

EVALUATION OF ALLELIC CONTENT IN AN EXPERIMENTAL ALDER (*ALNUS* SPP.) PLANTATION

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Voronova A., Lazdina D., Korica A., Veinberga I., Liepins K., Rungis D. 2015. Evaluation of allelic content in an experimental alder (*Alnus* spp.) plantation. *Acta Biol. Univ. Daugavp.*, 15 (1): 227 – 240.

Grey alder (*Alnus incana*) and black alder (*Alnus glutinosa*) are typical pioneer tree species that colonize abandoned farmlands and clear-cut areas in forests. F1 hybrids of these species were first described in Latvia in 1957 and which exhibited heterosis or hybrid vigour. Theoretically, seeds of hybrids will be variable and could even exhibit signs of outbreeding depression, and have lower fitness compared to the parent species. Hybrids of *A. incana* and *A. glutinosa* are difficult to distinguish from the parental species due to a continuous phenotypic variation of distinguishing characteristics, and introgression could lead to even higher variability in traits in further generations. Experimental plantations of *A. incana* x *A. glutinosa* hybrids seeds were established and were analysed by several parameters: form of leaf, bark, the crown shape, height, diameter and growth increment. As expected, high variability of phenotypic traits in the experimental alder plantation was observed. 187 trees samples were analysed with seven molecular markers that enable discrimination between grey and black alder species, revealing high genetic heterogeneity.

Key words: *Alnus incana* x *Alnus glutinosa*, hybrid content, phenotypic variation, outbreeding depression.

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INTRODUCTION

Grey alder (*Alnus incana* (L.) Moench) and black alder (*Alnus glutinosa* (L.) Gaertn) belong to the birch family (*Betulaceae*) genus *Alnus*. *Alnus* species are monoecious wind-pollinated trees with an outcrossing breeding system. Both species are distributed throughout continental

Europe ranging from southwest Spain to northern Scandinavia and northwest Russia. In Latvia, *A. incana* is the fourth most distributed tree species and covers 206511.3 ha of forest land, but *A. glutinosa* is less widespread (82165.4 ha) (State Forest Service data, 2015). Most *Alnus* species are tetraploids ($2n = 4x = 28$) (Oginuma et al. 2000). *Alnus incana* has been utilized for establishment

of forest plantations of former farmlands due to rapid growth and low requirements regarding soil productivity. Alders have the ability to enrich the soil with nitrogen fixing bacteria that are propagated by root nodules. Unlike grey alder, black alder (*Alnus glutinosa*) prefers wet soils and has a longer life span. The timber of the black alder is valued higher compared to that of grey alder due to superior mechanical and decorative qualities. According to phylogenetic studies both species belong to one branch of the *A. incana* complex and are related (Chen & Li 2004).

Hybridisation of *A. incana* and *A. glutinosa* species occur naturally and has been reported in Latvia, Belarus, Poland, The Czech Republic, Sweden, Ireland and other countries (Banaev & Bazant 2007). Hybridisation occurs usually due to environmental variables such as cold prolonged springs, which could alter the usual flowering times of the two species, which usually separated by one week (Banaev & Bazant 2007). First generation hybrids often exhibit hybrid vigour, and *A. incana* x *A. glutinosa* hybrids were characterized as fast growing, with greater drought and pythium rot (*Fomes igniarius* f. *alni*) resistance (Kundzins 1957, 1966, Pirag 1962). Alder hybrids were of interest in Latvia as they are faster-growing and having superior stem quality than the parent species (Kundzinsh & Pirags 1959). Because of the interest in hybrid production, controlled pollination of *A. incana* and *A. glutinosa* was carried out in Latvia, and the F1 hybrid material was utilised to establish experimental plantations in the 1950's by Kundziņš. Kundzins (1957) noted that seed germination was significantly higher when *A. incana* was used as the maternal parent (26 % germination), whereas when *A. glutinosa* was used as the maternal parent, seeds germination was only 1.5 %. Improved characteristics usually are attributable to F1 hybrids, but plant hybrids in next generations tend to backcross to one of the parent species, and several generations of introgression could lead to a complex mixture of parental species' genes and alleles and outbreeding depression (Whitlock et al. 1995, Hauser et al. 1998). From another point of view,

even if fitness of F2 and backcross individuals is highly variable and on average lower than that of the parental species, some individual plants from advanced generation backcrosses may show reasonable fitness, as shown for oilseed rape hybrid backcrosses (Hauser et al. 1998). Evolutionary studies suggest that almost 50 % of all plant species originated via hybridization speciation (Wood et al. 2009, Mallet 2007). Polyploidy may confer evolutionary advantages for plant species, including lessening of hybrid outbreeding depression (Stebbins 1950; Soltis & Soltis 2000). Hybrids between alder species are difficult to distinguish from each other due to continuous phenotypic variation of distinguishing characteristics (Pirāgs 1962, Parnell 1994, Banaev & Bazant 2007, Poikans 2014). The genetic status of hybrids is often unknown or unexamined, and classification based on morphological traits alone could lead to misclassification (Rieseberg & Ellstrand 1993, Fritz, 1999). Conventional selection of the best planting material from natural alder stands could be associated with a risk of selecting naturally occurring hybrid or introgressed trees. Distinguishing characteristics of *A. incana* x *A. glutinosa* hybrids are the number of pairs of leaf veins, leaf coefficient, upper angle of the leaf blade and others (Banaev & Bazant 2007).

Various molecular marker techniques have been utilised for identification of woody plant species and their hybrids. These include anonymous marker systems such as RAPDs (Random Amplified Polymorphic DNA) (Grattapaglia et al. 1992, Rossetto et al. 1997, Kumar et al. 1999, Nkongolo et al. 2005), AFLPs (Amplified Fragment Length Polymorphism) (Guo et al. 2006), ISSRs (Inter Simple Sequence Repeat) (Pharmawati et al. 2005). SCAR (Sequence Characterized Amplified Region) markers were developed from non-specific RAPD markers to achieve reproducibility (Hernandez et al. 2001, Das et al. 2005, Evans & James 2003, Gunter et al. 2003, Ghosh et al. 2011). SSR (*Simple Sequence Repeat*) are locus specific, co-dominant highly polymorphic markers that have been widely used for species identification

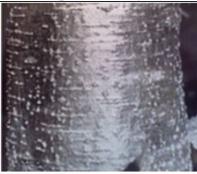
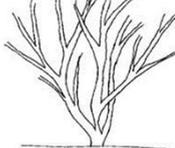
(Khasa et al. 2005, Arumugasundaram et al. 2011, Shirasawa et al. 2013). Analysis of whole-genome sequencing data from *Capsicum* spp. identified 19 SSR loci out of 2245 that could distinguish different species (Shirasawa et al. 2013), but identification of species-specific SSR loci from a limited amount of markers does not always result with success (Bruschi et al. 1999). PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) of plastid DNA can be used for evaluation of the origin of presumed allotetraploids, or to identify their maternal parents (Guo et al. 2006). The PCR-RFLP technique was used to develop one diagnostic SNP in chloroplast DNA for identification pine (*Pinus uliginosa* x *P. sylvestris*) hybrids (Wachowiak et al. 2005). PCR-RFLP markers are co-dominant and locus-specific, genotypes are easily scored and interpreted, these markers are highly reproducible.

In this study, an experimental trial plantation was genotyped with species-specific molecular markers developed previously to identify black and grey alder species and their hybrids (Rungis et al. 2010). The species-specific markers were developed from SNPs and indels (insertions/deletions) identified between *A. incana* and *A. glutinosa*. The experimental plantation was established utilising seeds collected from a mother tree seed plantation, which contained a mixture of *A. incana* and *A. glutinosa* individuals, and possibly some hybrid individuals. The seeds were collected from morphologically identified *A. incana* individuals, however, due to the uncertainty of the provenance of the mother tree seed orchard, individuals from this seed orchard were also genotyped with the species-specific markers. This enabled differentiation of *A. incana* and *A. glutinosa* individuals, and to determine the extent of hybridisation within the experimental plantation as well as the mother tree seed plantation. Early stage growth parameters (height and diameter) were measured within the experimental plantation and compared to data from similarly aged pure *A. incana* and *A. glutinosa* plantations.

MATERIAL AND METHODS

The seeds for establishment of the experimental plantations were collected in a mixed grey alder and black alder plantation in Aknīste district (Latvia) established 1948. The plantation was established with the aim of producing hybrid alder seeds; however, the information about the origin of the reproductive material is incomplete. Kundziņš (1957) noted that he used seed mixtures of both species and later identified hybrids in the experimental trial; in addition he reported that he performed *A. incana* x *A. glutinosa* controlled pollination tests. Kundziņš (1968) describes experimental pollination of both species, as well as hybrid pollination with both parental species (backcrosses) and F2 hybrid crosses. The trees were planted in a regular grid with alternating rows of grey and black alder. The seeds were collected in the autumn of 2008 from trees morphologically identified as grey alder. Because of the limited seed crop, the seeds were collected from the five phenotypically superior mother trees for establishment of an experimental/demonstration plantation at Skriveri - long term planting of a short rotation coppice as an agriculture crop. One-year old containerized seedlings were used for establishment of the experimental plantation in 2010. Measurements of tree height were done every year and trees became more different year by year. It was noticed that individual trees varies significantly by growth, shape of leaves and crown, as well colour of the bark (Fig. 1). For genetic analyses, 187 trees were sampled from the experimental alder plantation at Skriveri, Latvia and 30 individuals were sampled from the mother tree seed stand. 24 individuals from a grey alder plantation in Zente, Latvia were collected as a control for the molecular analyses. DNA from alder leaves was isolated using a CTAB-based method (Porebski et al. 1997), purified with RNase A (*Thermo Scientific*) and concentration was adjusted to 50 ng/μL.

Species-specific SNPs (Single Nucleotide Polymorphisms) were identified using the TILLING (Targeting Induced Local Lesions IN Genomes) method using the CEL I single strand endonuclease (Till et al. 2004). M13-tailed

Morphological parameters					
1					
2					
3					
4					
5					
Leaf shape					
	29%	5%	12%	19%	35%
Bark	Dark coarse (DC)		Light coarse (LC)		Light smooth (LS)
					
	24%	3%	73%		
Crown shape	Shrublike (S)		outspread (O)		compact (C)
					
	23%	47%	30%		

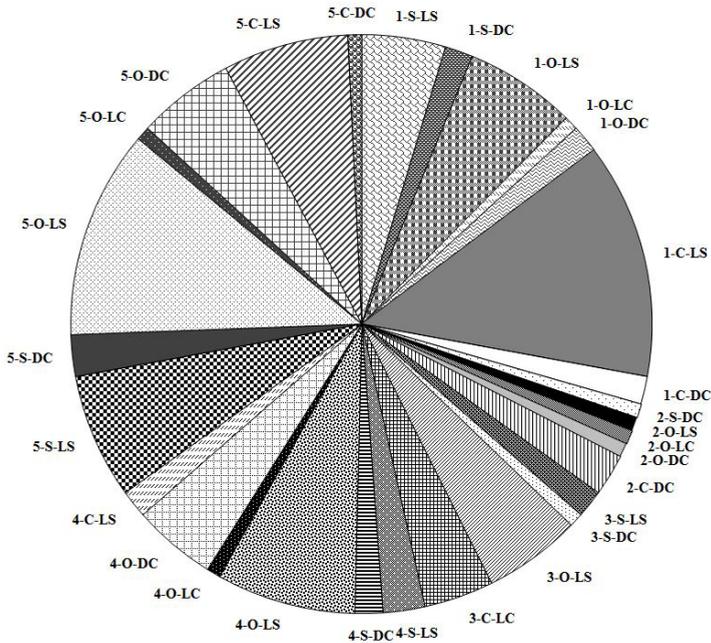


Fig. 1. Morphological characterisation of experimental hybrid plantation. 1- *A. glutinosa* leaf shape; 2 to 4 intermediate leaf shape ; 5 – *A. incana* leaf shape; S- Shrublike crown shape, O- outspread crown shape, C- compact crown shape; DC-Dark coarse bark, LC-Light coarse bark, LS- Light smooth bark.

Table 1. PCR primers used in the study, product size and polymorphisms used for the identification of *A. incana* and *A. glutinosa* specific loci. *product size is constantly larger than expected by gene bank sequence analysis; high similarity with *ptr1* gene is confirmed with sequencing

Nr.	NCBI GI Nb.	Primer sequence	Product size, bp	<i>A. incana</i> → <i>A. glutinosa</i> distinguishing polymorphism	Restriction enzyme	Cuts Exp. products size
2 F	GI: 3319650 pollen allergen <i>Alng4</i>	CAACAACGGTA-ATGGCTGAC	246	SNP G ²¹⁶ →A	<i>XapI (ApoI)</i>	<i>A. glutinosa</i> 215/31
s2R		GGAAAACCCACG-CATGTTAT				
12 F	GI: 31075167 dicarboxylate transporter <i>ptr1</i> gene	AATCATC-CAATGGGGTTCAA	570*	SNP A ³³⁰ →G	<i>Cail (AlwNI)</i>	<i>A. glutinosa</i> 333/237
12 R		CCTGGACAG-TAGCCAACCAT		SNP C ⁴¹⁹ →T	<i>HpyF3I (DdeI)</i>	<i>A. glutinosa</i> 419/151
s16F	GI:1289203 thiazole biosynthetic enzyme, <i>THI</i> gene	TGAACCACGA-CACCCAGTCGT-GCA	217	SNP C ⁹⁵ →G	<i>MspI (HpaII)</i>	<i>A. incana</i> 95/122
16R		ATCATCCCAGGCA-CAATCTC		SNP T ⁸⁹ →G	<i>BshNI (BanI)</i>	<i>A. incana</i> 88/129
5sF	GI:166425 5S rRNA gene	CGGATCCCAT-CAGAACTCCGCA	411	SNP G ¹⁸⁸ →A	<i>AdeI (DraIII)</i>	<i>A. incana</i> 187/224
5sR		C T A A C T - GAACTGGCCCG-CATAGTG				
10F	GI: 31559207 pathogenesis-related protein <i>PR10A</i> gene	GTTTCATGAAGGC-GGTTCACT	400	I N D E L A ¹⁹² ATTAT ¹⁹⁷ →A ¹⁹² T	<i>VspI (AseI)</i>	<i>A. incana</i> 190/210
10R		A C A C A T G C A T - GAAAGCCACA				
10RsB		AATGAAGCAGC-TAGGTTTAG	330	SNP, INDEL A ³¹⁵ CCTAGCTGCT ³³³	Product present in <i>A. incana</i>	
10RsM		GAATTAAGCAGC-TAGCTCGC	349	SNP, INDEL A ³¹⁵ GC-GAGCTAGCTGCT-TAT ³³³	Product present in <i>A. glutinosa</i>	

primers were constructed using 23 *Alnus glutinosa* or *Alnus incana* NCBI GenBank sequences using the Primer3 software (Untergasser et al. 2007). Initial PCR amplification was performed with unlabelled primers, followed by amplification with 5'FAM and 3'HEX M13 labelled primers. For species-specific SNP identification, PCR amplification products from four *A. incana* and four *A. glutinosa* individuals were hybridised by heating to 95°C followed by gradual cooling to room temperature. In addition, four hybrids

and two black and grey alder samples were analysed separately. After digestion with CEL I, fragment separation was performed using an ABI-Prism 3130xl Avant Genetic Analyzer (*Applied Biosystems*). Gene fragments where SNPs were present in mixed and hybrid samples, but not in pure samples (heterozygotes), were Sanger sequenced from both species (7 grey alder, 6 black alder, 5 hybrid individuals). Species-specific PCR-RFLP (PCR restriction length polymorphism) assays were developed, which

were validated using 19 black alder, 22 grey alder and 21 potential hybrid alders from Latvia and Estonia and 36 unknown samples.

Seven species-specific polymorphic loci were used for genotyping of the experimental plantation. One single sequence repeat (SSR) marker (L3.1) (Kulju *et al* 2004) was previously reported as distinguishing grey and black alder (Zhuk *et al.* 2008). The six remaining species-specific markers were reported previously (Rungis *et al.* 2010) (Table 1). Screening for the *A. incana* and *A. glutinosa* specific loci was performed using PCR amplification with subsequent restriction and electrophoretic fragment size separation (Fig. 2). PCR was performed in final volume of 20 ml with the same PCR protocol for all markers: 50-100 ng template genomic DNA, final concentration of 1x Dream Taq buffer (*Thermo Scientific*), 2 mM MgCl₂, 0.2 mM dNTP mix (*Thermo Scientific*), 1 mM of each primer, 0.8 U DreamTaq polymerase (*Thermo Scientific*), 0.4mg/ml BSA (*Thermo Scientific*). The following PCR amplification program was used: 95°C-3 min; 30 cycles of 95°C-30 sec., 55°C-30

sec., 72°C-40 sec; and final elongation at 72°C for 7 min. The same program was used for the 5S ribosomal RNA gene amplification. PCR program conditions for the thiazole biosynthetic enzyme (16 F/R) was as follows: 95°C-3 min; 38 cycles of 95°C-30 sec., 50°C-30 sec., 72°C-40 sec; and final elongation in 72°C for 10 min. For the *ptr1* gene (12F/R), the PCR amplification program was same with the exception of an annealing temperature of 55°C. Restriction was performed in a final volume of 15 µL containing 5 µL of PCR product, 5 U of enzyme and 1x appropriate digestion buffer (*Thermo Scientific*). Reaction was incubated at 37°C for one hour; reaction was stopped by adding of 1x DNA Loading Dye & SDS solution (*Thermo Scientific*). Products were separated using electrophoresis in 1x TAE buffer and 1.7 % TopVision agarose gel (*Thermo Scientific*) with ethidium bromide.

RESULTS

Proportion of *A. incana* and *A. glutinosa* alleles in each analysed individual was calculated.

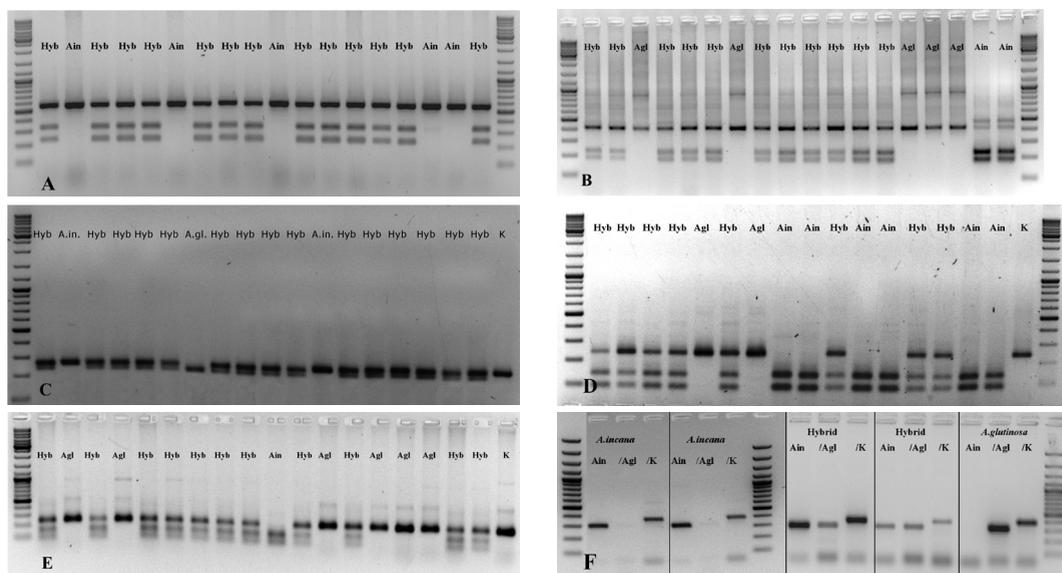


Fig. 2. Polymorphism detection using electrophoretic separation of restriction enzyme digested PCR products and species-specific primers. A) *Ptr1* (12F/R), *Cail*; B) 5S rRNA gene, *AdeI*; C) *Aln g 4* gene (2F/R), *XapI*; D) thiazole biosynthetic enzyme (s16 F/R), *MspI*; E) thiazole biosynthetic enzyme (s16 F/R), *BshNI*; F) *pr10A* gene (10 sB, 10sM, 10 F/R).

Two SNPs in the thiazole biosynthetic enzyme gene (*THI*) located 6 bp apart from each other were previously identified as distinguishing both species. Genotyping of phenotypically identified species samples indicated that the nucleotide variant G⁸⁹ was found only in *A. glutinosa* individuals, but the heterozygous variant T⁸⁹/G was found in *A. incana* samples, as well as the homozygous variant T⁸⁹. The variant C⁹⁵ was specific for *A. incana* individuals, but the heterozygous variant G⁹⁵/C was found in *A. glutinosa*, as well as the homozygous variant G⁹⁵. Five individuals were identified that had the rare genotype G⁸⁹/C⁹⁵, which were considered as hybrids. Both SNPs are synonymous, i.e. they do not affect the amino acid sequence of the protein encoded by the gene. Of 24 individuals from a natural *A. incana* stand in Zente genotyped with the *THI* gene marker, 15 genotypes were T⁸⁹/G, C⁹⁵, 12 genotypes were T⁸⁹, C⁹⁵ (both genotypes were previously found in *A. incana*) and two samples had the rare genotype G⁸⁹/C⁹⁵ (considered as hybrid). Samples, where both SNP loci were heterozygous were considered as hybrids. The 24 analysed individuals from the *A. incana* stand had the expected *A. incana* specific SNPs for all other markers analysed.

From the 187 individuals genotyped from the experimental alder plantation, 90 unique

haplotypes were identified, taking into account all SNP loci studied, the largest group with identical genotypes consisted of five trees. The proportion of *A. incana*/*A. glutinosa* alleles per sample were calculated with assumption of codominant data: heterozygotes were counted as one allele from each species, but homozygotes as two alleles from the corresponding species, and the total proportion of species-specific locus was calculated. From the experimental plantation, seven trees had 100 % *A. incana* alleles and two trees were heterozygous at all five loci. No individuals with 100 % *A. glutinosa* alleles were identified in this sample set. 31 individuals had an equal proportion of alleles from both species, but with an uneven distribution (i.e. they were not F1 hybrids). 100 individuals had 60-90 % *A. glutinosa* alleles and 47 individuals had 60-90 % of *A. incana* alleles. The 30 individuals analysed from the mother tree seed plantation identified one individual with 100 % *A. glutinosa* alleles and three individuals with 100 % *A. incana* alleles. 23 individuals had 60-80% *A. incana* alleles and three individuals were heterozygotes at each allele. The control *A. incana* stand (24 individuals) have 22 individuals with 100% *A. incana* alleles, and two individuals with 90% *A. incana* alleles. No correlations were observed between growth parameters and allelic proportion the correlation coefficient for tree height in 2014

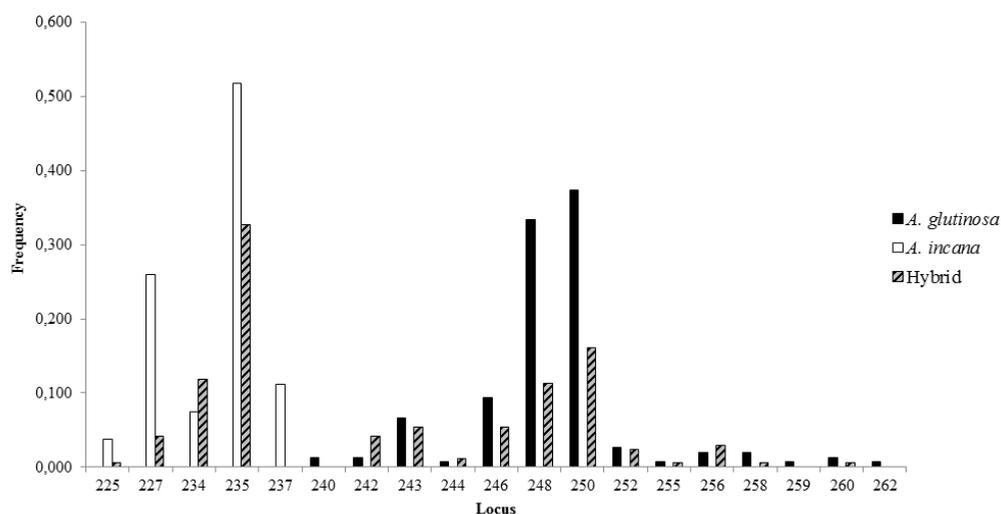


Fig. 3. Allele frequency of SSR marker L3.1 alleles in the experimental plantation (187 individuals).

and *A. glutinosa* allele proportion was 0.038, collar diameter and *A. glutinosa* allele proportion 0.126, growth increment and *A. glutinosa* allele proportion 0.0598.

The SSR marker L3.1 differentiates *A. incana* and *A. glutinosa* species by repeat size difference, with *A. incana* individuals having allele sizes of 237bp or smaller and *A. glutinosa* individuals having allele sizes of 240bp or more. Genotyping of the individuals from the experimental plantation revealed a large variation in allele representation and frequency (Fig. 3). In the maternal tree seed plantation, variation at the L3.1 locus was lower

and no individuals with only *A. glutinosa* alleles were identified (Fig. 4).

The genotyping data was encoded in a binary manner (*A. incana*, *A. glutinosa* and hybrid) as well as species-specific allele frequencies and sorted into five groups based on growth increment and diameter. Allele frequencies were analysed with GenAlEx 6.4 (Peakall & Smouse 2006). No association was observed between *A. incana* and *A. glutinosa* or hybrid allele frequencies and height or diameter groups (Fig. 5). Mean height (*Hv*) of ghybrid 5-year seedlings was not correlated with *A. glutinosa* allele content. The

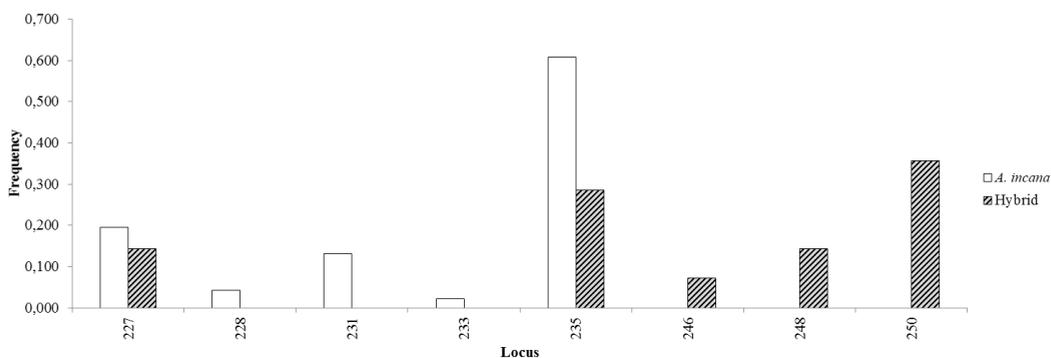


Fig. 4. Allele frequency of SSR marker L3.1 alleles in the mother tree seed plantation (30 individuals).

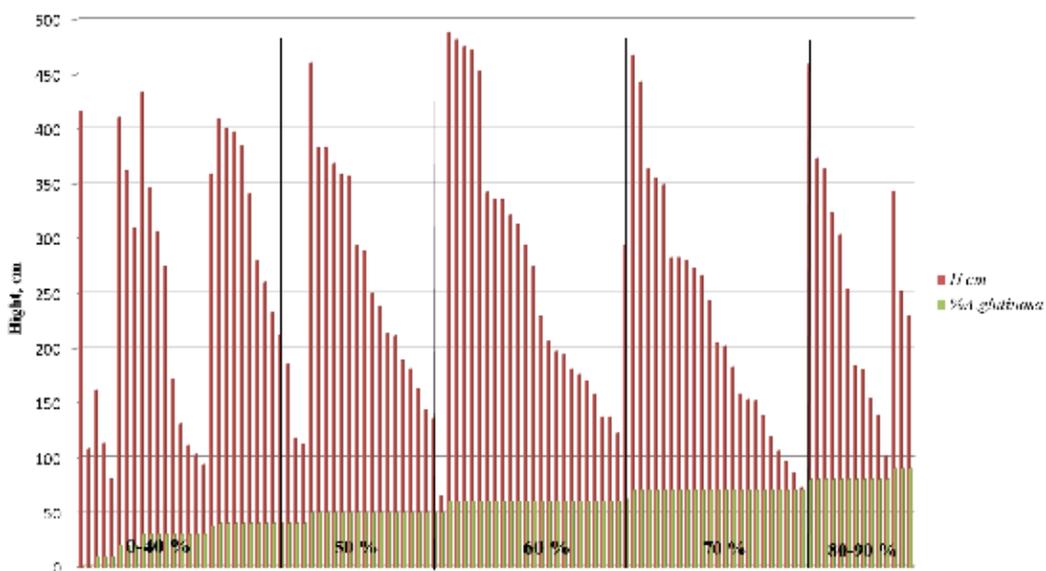


Fig. 5. Hybrid 5-year seedlings mean height (*Hv*) and *A. glutinosa* allele content.

Table 2. Mean height and diameter values by seedling age and comparison with reference *A. incana* and *A. glutinosa* data

Seedlings age	<i>A. incana</i> x <i>A. glutinosa</i> experimental plantation hybrids		Regenerated seedlings <i>A. incana</i> (Daugaviete 2010)		<i>A. incana</i> container seedlings (Liepins, unpublished data)	<i>A. glutinosa</i> container seedlings (Liepins, unpublished data)
	<i>Hv</i> , m	<i>Dv</i> , cm	<i>Hv</i> , m	<i>Dv</i> , cm	<i>Hv</i> , m	<i>Hv</i> , m
4 - y e a r seedlings	1.71±0.93	2.096±0.48	4.38±0.64	2.94 ±0.63	3.37-3.61	2.73
5 - y e a r seedlings	2.87±1.033	5.64±2.45	4.81±0.54	4.07 ±0.53	4.61-4.87	3.97

height of individuals with an *A. glutinosa* allele content of 0% to 40 % was 2.54 ± 1.19 m; with an equal *A. glutinosa*/*A. incana* allele content $Hv=2.60 \pm 1.04$ m; and with an *A. glutinosa* allele content from 60% to 90% $Hv=2.57 \pm 1.13$ m. The mean frequency of *A. incana*, *A. glutinosa* and hybrid loci in groups of different height in 2014 is shown in Fig. 6. The mean amount of heterozygotes is elevated in the group with the highest tree height, but not significantly.

Mean tree height (*Hv*, m) and diameter of root collar (*Dv*, cm) were compared to reference data for *A. incana* growth in individuals of the same age (Daugaviete 2010), where regeneration of vegetative shoots were measured. *Hv* also was compared with data from plantations where former farmlands were regenerated with *A. incana* or *A. glutinosa* containerised seedlings (Liepins & Liepins 2010). *Hv* in the experimental plantation in 2013 (4-years old) was 1.71 m (109 individuals measured); *Dv*=2.096 cm (34 individuals measured). *Hv* in 2014 (5-years old) was 2.87 m, and *Dv*=5.64 (343 individuals measured) (Table 2). Tree height in the experimental plantation varied from 0.24-3.8 m in 2013 and from 0.4-5.03 m in 2014. The mean values for the experimental plantation were lower than the values from the reference data. However, the variation was much higher in the experimental plantation, which is most probably a result of the advanced backcross hybrid status of the seedlings used to establish the experimental plantation.

DISCUSSION

Most hybrid species are weakly isolated from the parental species and tend to re-assimilate with one or the other parental species due to asymmetric levels of interspecific gene flow (Doyle, 2000). *A. incana* x *A. glutinosa* hybridisation occurs naturally in Latvia, and it is probable that introgressed hybrid alder trees are already constituents of natural populations. Grey and black alder habitat preferences and flowering times differ, but in areas with changeable microclimatic conditions hybrid trees could possibly have a competitive advantage. The origin of tetraploid *Alnus* species has not been studied, but some diploid species are known *Alnus hirsute* var. *microphylla*, *A. pendula*, and *A. serrulatooides* (Oginuma *et al.* 2000). Allotetraploids are characterized by disomic inheritance (Stebbins 1950, 1971, Ramsey & Schemske 2002).

Mean height and diameter measurements of the 4-year old individuals in the experimental plantation were lower than reference mean measurements for the parental species (*A. incana*) in the naturally regenerated stands (Daugaviete 2010) and *A. glutinosa* and *A. incana* plantations established with seedlings (Liepins, unpublished). However for 5-year old individuals in the experimental plantation, the mean height is still lower than the reference data, but the mean diameter was larger. Variation of data was higher compared with parental species

stands. Mean shoot height at a similar age for *A. glutinosa*, which has a slower growth rate (Liepins & Liepins, 2010), was larger compared with the experimental hybrid plantation. The growth parameter data from the experimental plantation may not be directly comparable to the data from the reference plantations of *A. incana* and *A. glutinosa* due to differences in environmental and other experimental conditions. However, comparison of the data can give a general indication of the comparative growth potential of the experimental plantation, and in particular, the amount of variation found between individuals in comparison to *A. incana* or *A. glutinosa* plantations. This data indicates that *Alnus* hybrid backcrosses could suffer from outbreeding depression. However several individual trees have growth parameters comparable to the parent tree species at the same age. Mean diameter of the root collar of 5-year old seedlings was similar between the experimental hybrid plantation and the *A. incana* naturally regenerated stand. It was also reported by Pirags (1962) that mean diameter at breast height of hybrid alder was higher than in parent species. No correlation found between growth parameters and allelic proportions in the various hybrids identified. Growth rate is known to change with ages in various tree species. An especially

large variation is expected in the experimental plantation of introgressed hybrids analysed in this study. Therefore measurements of growth over a number of years are necessary for precise conclusions regarding the growth potential of this hybrid material. Analysis of various fitness traits could also increase the amount of information available regarding the potential of the utilised planting material, however, this increases the cost of planting material and the success rate of vegetative propagation can be variable between individuals and species

According to the available data, the mother tree seed plantation was expected to consist of F1 hybrids and/or a mixture of both alder species (Kundzins 1957). The molecular analysis from this study suggests the presence of hybrid backcrosses already within the mother tree seed plantation, meaning that the analysed experimental plantation, which was established with seed from this plantation, consists of advanced generation (F3+) introgressed hybrids. Kundzins (1968) describes test pollination experiments where he uses not only *A. incana* x *A. glutinosa*, but also different combinations of the hybrid backcrosses to both parent species, as well as hybrid x hybrid crosses. He concludes that better growth was in

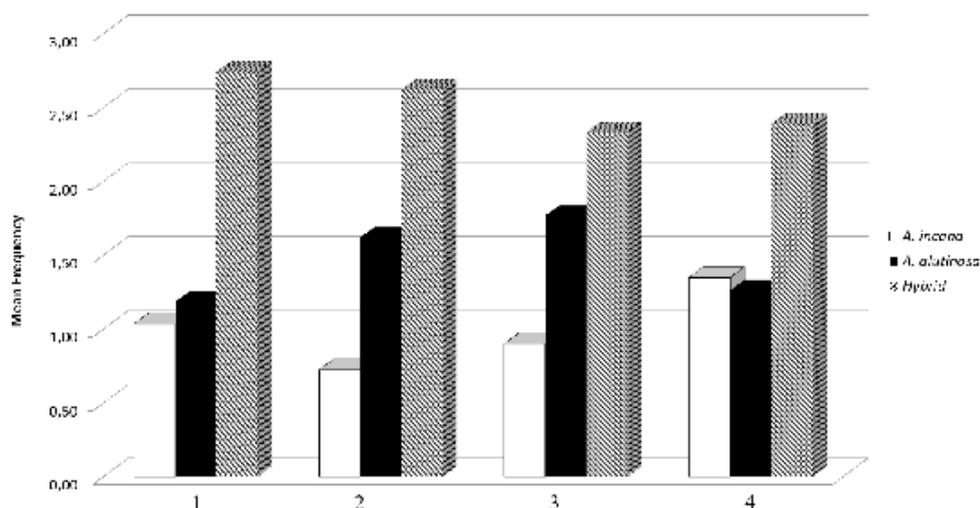


Fig. 6. Mean frequency of *A. incana*, *A. glutinosa* and hybrid loci in groups of different height in 2014. Height groups: 1: 3.55-4.88 m; 2: 2.50-3.49 m, 3: 1.52-2.43 m, 4: 0.65-1.44 m.

the first generation of hybrids, as well as *A. incana* x F1 hybrid trees, but trees from best hybrid x hybrid seeds (F2) exhibit lower growth than parent species. Therefore our results correspond to this information that the seed plantation also contains advanced hybrids and/or hybrid backcrosses to *A. incana*. Morphological analyses of the mother trees indicated that they were *A. incana*, however molecular marker analysis showed the advanced generation hybrid status of individuals from the mother tree seed plantation. The molecular marker analysis of the mother tree seed plantation confirmed the predominance of *A. incana* alleles in the seed plantation. Molecular marker analysis indicates that the mother trees from which seeds were collected had a larger proportion of *A. incana* alleles, but they contained a small proportion of *A. glutinosa* alleles. Open pollination and outcrossing can lead to the presence of backcrosses in the next generation, segregation of traits and increased phenotypic variation. A higher proportion of *A. glutinosa* alleles in the experimental plantation suggest pollination of mother seed trees with black alder or hybrid pollen, in addition, one tree with a predominance of *A. glutinosa* alleles was identified in the mother tree seed plantation. This is in agreement with the previously published data about the increased seed germination of hybrids with *A. incana* as the maternal parent (Kundzins 1957).

In addition to the previously reported *Alnus* species-specific SSR marker (Rungis et al. 2010), in this report we describe several *Alnus* species specific PCR-RFLP molecular markers that were used for identification of *Alnus glutinosa*, *Alnus incana* and their hybrids. Markers were constructed using coding gene regions and species-specific SNPs were identified using the TILLING technique. Markers were validated using samples from an *A. incana* plantation with known provenance, as well as morphologically identified individuals from each species and potential F1 hybrids (Rungis et al. 2010). Further analysis and research with these species-specific markers is required to determine the stability and precision of these markers. There is some evidence for recombination or assortment

within species for these markers, and analysis of additional putative *A. incana* and *A. glutinosa* individuals will enable assessment of the extent of variation of these markers within *Alnus* species. These species-specific markers, in conjunction with other species identification methods will also enable investigation of the extent of natural hybridisation of these two species.

CONCLUSIONS

Analysis of an experimental hybrid alder (*A. incana* x *A. glutinosa*) plantation revealed a high degree of variation in the allelic proportion of each parental species between individuals. Growth traits also demonstrated a high degree of variation in comparison to equivalent aged *A. incana* and *A. glutinosa* plantations. This high degree of variation can be attributed to segregation of alleles in advanced generation hybrid individuals. The mother tree seed plantation was also shown to contain advanced generation hybrids. Monitoring of the experimental plantation may reveal fitness advantages for these hybrid individuals in response to environmental or other conditions. The species-specific markers described in this report will be useful for further investigation of the extent and direction of natural hybridisation between *A. incana* and *A. glutinosa*.

ACKNOWLEDGEMENTS

This research was funded within the ERDF project Nr. 2014/0025/2DP/2.1.1.1.0/14APIA/VIAA/101. Development of identification technologies for vegetatively propagated short rotation tree species.

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Received: 27.04.2015.

Accepted: 08.06.2015.

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