Within- and Among-stand Genetic Diversity of Common Aspen (*Populus tremula* L.) in Latvia

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Abstract

Special areas for *in situ* protection of *Populus tremula* autochthonous populations – gene reserve units – are mainly chosen based on phenotypic characteristics of trees, species biology and abundance, but detailed information on the genetic composition of stands is lacking. The aim of this study was to assess the genetic diversity in common aspen stands in Latvia to provide information on the optimum area and designation of gene conservation units.

A total of 20 pure common aspen stands from 8 populations were analysed using 6 SSR markers at the age of 6 to 8 years after first pre-commercial thinning. The average number of ramets per clone was only 1.4 and most of the analysed individuals (80±4.9%) had a unique genotype. The maximum distance between identified ramets of most clones was 10 to 20 m. However, the maximum distance between identified ramets of some clones exceeded 80 m, reaching as much as 169.4 m.

Analysis of molecular variance (AMOVA) shows that 7% of the total genetic variation was between populations and 93% within populations ($P < 0.001$).

A comparison of genetic diversity parameters was made between the two regions designated as genetic resource units and the other analysed regions. The mean number of alleles was lower in the genetic resource stands (8.333 vs. 11.333). The effective number of alleles (Ne) and heterozygosity was also slightly lower in the genetic resource units (N_e – 3.513 vs. 3.967; H_o – 0.597 vs. 0.655; H_e – 0.705 vs. 0.731). To account for the sample size difference, a rarefaction approach implemented in the software package FSTAT was used to compare allelic richness and observed heterozygosity, and no significant differences were found between the two groups.

Cluster analysis of genetic distances demonstrated some congruence with the geographical distances between regions: regions in the western part of Latvia formed a separate group from those located in the eastern part of the country.

Key words: genotypic diversity, heterozygosity, gene reserve units, population, genetic distance.

Introduction

Common aspen (*Populus tremula* L.) is distributed across a diverse range of climatic conditions in Europe: from Greece in the south almost to the northern tree line in Scandinavia (EUFORGEN 2009). Its economic value is not very high (main use: energy-wood and specific niche products) and the quality of it is often reduced by browsing damage and rot. Therefore, in the Baltic Sea Region, aspen does not occupy large areas. In Latvia, according to National forest inventory data, aspen-dominated stands cover 244.71 thousand ha or 7.74% of the total forest area. However, common aspen is often present in admixture with other tree species (e.g. Norway spruce) and has a high importance in protection of biodiversity in boreal and also in hemiboreal conditions (Latva-Karjanmaa et al. 2007).

Common aspen can propagate both generatively by seed and vegetatively by root suckers. Seeds of this tree species are small and capable of long-range dispersal, however, their germination rate drops sharply after 2-3 days and the survival of seedlings is comparatively low (Latva-Karjanmaa et al. 2003, Mangalis 2004). Abundance of seed is an evolutionary adaptation ensuring that at least some of them will reach a favourable germination spot (e.g. open mineral soil after forest fire) and grow to reproductive age (Suvanto and Latva-Karjanmaa 2005). Decreasing area and frequency of natural disturbances are mentioned as some of the main causes of reduction of genetic diversity of common aspen (Myking et al. 2011).

Survival and distribution over short distances is ensured by root suckers: after harvesting or natural felling of trees, they can form young stands with densities up to 100 thousand trees per ha (Smilga 1968). Vegetative regeneration over several consecutive generations can lead to reduction of within-stand genetic diversity due to intensive competition between clones and survival of a (few) fittest clones

(Eriksson 1989). Additionally, aspen is shade intolerant, therefore the probability of new genotypes (seeds) sprouting (and surviving) in very dense stands is reduced (Jelinski and Cheliak 1992). Expansion of a particular clone is limited by diseases, aging, and accumulation of harmful mutations as well as competition between clones (Steinger et al. 1996), and also heterogeneity of growing conditions, ensuring differential survival and expansion among genotypes (Krasny and Johnson 1992, Peterson and Jones 1997).

Regeneration by root suckers is the most commonly used regeneration method after clear-cutting of common aspen stands in Latvia. There is a risk that in the long-term this practice might lead to a reduction of genetic diversity. For a related *Populus* species, American aspen (*Populus tremuloides* Michx.), a single clone represented by 47 thousand ramets occupying 43.3 ha was found (Kemperman and Barnes 1976). Reduction of the number of clones, similarly to fragmentation of stands, leads to a depletion of genetic diversity due to genetic drift and inbreeding (Aguilar et al, 2008). This process affects both selectively neutral and adaptive variation (Ennos et al. 1998). Past and present changes in genetic diversity are usually analysed using molecular markers linked to selectively neutral parts of the genome, however, the main practical interest is to maintain diversity of adaptive traits, ensuring the capacity of populations to adjust to changing conditions (e.g. climatic conditions) and survive (Alfaro et al. 2014). Therefore, special areas for *in situ* protection of autochthonous populations and rare genotypes are designated in many countries (Soutar and Spencer 1991, Eriksson et al. 1993, Ennos et al. 1998, Millar, Ledig and Riggs, 1990, Young et al. 2000). In Latvia, based only on phenotypic evaluation (stem quality, vitality) three genetic conservation units (groups of common aspen stands), with a total area 162.4 ha, located in the eastern part of the country (Limbaži GRS, Žīguri GRS and Jekabpils GRS municipalities, see Figure 1) have been designated as aspen genetic resource stands.

EUFORGEN (European forest genetic resources program) guidelines for the protection of genetic resources for common aspen, emphasize the need for active management of the designated stands, including creation of small clearcuts and soil scarification as measures to promote regeneration via seeds and suppression of regeneration via root suckers (von Wühlisch 2009). These guidelines, as well as other documents, emphasize the need for detailed information on the genetic composition of stands designated for genetic resources protection (Ennos et al. 1998) that can be used both in the selection process of the stands as well as in monitoring of changes over time. However, such information is seldom available and indirect criteria, linked to species biology and abundance, are often used for selection of these stands.

The aim of this study was to assess the genetic diversity in common aspen stands in Latvia to provide information on the optimum area and designation of gene conservation units. DNA markers were used to determine the number of clones and genetic diversity in young, naturally regenerated commercial common aspen stands. Genetic diversity and differentiation of currently designated aspen gene conservation units was compared with other analysed stands, to determine the amount of genetic diversity conserved in the current aspen gene conservation units and to assess the requirement for designation of additional aspen gene conservation units.

Materials and Methods

Aspen stands were selected in forest districts with a comparatively high proportion of aspen stands, and to ensure coverage of various regions within Latvia (Figure 1). The selected stands were young, naturally regenerated stands, and included two currently designated common aspen genetic resource stands (Limbaži GRF and Žīguri GRF regions).

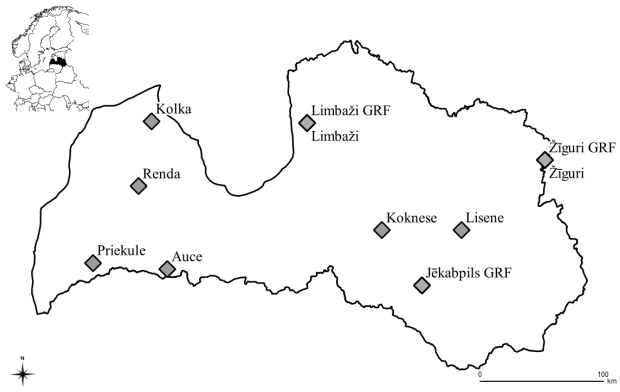

Figure 1. Location of gene reserve forests (GRF) and sampled populations of common aspen

Within the selected forest districts (regions), pure aspen stands aged from 6 to 8 years with an area e" 1 ha, in forest types *Oxalidosa*, *Aegopodiosa, Mercurialiosa mel.* were randomly selected based on forest inventory data (88% of aspen stands in Latvia are located in these forest types according to the National Forest Inventory data). In the majority of regions, samples were collected from 3 stands, however in the Priekule and Renda districts, samples were collected from a single stand in each district. Altogether samples were collected from 20 aspen stands: 16 in *Oxalidosa* forest type, 3 in *Aegopodiosa* and 1 in *Mercurialiosa mel* (Table 1).

In each stand, transects were established at regular distances and one sample tree selected every 10-15 m tray to cover overall aspen occupied area. GPS coordinates and samples for DNA extraction were taken from every sampled tree. A total of 779 individuals tree were used for clonal analysis. Each region was on average represented by 97 individuals.

Table 1. Characteristics of studied common aspen population

Region	Geographical coordinates	Number of stands	Total area, ha	Forest type	Age, years
Renda	57º14'N 22º19'E	1	3.8	*Mercurialiosa mel.*	8
Priekule	56º38'N 21º55'E	1	4.3	*Oxalidosa*	8
Lisene	56º40'N 26º47'E	3	9.8	*Oxalidosa, Mercurialiosa mel.*	8
Koknese	56º42'N 25º33'E	3	10.3	*Oxalidosa*	8
LimbažiGRS	57º29'N 24º36'E	3	6.1	*Aegopodiosa*	7
ŽīguriGRS	57º09'N 27º47'E	3	13.5	*Oxalidosa*	7
Dundaga	57º31'N 22º31'E	3	9.3	*Oxalidosa*	6
Auce	56º27'N 23º00'E	3	6.5	*Oxalidosa*	6

DNA was isolated from aspen leaves using a modified CTAB-based method (Doyle and Doyle 1987): polyvinyl pyrrolidone (PVP) and mercaptoethanol was added to the extraction buffer before use (Porebski et al. 1997). Samples were genotyped with six Simple Sequence Repeat (SSR) markers previously developed for black poplar (*Populus nigra* L.): WPMS05 (van der Schoot et al. 2000), WPMS14, WPMS15, WPMS20, WPMS18 and WPMS16 (Smulders et al. 2001). Forward primers were synthesised with a 6-FAM, HEX or NED fluorescent label in order to facilitate visualisation on an ABI Prism 3130x-Avant Genetic Analyzer (*Applied Biosystems*).

SSR amplification reactions were performed separately for each marker in a 20 mL reaction mixture containing 50 ng of DNA, 1x Taq buffer (*Fermentas*), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.1 M of each primer, 0.08 mg BSA (*Fermentas*), 0.5 U Taq polymerase (*Fermentas*). PCR conditions were as follows: initial denaturation for 3 min at 95 ºC, 38 cycles of denaturation at 95 ºC for 30 sec, annealing at 55 ºC for 30 sec, extension at 72 ºC for 30 sec; and one cycle of final extension at 72 ºC for 10 min. Amplification products were separated on an ABI Prism 3130x-Avant Genetic Analyzer (*Applied Biosystems*), and analysed with GeneMapper v4.0 (*Applied Biosystems*).

Clonal identity was determined by simple fragment matching using all genotyped individuals, and the number of clones and distances between ramets was calculated. Genotypic diversity was determined as the G/N ratio dividing the number of identified genotypes (G) in a stand with the number of sampled individuals (N) (Namroud et al. 2005). For characterize individual clone (clone size) spread in forest stand, we calculated the maximum distance between ramets of a single clone using GPS coordinates, and also calculated the average distance between ramets of a single clone in populations.

Sixty-one triploid individuals were identified, however, some of them were genetically identical ramets, and there were 22 distinct triploid genotypes identified. To allow genetic analysis using co-dominant markers, these triploid individuals were removed from the data set prior to further analysis. Identical diploid genotypes were not removed from the dataset prior to further analysis. Diploid genotypes were analysed using the Micro-checker software (Van Osterhout et al. 2004) to detect potential scoring errors and null alleles. The data were analysed by regions (8 regions) as well as by individual stands (20 stands). Genetic diversity parameters were analysed using GenAlEx 6.5 (Peakall and Smouse 2006) using codominant diploid genotypes. The Probability of Identity (*PI*) was calculated, which provides an estimate of the average probability that two unrelated individuals, drawn from the same randomly mating population, will by chance have the same multilocus genotype. Pairwise relatedness was estimated using the method described in Ritland (1996), and implemented in the GenAlEx program. The 95% confidence intervals about the mean value confidence limits and the 95% confidence intervals about the null hypothesis of 'No Difference' across the populations were determined by bootstrap resampling (999 permutations). Genetic distances (Nei's 1972) were calculated and bootstrap analysis of 1000 neighbour-joining dendrograms was performed using PowerMarker software (Liu and Muse 2005). Dendrograms were visualised using DarWin software (Perrier and Jacquemoud-Collet 2006). The software Fstat (Goudet 2001) was used to compare allelic richness, heterozygosity and relatedness between the genetic resource stands and other analysed stands (1000 permutations).

Results

Identification and analysis of clones

Average number of individuals with unique genotypes per region was 71. The probability of identity calculated for the six SSR markers was 1×10^{-6}, indicating that these markers had sufficient resolution to reliably identify ramets belonging to one clone, which were naturally propagated via root suckers. An average of 80% of analysed individuals were represented by a single genotype (Table 2), ranging from 72% in Limbaži GRS to 91% in Koknese. These regions had also the smallest (Limbaži GRS) and largest (Koknese) values of genotypic diversity index: 0.64 and 0.86, respectively, and quite similar expected heterozygosity (H_e) 0.673 in Limbaži GRS and 0.714 in Koknese, only the Renda stand has lower He 0.505 (Table 4). The number of ramets per clone was low (average 1.4) (Table 2), and only 14 clones (from 655) had more than 5 ramets, with a maximum of 17 ramets for one clone from Renda region.

Calculated average number of clones per hectare ranged from 6.1 to 26.8 and average distance between single clone ramets was 48±9.5 m. The smallest average distance was found in Lisene region (22 m), and the largest one in Koknese (85.6 m). Most part of identified clones (42±15.5%) maximum distance between ramets were 10 to 20 m, and then this proportion decreasing. Distance between 20 to 30 m spread (16±14.4%) and for the rest was further than 30 m (Figure 2).

Table 2. Clonal composition and genotypic diversity of common aspen populations

Population	Number of		Clones per ha	Individuals represented by a single genotype		Ramets per clone	Genotypic diversity index
	Individuals	unique genotypes		number	proportion, %		
Lisene	105	87	8.1	70	80.5	1.2	0.83
Koknese	90	77	7.9	70	90.9	1.2	0.86
LimbažiGRS	101	65	10.7	47	72.3	1.6	0.64
ŽīguriGRS	107	82	6.1	65	79.3	1.3	0.77
Kolka	95	59	6.3	59	69.0	1.6	0.60
Auce	102	74	9.9	60	81.1	1.4	0.73
Renda	84	67	26.8	52	77.6	1.3	0.80
Priekule	95	73	17.0	61	83.6	1.3	0.77
Mean	97	71	12.8±5.74	57±11.1	80±4.9	1.4±0.26	0.7±0.10

Notable differences among regions were found: in Koknese, Limbaži GRS and Auce, a relatively high portion of clones with more than one ramet (15±16.8%) had a maximum distance between ramets exceeding 80 m. Clone with larger number of ramets have greater distribution area, because strong and significant correlation between number of ramets per clone and distance between the two outermost ramets ($r = 0.77$, $P < 0.05$) was found.

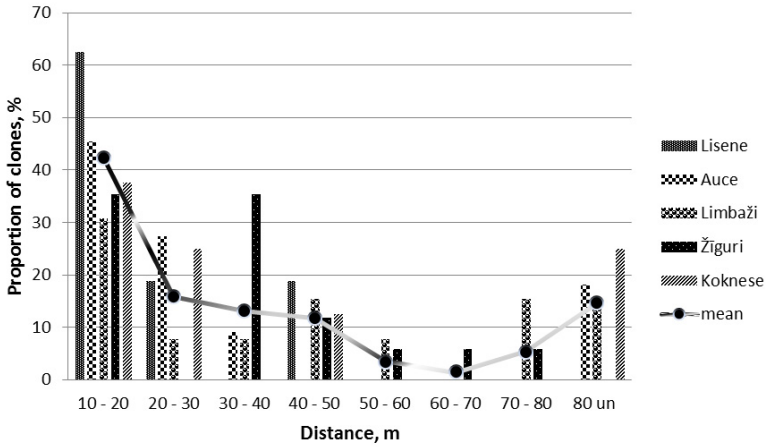

Figure 2. Distribution of clones according to maximum distance between their ramets

*Only genotypes with more than one ramet included

Genetic diversity and differentiation

The number of alleles per SSR locus ranged from 8 (WPMS15 and WPMS18) to 15 (WPMS05). Observed heterozygosity of markers ranged from 0.464 (WPMS05) to 0.793 (WPMS16). The F_{is} values for the loci ranged from -0.089 to 0.336 (Table 3). Analysis with the Micro-checker software of the entire dataset showed that the loci WPMS05, WPMS18 and WPMS20 had an excess of homozygous genotypes, indicating the possibility of null alleles. However, when the Micro-checker analysis was repeated on each region separately, then the excess of homozygotes was not detected in all regions for all markers. An excess of heterozygotes was detected in one region (eight) for marker WPMS15, three regions for marker WPMS20, four regions for marker WPMS18 and seven regions for marker WPMS05. All loci were retained for further analysis, with the caveat that the presence of null alleles may increase estimates of F_{st}.

The mean number of alleles varied from 4.333 in Renda to 9.167 in Priekule. The number of alleles with a frequency higher than 5% ranged from 2.833 in Renda to 4.833 in Auce and Kolka, indicating that more than half of the alleles are present at low frequencies. The estimated number of effective alleles (*Ne*) – describing the number of equally frequent alleles needed to achieve a given level of gene diversity – was similar among regions (Table 4). The number of unique alleles was determined for each region and no unique alleles were observed in Žīguri GRS, Kolka and Renda regions.

Table 3. Characteristics of SSR markers used in common aspen genotyping

Marker	Allelic size range, bp	No. of alleles	H_o	H_e	I	F_{st}	F_{is}
WPMS05	271-344	13	0.464	0.700	1.504	0.058	0.336
WPMS14	207-246	14	0.654	0.625	1.434	0.050	-0.047
WPMS15	182-203	8	0.759	0.697	1.336	0.023	-0.089
WPMS20	205-239	12	0.550	0.591	1.227	0.122	0.068
WPMS18	207-237	8	0.619	0.713	1.396	0.063	0.133
WPMS16	144-207	15	0.793	0.765	1.678	0.069	-0.037

Notes: H_o is the observed heterozygosity, H_e is the expected heterozygosity, I is the Shannon information index, F_{st} is the fixation index; F_{is} is the inbreeding coefficient.

Pairwise F_{st} values between regions ranged from 0.013 (Auce-Lisene) to 0.180 (Priekule-Renda) (Table 5). Analysis of molecular variance (AMOVA) shows that 7% of the total genetic variation was between regions and 93% within regions ($P < 0.001$). Analysis of the data by stands rather than regions showed that the pairwise Fst values increased (max. = 0.247, min. = 0.016, Table 6). AMOVA also showed that 10% of the total genetic variation was between stands and 90% within stands ($P < 0.001$).

Table 4. Mean allelic patterns in populations of common aspen

Population	Auce	Lisene	LimbažiGRS	ŽīguriGRS	Kolka	Koknese	Priekule	Renda
Na	7.833	8.167	7.167	7.000	8.167	8.000	9.167	4.333
Na Freq ≥5%	4.833	4.333	4.000	4.333	4.833	4.000	4.333	2.833
Ne	3.431	3.604	3.142	3.596	3.705	3.663	3.807	2.227
I	1.491	1.528	1.385	1.483	1.546	1.508	1.578	0.916
No. of unique alleles	0.500	0.167	0.167	0.000	0.000	0.167	0.833	0.000
He	0.702	0.720	0.673	0.713	0.714	0.710	0.717	0.505

Notes: N_a is the number of different alleles, N_a Freq ≥ 5 % is the number of different alleles with a frequency ≥ 5 %, N_e is the number of effective alleles, *I* is the Shannon's information index, No. of unique alleles is the number of alleles unique to a single population, H_e is the expected heterozygosity.

Table 5. Pairwise region F_{st} values

	Auce	Lisene	LimbažiGRS	ŽīguriGRS	Kolka	Koknese	Priekule	Renda
Auce	-	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Lisene	0.013	-	0.001	0.001	0.001	0.001	0.001	0.001
Limbaži	0.037	0.028	-	0.001	0.001	0.001	0.001	0.001
Žīguri	0.023	0.020	0.024	-	0.001	0.001	0.001	0.001
Kolka	0.018	0.013	0.043	0.019	-	0.001	0.001	0.001
Koknese	0.041	0.028	0.056	0.037	0.025	-	0.001	0.001
Priekule	0.093	0.083	0.098	0.087	0.078	0.112	-	0.001
Renda	0.089	0.095	0.141	0.112	0.092	0.143	0.180	

Notes: F_{st} values below diagonal, probability, *P*(rand >= data) based on 999 permutations is shown above diagonal.

Table 6. Pairwise stand F_{st} values

Site	Auc1	Auc2	Auce3	Lis1	Lis2	Lis3	Limb1	Limb2	Limb3	Žīg1	Žīg2	Žīg3	Kolk1	Kolk2	Kolk3	Kok1	Kok2	Kokn3	Priek	Rend
Auc1	-	**	**	**	**	**	**	**	**	**	**	*	**	**	**	**	**	**	**	**
Auc2	0.17	-	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Auc3	0.07	0.22	-	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Lisen1	0.04	0.15	0.04	-	**	**	**	**	**	**	**	**	*	**	**	**	**	**	**	**
Lisen2	0.05	0.18	0.05	0.05	-	*	**	**	*	**	**	**	**	**	**	**	**	**	**	**
Lisen3	0.03	0.17	0.05	0.05	0.02	-	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Limb1	0.07	0.21	0.14	0.08	0.12	0.10	-	**	**	**	**	**	**	**	**	**	**	**	**	**
Limb2	0.08	0.18	0.10	0.08	0.10	0.06	0.06	-	**	**	**	**	**	**	**	**	**	**	**	**
Limb3	0.05	0.20	0.06	0.05	0.02	0.03	0.08	0.07	-	**	**	**	**	**	**	**	**	**	**	**
Žīg1	0.04	0.16	0.06	0.04	0.04	0.04	0.07	0.05	0.04	-	**	**	*	**	*	**	**	**	**	**
Žīg2	0.06	0.18	0.04	0.05	0.06	0.07	0.10	0.09	0.06	0.05	-	**	**	**	**	**	**	**	**	**
Žīg3	0.03	0.23	0.06	0.06	0.04	0.04	0.07	0.08	0.05	0.05	0.06	-	**	**	**	**	**	**	**	**
Kolk1	0.03	0.15	0.06	0.02	0.03	0.04	0.09	0.09	0.05	0.02	0.05	0.05	-	**	*	**	**	**	**	**
Kolk2	0.07	0.20	0.06	0.07	0.04	0.05	0.14	0.10	0.06	0.06	0.06	0.08	0.07	-	**	*	*	**	**	**
Kolk3	0.05	0.19	0.05	0.06	0.05	0.05	0.13	0.12	0.05	0.03	0.06	0.07	0.02	0.06	-	**	**	**	**	**
Kokn1	0.11	0.20	0.10	0.10	0.05	0.06	0.17	0.14	0.08	0.08	0.09	0.13	0.09	0.02	0.09	-	**	**	**	**
Kokn2	0.09	0.25	0.10	0.10	0.07	0.09	0.14	0.13	0.10	0.09	0.07	0.07	0.10	0.03	0.10	0.08	-	**	**	**
Kokn3	0.08	0.20	0.13	0.11	0.08	0.05	0.12	0.13	0.10	0.08	0.13	0.08	0.07	0.12	0.08	0.13	0.13	-	**	**
Prie	0.12	0.22	0.09	0.11	0.10	0.08	0.17	0.11	0.09	0.08	0.10	0.12	0.09	0.10	0.09	0.13	0.16	0.14	-	**
Ren	0.11	0.20	0.14	0.10	0.12	0.13	0.20	0.22	0.13	0.13	0.15	0.13	0.10	0.16	0.11	0.18	0.18	0.22	0.18	-

Notes: F_{st} values below diagonal, probability, *P*(rand >= data) based on 999 permutations is shown above diagonal: (*<0.05; **=0.01); Abbreviations of the sites – first 3-4 letters of the name of geographical location, see Figure 1.

The mean pairwise relatedness values (Ritland, 1996) within each region were significantly higher than across the entire dataset ($P < 0.001$). In particular, the mean pairwise relatedness values were higher in Priekule and Renda regions (Figure 3). These were the regions, where only one stand was sampled, in contrast to the other regions, where 3 stands per region were sampled. The mean pairwise relatedness estimates within each stand were also significantly higher than across the entire dataset ($P < 0.001$), with the exception of the stands Lisene 2 ($P = 0.003$), Kolka 2 ($P = 0.051$), Koknese1 ($P = 0.020$) and Koknese 2 ($P = 0.025$) (Figure 4). The mean pairwise relatedness values within each stand were higher, and more similar to Priekule and Renda stands/regions. The Auce 2 stand had a much higher mean due to the fact that only 9 unique genotypes were found in this stand.

A comparison of genetic diversity parameters was made between the two regions designated as genetic resource stands and the other analysed regions. The mean number of alleles was lower in the genetic resource stands (8.333 vs.

11.333); however, the mean number of alleles with a frequency over 5% was the same (4.667). The effective number of alleles (N_e) and heterozygosity was also slightly lower in the genetic resource stands (*Ne* – 3.513 vs. 3.967; H_o 0.597 vs. 0.655; H_e – 0.705 vs. 0.731). There were two unique alleles in the genetic resource stands, and 20 unique alleles found only in the other analysed regions. However, most of these were low frequency alleles, only one was with a frequency over 5% (f = 0.081). To account for the sample size difference, a rarefaction approach implemented in the software Fstat was used to compare allelic richness and observed heterozygosity, and no significant differences were found between the two groups.

Cluster analysis of genetic distances demonstrated some congruence with the geographical distances between regions: regions in the western part of Latvia (Kolka, Renda, Auce) formed a separate group from those located in the eastern part of the country (Limbaži GRS, Žīguri GRS, Koknese) (Figure 5). However, the bootstrap values indicated a low level of support for these clusters. Phylogenetic analysis of genetic distances between stands was even less clear, with all branchpoints having less than 50% bootstrap support (data not shown).

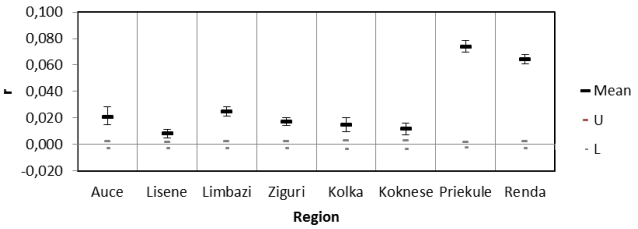

Figure 3. Mean within region pairwise relatedness values (estimated according to Ritland, 1996). Upper (Uv error) and lower (Lv error) error bars bound the 95% confidence interval about the mean values as determined by bootstrap resampling. Upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of 'No Difference' across the populations as determined by permutation

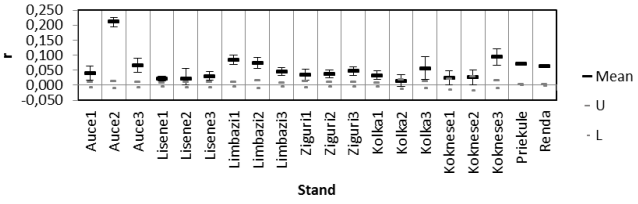

Figure 4. Mean within stand pairwise relatedness values (estimated according to Ritland, 1996). Upper (Uv error) and lower (Lv error) error bars bound the 95% confidence interval about the mean values as determined by bootstrap resampling. Upper (U) and lower (L) confidence limits bound the 95% confidence interval about the null hypothesis of 'No Difference' across the populations as determined by permutation

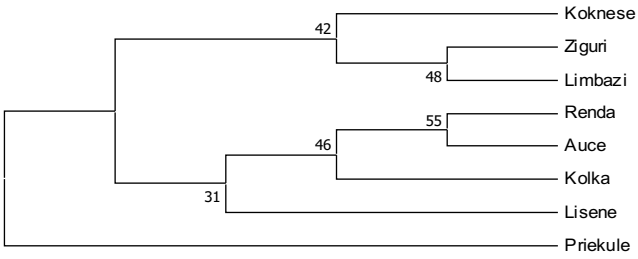

Figure 5. Consensus dendrogram of 1000 neighbour joining trees of Nei (1972), genetic distances between regions of common aspen. Numbers at branchpoints indicate bootstrap support (%)

Discussion

The number of clones per hectare influences the genotypic diversity of stands, but only few studies have assessed this for common aspen. Easton (1997) using seven isoenzyme markers identified 21 clones in an area of 4.6 ha: on average 4.6 clones ha^{-1}. An identical result was reported by Culot et al. (1995) in an area of 3 ha also using allozymes markers. These results were notably lower than the average in our study, 12.8±5.74 clones ha^{-1}. The differences could be attributed to: a) variation among stands (characteristics of the particular stands analysed) – also in our study the number of clones per hectare varied greatly (6.1 – 26.8 ha^{-1}); b) sensitivity of clone identification could be higher using SSR markers compared to the less polymorphic isozyme markers (Rajora and Rahman 2003). Using 9 SSR markers in nine stands (altogether 219 sample trees) Suvanto and Latva-Karjanmaa (2005) found 12.7 clones ha^{-1} in boreal conditions in Finland (61º 12'-64º 12' N, 25º 04'-29º 51' E), which was similar to that reported here. Comparison among studies might be influenced not only by the method of clone identification, but also by the sampling density. Since none of the studies (including ours) genotyped all trees in stands, but relied on selected sample trees, and there was on average only 1.4 ramets per clone (ranging from 1.2 to 1.6), it is likely that the results underestimate the true within-stand genotypic diversity.

For most (42 ± 15.5%) of the genotype with more than one ramet (representing on average 20% from all clones), the maximum distance between ramets was 10 to 20 m – close to a distance between the sample trees. This coincides with the distance, which is the greatest number of one stump root suckers (Suvanto and Latva-Karjanmaa 2005). This result indicates that increasing sampling density might lead not only to increased number of identified genotypes, but also an increase in the number of ramets per clone. Some of the population (Koknese, Limbaži GRS, Auce) had a higher proportion of clones with the maximum distance between their ramets exceeding 80 m (Figure 2). In some cases, ramets from one clone were identified up to 136.3 m (Auce) and 169.4 m (Limbaži GRS) apart. These results notably exceed

the previously reported distance of 60 m (Suvanto and Latva-Karjanmaa 2005) and indicates that several clones in these stands might have a selective advantage and/or are present in particular site for a comparatively longer time (several generations). Average distance between ramets in Koknese was 85.6 m, noticeably exceeding the average across sample stands: 48 ± 9.5 m. However, in Koknese, Limbaži GRS and Auce regions the proportion of genotype represented by a single ramet is high: from 72% (Limbaži GRS) to 91% (Koknese), therefore firm conclusions on the dominance of several clones cannot be inferred. A notably lower proportion of clones represented by a single ramet have been found previously: from 16 % (Suvanto and Latva-Karjanmaa 2005) up to 64% (Easton 1997). Consequently, also the average number of ramets per clone was higher than in our study, from 2.0 (Suvanto and Latva-Karjanmaa 2005) and for comparison using allozymes markers 1.8 (Easton 1997) to 9.8 (Culot et al. 1995).

Genotypic diversity is influenced by the influx of new genotypes, competition among genotypes and the history of stand. Influx of new genotypes is ensured by seeds (Soane and Watkinson 1979) that can fly long distances and regeneration from seeds is reported to be relatively successful for common aspen (Worrell et al. 1999, Suvanto and Latva-Karjanmaa 2005). Intense competition, reducing the number of genotypes, occurs in the first years of tree growth. For example, a 50% reduction in the number of sprouts during the first vegetation season was found for American aspen (Krasny and Johnson 1992) followed by a further notable reduction in the number of genotypes during early phases of stand development (Watkinson and Powell 1993). Additional factors contributing to this process are competition between species and browsing damages (Edenius and Ericsson 2007). The influences of these factors were not assessed in this study, since the material was collected after the first pre-commercial thinning. A gradual reduction of the number of genotypes and a wider spread of ramets of a particular (the fittest) clone is expected, if aspen stands are naturally regenerating over several generations and its roots can reach further form initial stump. Our data were not suitable for the analysis of this tendency, since inventory information for only one (previous) generation was available. Lisene was the only region originating from spruce and birch dominated stands with aspen admixture (≤40%) in the previous generation (the rest developed from aspen-dominated stands), and had the smallest average distance between ramets (22 m), however, its genotypic diversity (0.83) is similar to that of Koknese (0.86) (Table 2) – population with the largest average distance between ramets.

The genetic diversity detected using the six SSR markers was high, and the number of alleles per locus was greater than reported previously for 23 genotypes of *Populus nigra* (Smulders et al. 2001). The observed and expected heterozygosity values were also higher than reported previously in *P. tremula* (Suvanto and Latva-Karjanmaa 2005). Although the utilised markers were developed in a different *Populus* species, there was little indication of a high frequency of null alleles. The presence of null alleles was suggested for some loci, but not in all analysed regions, and only the locus WPMS05 had an excess of homozygotes over the expected values in seven of the eight analysed regions. The pairwise F_{st} values between regions were higher for Renda and Priekule regions. Samples were collected from only one stand in these two regions, in contrast to the other regions, where samples were collected from three stands. Genetic diversity parameters such as number of alleles and heterozygosity were lower in Renda region, but were similar to the other regions for Priekule region. However, the pairwise relatedness values were higher in these two regions, which could explain the differentiation of these two regions in contrast to the other regions. Differentiation between the other analysed regions (where three stands per region were analysed) did not show the same degree of differentiation, and the pairwise F_{st} values were all lower than 0.030. A similar situation was found, when the data was analysed at stand level – the most differentiated stands were the ones with the highest mean pairwise relatedness.

Comparison of the existing genetic resource stands (Limbaži GRS and Žīguri GRS regions) and the other regions did not detect any significant differences in genetic diversity parameters such as allelic richness and heterozygosity. The mean number of high frequency alleles (>5%) was the same, but there was a higher number of unique low frequency alleles in the other regions. This is probably due to the sample size differences between the groups, but has implications for genetic resource management. One of the aims of genetic resource conservation is to maximise the amount of genetic diversity that will be available for future utilisation. This includes the conservation of rare allelic variants, which could potentially have a high adaptive value, particularly in changing environments. Therefore, the designation of *P. tremula* genetic resource stands should maximise the number of stands with a sufficient amount of genetically distinct individuals in order to conserve these rare allelic variants. The use of a small number of neutral markers can reveal large scale population differentiation, and changes in genetic diversity, but often differences in adaptive traits are more difficult to determine at a molecular level (i.e. $F_{st} < Q_{st}$). The neutral markers utilised in this study were effective at identifying clones and relatedness within naturally regenerated aspen stands. Differences in relatedness that were detected could be related to sampling strategy (i.e. the higher pairwise relatedness in region, where only one stand was sampled) or the characteristics of a particular stand (i.e. Auce 2, where only 9 clones were identified). Therefore, selection of genetic resource stands should be done using a combination of criteria, including agro-climatic factors, potentially useful traits for breeding as well as analysis with

molecular markers to analyse the genetic diversity and relatedness of the selected stands. Our results indicate that for effective conservation of genetic diversity of common aspen in gene reserve units, a higher number of smaller stands distributed in different parts of the country should be preferred over the existing strategy of a few larger areas only in the eastern part. Successful conservation of genetic units would require active management. Removal of late successional tree species and clear cutting of small areas and soil scarification would provide suitable conditions for natural seeding from adjacent stands would be successful (von Wühlisch 2009).

In order to estimate optimal size of the gene reserve forest as well as to characterize the influence of sampling on the estimated values of genetic diversity and to establish a sampling strategy for monitoring of changes in the diversity measures within gene reserve units, further studies should include larger areas (stands) with smaller distances between sampled trees (ideally – genotyping all trees). Also, changes in genetic diversity over time (age of the stand and regeneration) should be addressed.

Conclusions

The number of clones in young (6-8 years), naturally regenerated *Populus tremula* stands identified in this study is similar to that found in older managed and old growth stands (35-152 years) in Finland, and is lower than found in other poplar species. Overall genetic diversity is high, and population differentiation is low. However, a multifaceted approach is required for selection of genetic resource stands for *Populus tremula*, which takes into account adaptive potential and genetic diversity and relatedness.

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