* species, and only 2 birch SSR markers have been tested on *Alnus* species (Kulju *et al*. 2004). Species-specific DNA markers would be desirable for identification of naturally occurring *Alnus* hybrids as their identification by morphological features is difficult due to a lack of clearly identifiable intermediate phenotypes. One possibility is that this spectrum of morphological features is due to the presence of various generations of hybrids with different mating patterns (*e.g.* hybrid x hybrid and hybrid backcrosses). To elucidate this, it will be necessary to identify multiple species-specific markers in order to more accurately assess the proportion of genetic material from each species in a potential hybrid. Our aim was to identify previously developed SSR markers that could be used in *Alnus* in future studies of population structure and genetic diversity, as well as to discover species-specific markers to assist in identifying naturally occurring *Alnus* hybrids. Considering that within the *Betulaceae* family, *Alnus* and *Betula* are sister genera (Chen *et al*. 1999), we took 15 previously developed birch SSR markers (Kulju *et al*. 2004), and tested these on *A. glutinosa* and *A. incana* accessions, as well as potential hybrids identified in natural forests stands.

Materials and methods

Plant material: *A. glutinosa* and *A. incana* accessions were collected from both natural stands and nursery stock. As no studies regarding genetic diversity and population structure of Latvian *Alnus* species have been conducted, a random sampling of individuals from various regions of Latvia was taken. When collecting multiple samples from a single forest stand, trees more than 50m apart were sampled, in order to diminish the possibility of sampling members of the same clone. Some *A. glutinosa* individuals were collected from the nursery stock, however, these were not elite or selected germplasm, but germinated seed from natural *A. glutinosa* stands. Potential hybrids were identified by visual examination, and the GPS coordinates of these recorded. The morphological features used to identify potential hybrids were type of bark, leaf shape and vein pattern and the presence of a tuft in the angles of leaf veins. 14 *A. incana* individuals, 11 *A. glutinosa* individuals and 9 potential hybrids were collected for DNA extraction.

DNA extraction: DNA was extracted from leaves using the Genomic DNA Purification Kit (Fermentas) with modifications.

SSR markers: SSR markers were from Kulju *et al* (2004). The forward primer was synthesised with a 6-FAM, HEX or NED fluorescent label to allow visualisation of amplification products on a fluorescent sequencer. PCR conditions were the following: 95 ^{0}C for 5 min, 35 cycles of 95 ^{0}C for 30 sec, 50 ^{0}C – 30 sec, 72 ^{0}C – 30 sec; 72 ^{0}C - 7 min; in a total volume of reaction 20 ml containing 50 ng template DNA, 1.5x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 U *Taq* polymerase (*Fermentas*), 0.5 mM of forward (labelled) and reverse primers (*Applied Biosystems*). 0.5 µL of each PCR product was mixed with 10µL formamide and 0.7µL GS350 size standard. After denaturation, the samples were run on an ABI 3100*xl* capillary sequencer, and genotyped using GeneMapper software.

Genetic analysis: SSR markers were scored as diploid co-dominant markers, and analysed using GenAlEx software (Peakall and Smouse 2006). Sequence alignment and comparison were done using the BLAST software available on the NCBI website (*http://www.ncbi.nlm.nih.gov/blast/Blast.cgi*).

DNA sequencing: Individuals homozygous at locus L3.1 were sequenced. PCR products were purified and directly sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (*Applied Biosystems*).

Results

We sampled a limited number of *A. incana* and *A. glutinosa* individuals as we had no *a priori* knowl-

edge of potential population structure in Latvian *Alnus* species. However, results from other DNA marker studies in Latvian forest tree species indicate that population differentiation is low, and genetic diversity is high (Rungis *et al*, unpublished results), which would imply that large sample sizes are not required to capture a large proportion of genetic diversity. Our primary aim was to identify *Betula pendula* SSR markers that could be utilised in *Alnus* species, and therefore future studies will address fundamental issues of genetic diversity and population structure in Latvian *Alnus* species.

Of the 15 birch SSR markers tested, 8 amplified clearly scorable products. PCR amplification in general was less successful in *A. incana* than in *A. glutinosa*. Of the 8 informative SSR primer pairs, PCR products were observed in only 62% of *A. incana* individuals and 88% of *A. glutinosa* individuals. While evidence of null alleles was found in the analysis of the allele frequencies, this lower PCR success rate was also observed for a variety of different primers and DNA samples (unpublished results). As the DNA extraction protocol was identical for both species, it is possible that the DNA extracted from *A. incana* contains higher concentrations of PCR-inhibitory compounds.

Analysis of the combined data from both *Alnus* species revealed that the average number of alleles in *Alnus* was similar to *Betula* (7.75 vs. 8.00), as was the expected heterozygosity (0.696 vs. 0.711). The size range of alleles amplified with each marker overlapped with the size ranges reported in *Betula* (Kulju *et al.* 2004), with the exception of locus L7.8 where the fragments amplified from *Alnus* species were larger (314-349 bp) than in *Betula* (295-307 bp). However, the observed heterozygosity levels were much lower in *Alnus* (0.488 vs. 0.734). This dramatic decrease in the observed heterozygosity (and corresponding increase in the inbreeding coefficient (F)), may indicate the presence of null alleles. This was most pronounced in *A. incana* (average F – 0.368), but was also apparent in *A. glutinosa* (average F – 0.237).

The SSR marker data were also analysed for both species separately (Table 1). The success of amplification (N) was similar for all markers except L2.7 and L5.5, where the number of individuals successfully genotyped was much lower in *A. incana.* The number of alleles detected (Na) was also similar between species, except for marker L2.7, where the lower number of alleles in *A. incana* is probably due to the lower PCR success rate. As mentioned above, overall, the measures of heterozygosity (Ho and F), were lower in *Alnus* species; however this was not evenly distributed in markers between species. For example, no heterozygous loci were observed for marker L3.4 in *A. glutinosa*, while in *A. incana*, the observed heterozygosity was 0.36. Conversely, while no heterozygous loci were observed for marker L5.5 in *A. incana*, in *A. glutinosa*, the observed heterozygosity was 0.75. Other markers with large differences in observed heterozygosity between species were L2.7 and L3.1. In some cases this may be due to low PCR success rate in *A. incana* (markers L2.7 and L5.5 only amplified *A. incana* 3 individuals). However, markers L3.1 and L3.4 amplified similar number of individuals from both species.

Table 1. SSR marker polymorphism and heterozygosity

	N		Na		Ne		Ho		He		F	
Locus	***A. in***	***A. gl***	***A. in***	***A. gl***	***A. in***	***A. gl***	***A. in***	***A. gl***	***A. in***	***A. gl***	***A. in***	***A. gl***
L7.8	11	11	7	7	4.84	5.38	0.82	0.73	0.79	0.81	-0.03	0.11
L2.7	3	10	4	10	3.60	7.41	0.33	0.80	0.72	0.87	0.54	0.08
L3.1	12	11	6	6	2.36	3.61	0.25	0.64	0.58	0.72	0.57	0.12
L3.4	11	11	3	2	2.07	1.20	0.36	0.00	0.52	0.17	0.30	1.00
L5.5	3	8	2	3	1.80	2.84	0.00	0.75	0.44	0.65	1.00	-0.16
L5.4	13	11	2	4	1.45	2.60	0.23	0.36	0.31	0.62	0.26	0.41
L2.3	8	8	3	3	2.33	1.29	0.38	0.13	0.57	0.23	0.34	0.45
L022	8	7	4	6	2.56	4.45	0.63	0.86	0.61	0.78	-0.03	-0.11
average	8.63	9.63	3.88	5.13	2.63	3.60	0.37	0.53	0.57	0.60	0.37	0.24

N – no. of individuals, Na – no. of alleles, Ne – no. of effective alleles, Ho – observed heterozygosity, He – expected heterozygosity, F – inbreeding coefficient

The results were also analysed to determine if there were any species-specific alleles identified by the successfully amplifying primer pairs. The combined genotypes of the 8 SSR markers clearly differentiated the two species in population assignment tests and principal coordinate analysis (data not shown). While private alleles were found for all markers in one or both species, there were no fixed alleles polymorphic between the two species. Only one marker (L3.1) was found to clearly differentiate between *A. glutinosa* and *A. incana*, with alleles in *A. glutinosa* 240bp and larger, and alleles in *A. incana* smaller than 240bp (Table 2). The amplified fragments from each species were sequenced to determine the basis of this size differenti-

ation. It was found that there were no insertions or deletions outside of the SSR region, and that the length polymorphism was entirely due to addition or loss of repeats. The marker was subsequently tested on the potential hybrids identified using morphological indicators. This marker correctly identified all samples morphologically identified as *A. incana* or *A. glutinosa*. Nine individuals were identified as potential hybrids according to morphological features; of these genotyping with marker L3.1 identified five as hybrids, three as *A. incana* and one as *A. glutinosa* (Table 2). However, these potential hybrids were not morphologically uniform, and it is possible that they were not morphologically uniform, and it is possible that they were not all pure, 1st generation hybrids.

Table 2. Genotypes of *A. incana* and *A. glutinosa* of putative hybrids with marker L3.1

Individual	Morphological spp. designation	L3.1 genotype (length in base pairs)		Marker spp. designation
aln74	hybrid	227	227	*A. incana*
aln75	hybrid	227	227	*A. incana*
aln76	hybrid	227	248	hybrid
aln84	hybrid	227	248	hybrid
aln85	hybrid	227	248	hybrid
aln86	hybrid	227	227	*A. incana*
aln87	hybrid	227	248	hybrid
aln88	hybrid	227	246	hybrid
aln90	hybrid	246	246	*A. glutinosa*
Baln09	*A. incana*	227	227	*A. incana*
Baln11	*A. incana*	236	236	*A. incana*
Baln12	*A. incana*	233	233	*A. incana*
Baln13	*A. incana*	231	231	*A. incana*
Baln18	*A. incana*	PCR failed		-
Baln21	*A. incana*	227	227	*A. incana*
Baln22	*A. incana*	227	227	*A. incana*
Baln26	*A. incana*	PCR failed		-
Baln54	*A. incana*	227	235	*A. incana*
Baln61	*A. incana*	227	229	*A. incana*
Baln63	*A. incana*	227	227	*A. incana*
Baln66	*A. incana*	227	233	*A. incana*
Baln67	*A. incana*	227	227	*A. incana*
Baln70	*A. incana*	227	227	*A. incana*
Maln02	*A. glutinosa*	248	248	*A. glutinosa*
Maln04	*A. glutinosa*	240	247	*A. glutinosa*
Maln06	*A. glutinosa*	240	248	*A. glutinosa*
Maln15	*A. glutinosa*	250	273	*A. glutinosa*
Maln17	*A. glutinosa*	240	248	*A. glutinosa*
Maln19	*A. glutinosa*	240	250	*A. glutinosa*
Maln33	*A. glutinosa*	244	247	*A. glutinosa*
Maln35	*A. glutinosa*	248	248	*A. glutinosa*
Maln39	*A. glutinosa*	248	248	*A. glutinosa*
Maln51	*A. glutinosa*	244	250	*A. glutinosa*
Maln53	*A. glutinosa*	248	248	*A. glutinosa*

Alleles of length less than 240 base pairs (bp) were found in all *A. incana* individuals, alleles 240bp and larger in all *A. glutinosa* individuals. Hybrids would be expected to have one allele smaller than 240 bp, and one allele 240 bp or larger. Alleles 240 bp and larger are shaded grey

The birch sequences from which the SSR markers were developed are available on GenBank (accession numbers in Kulju *et al*, 2004). In order to determine if these were expressed sequences, they were BLASTed against the GenBank expressed sequence tags (EST) database. Three of the birch SSR sequences found matches in the EST database: L1.10 (e value 6e-25, 83% sequence identity), L022 (e value 3e-123, 85% sequence identity), L7.4 (e value 2e-30, 84% sequence identity), indicating that they were probably derived from expressed sequences in birch. However, this did not correspond to the success of cross-species amplification, as 2 of the loci with similarity to expressed sequences did not amplify scorable fragments from the *Alnus* species.

Discussion and conclusions

The cross-species PCR amplification success rate is high (8 from 15), particularly as most of the markers do not appear to be derived from coding sequences. Our results show that null alleles are very likely to be a problem when using these markers in *Alnus*. This high incidence of null alleles is not surprising, especially considering that these SSR markers were developed from genomic libraries. However, the fact that observed heterozygosity for some markers differed between *A. incana* and *A. glutinosa* indicates that in some cases these null alleles may be species specific for a particular marker. Further studies using progeny arrays to determine Mendelian segregation in each species would be required to fully characterise these markers and their applicability to either one or both *Alnus* species included in this study.

The traditional method of SSR marker development involves genomic library construction, followed by repeat identification by hybridisation, and sequencing of clones. This method can be time consuming and expensive, and the return of useful SSR markers is often low (Squirrel *et al*. 2003). Currently there is not much research in *Alnus* utilising DNA markers, particularly population studies. However, given that interest in the utilisation of *Alnus* as plantation species is increasing, perhaps sufficient funding to develop SSR marker in *Alnus* will be found.

One solution to develop novel SSR markers is to data-mine existing sequence databases in order to identify SSR containing sequences, which can then be utilised for primer design. However, development of *Alnus* specific markers in this manner is not currently feasible as GenBank only contains 152 DNA sequences

from various *Alnus* species (accessed on 22/04/08). An alternative is to utilise an anonymous marker system (*e.g.* AFLPs), where prior sequence knowledge is not required. However, most anonymous marker systems are dominant (*i.e.* heterozygotes are not detected), and this reduces the information content of these marker systems.

Perhaps the most feasible solution for developing SSR markers for use in *Alnus* species is to utilise EST sequences from related species (such as *Betula*). EST-SSRs are more transferable across species, and while they are generally less polymorphic than genomic SSRs, they also have a lower incidence of null alleles (Rungis *et al*. 2004). Another solution would be to sequence the *Betula* SSR marker loci identified in this study in both *A. incana* and *A. glutinosa*, which would allow the identification of any sequence polymorphisms which may give rise to the null alleles putatively identified. This would allow the development of SSR marker primers better suited for use in *Alnus* species, as well as identification of potential species-specific sequences, which would be of use in the identification of hybrids.

Only one of these SSR markers (L3.1) identified clear species-specific allelic separation. When this marker was applied to the morphologically identified species individuals and putative hybrids, the SSR marker results confirmed the species designations, but were not entirely correlated for the putative hybrids. It is possible that the individuals where the morphological designation did not correspond to the DNA marker species designation are not pure hybrids, but backcrosses of hybrids to one of the species. The extent of natural hybridisation in Latvia is not known, and has not been investigated using DNA markers. However artificial hybridisation studies have found that hybridisation was only successful when *A. incana* was used as the maternal parent (Кундзиньш 1968). As *A. incana* flowers a week earlier that *A. glutinosa* (Banaev and Bažant 2007), this would limit the extent of natural hybridisation. The identification of hybrids by morphological features is difficult, as these characteristics are found in a continuum between pure *A. incana* and *A. glutinosa* types. Development of multiple species-specific DNA markers is necessary to determine the extent of natural hybridisation. Additionally, it will be possible to determine if putative natural hybrids which are more similar to one species morphologically, are 1st generation hybrids, or rather backcrosses to one of the species.

In conclusion, these *Betula* markers should be used with caution for population studies in both *A. glutinosa* and *A. incana*, due to the likely presence of null alleles. We identified one species-specific SSR marker which confirmed some of the morphologically identified hybrid designations. Additional species-specific markers will be required to further investigate the genetic composition of these phenotypically diverse, morphologically identified hybrids. Development of *Alnus* SSR markers utilising current DNA sequence databases will be difficult due to the small amount of publicly available *Alnus* sequence data. For more extensive population and diversity studies in *Alnus* species, it will be more useful to utilise an anonymous DNA marker technique such as AFLPs until such time as SSR markers are developed for *Alnus* species. However, these *Betula* SSR markers investigated in this study are useful for clonal identification and other DNA fingerprinting studies in *Alnus*, where the presence of null alleles will not adversely influence the results to the same extent as in population studies.

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МЕЖВИДОВАЯ АМПЛИФИКАЦИЯ МИКРОСАТЕЛИТНЫХ ДНК МАРКЕРОВ *BETULA PENDULA ROTH* В ИССЛЕДОВАНИЯХ *ALNUS* SPP.

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Резюме

Виды черной и серой ольхи *(Alnus glutinosa* L. и *Alnus incana* L. Moench*)* распространены на территории Латвии, но не выращиваются в комерческих целях. Ольха отличается неприхотливостью, быстрым ростом и способностью обогощения почвы азотом. Гибрид ольхи (*Alnus glutinosa* L. x *Alnus incana* L. Moench) особенно интересен для выращивания, так как естественные гибриды характеризуются еще более быстрым и высоким приростом древесины. Молекулярные ДНК маркеры не были разработанны и широко не применялись для исследований генома ольхи, в частности, в популяционных исследованиях. Задачей нашей работы являлось исследование возможностей применения ранее разработанных молекулярных ДНК маркеров в родственных видах для выявления структуры популяции и генетического разнообразия ольхи, так же, как и разработка видо-специфичных маркеров для идентификации гибрида *ольхи.* Амплификация пятнадцати ДНК молекулярных маркеров, ранее разработанных для березы на основе простых микросателитных повторов (SSR), была проведена среди серой, черной ольхи и потенциальных их гибридов, идентифицированных в естественных биотопах. Результат межвидовой амплификации был успешен в восьми случаях из пятнадцати, все же присутствие нулевых аллелей могут значительно усложнить применение неспецифичных микросателитных ДНК маркеров для исследований видов ольхи. Определено, что продукты амплификации одного из микросателитных ДНК маркеров (L 3.1) разделяет виды серой и черной ольхи. Это разделение было подтверждено анализом морфологически определенных видов, но не однозначно коррелировало в случае их гибридов. Предпологается, что идивиды, для которых морфологическое описание полностью не соответствовало анализу ДНК маркеров, образовались не в результате гибридизации, а обратного скрещивания гибрида с одним из видов ольхи. Разработка дополнительных видо-специфичных маркеров требуется для дальнейшего исследования генетического состава фенотипически различных гибридов ольхи.

Ключевые слова: *Alnus*, SSRs, межвидовая амплификация, *Betula.*