Genetic Diversity and Differentiation of Even-aged Norway Spruce Stands in Latvia

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Abstract

Norway spruce (*Picea abies* L. Karst.) is an important species in Latvia both ecologically and economically, and has been subjected to silvicultural management in Latvia since at least the middle of the 19th century. Forest regeneration activities starting in the 1960s resulted in the establishment of spruce stands with uncertified and often undocumented reproductive material. These spruce stands were often established by sowing, and no research or clear guidelines regarding the optimal density were available. As a result, many spruce stands were established and maintained at a high density. The growth of young spruce stands is initially slow, with annual height increment of 10-20 cm until the trees reach height of approximately two meters and in favourable growth conditions this stage is followed by a rapid increase in all stand parameters. However, the growth of some even-aged pure spruce stands abruptly declines at the age of approximately 40 years, while in other stands of similar age and composition this decrease or collapse is not observed. A comprehensive survey of even-aged spruce stands in Latvia has been undertaken, and factors influencing this decline in the growth potential of even-aged spruce stands have also been investigated, however, the genetic diversity and differentiation of even-aged spruce stands has not been investigated. A total of 19 SSR markers were utilised to genotype the 7 even aged spruce stands with differing growth potential. The genetic analysis and comparison of the perspective and non-perspective even-aged spruce stands indicated that the genetic diversity was not decreased in the non-perspective stands, and that genetic differentiation between stands and groups with differing growth potential assessments was low. The results obtained in this study suggest that the growth potential of even-aged Norway spruce stands is more dependent on the influence of environmental factors and management regime than genetic factors. This is a positive message, as in this case the conditions may be changed by the application of suitable management regime.

Keywords: forest management, silviculture, microsatellite markers, regeneration, stand density, thinning

Introduction

Norway spruce (*Picea abies* L. Karst.) is the third most widespread tree species in Latvia, and is important both ecologically and economically, and according to forest statistics, spruce forests cover 604 thousand ha or 18% of the total forest area. Norway spruce is an economically important tree species: in 2016, 16% of volume yield from harvesting was spruce timber and pulpwood (18% in state forests, 14% in other forests) (Anon., 2017). The average volume yield in spruce stands is considerably lower than in the pine stands, only 203 m^3 ha^{-1}, compared to 245 m^2 ha^{-1} (Anon 2017).

Norway spruce has been subjected to silvicultural management in Latvia since at least the middle of the 19th century, according to forest inventories from this period. Spruce stand thinning activities were not initiated until the beginning of the 20th century, as a result of the emerging market for pulpwood. Furthermore, as the economic importance of spruce increased, forest regeneration activities were introduced, which resulted in the establishment of spruce stands with uncertified and often undocumented reproductive material. Starting from the 1960s, artificial regeneration of spruce rapidly increased, often also in areas that were more suited for Scots pine. This increase in the proportion of spruce in Latvian forests was not only a result of economic factors, but also was influenced by other considerations, such as the intensive ungulate browsing pressure on juvenile pine stands (Saliņš 2002). These spruce stands were often established by sowing, and no research or clear guidelines regarding the optimal density were available. As a result, many spruce stands were established and maintained at a high density. However, subsequent research has determined that maintain spruce stands at high densities does not reduce browsing damages or

increase growth (after thinning) (Saliņš 2002), and in fact can reduce the quality and health status of the stand due to excessive mutual competition among the trees leading to lower nutrient availability to individual trees, lower stand resistance to climate extremes and increased infection by *Heterobasidion spp.*, further resulting in growth and stem quality reduction (Venn and Solheim 1994, Saliņš 2002, Zālītis and Špalte 2001, Dobbertin 2005, Zālītis and Jansons 2009, D'Amato et al. 2013, Zālītis et al. 2017).

Currently, the even aged stands of spruce with little or no admixture species cover approximately 38,800 ha, which accounts for about 40% of all pure stands of spruce in state-owned forests. Long-term experiments have shown that the growth of young spruce stands is initially slow, with annual height increment of 10-20 cm until the trees reach height of approximately two meters. In favourable growth conditions this stage is followed by a rapid increase in all stand parameters, often with the annual volume growth as high as 20 $m^3ha^{-1}year^{-1}$ in 30-50-year-old stands (Zālītis and Lībiete 2003, Zālītis and Lībiete 2005). However, the growth of some even-aged pure spruce stands abruptly declines at the age of approximately 40 years, while in other stands of similar age and composition this decrease or collapse is not observed (Lībiete and Zālītis 2007).

A comprehensive survey of even-aged spruce stands in Latvia has been undertaken, and factors influencing this decline in the growth potential of even-aged spruce stands have also been analysed (Zālītis and Lībiete 2005, Lībiete and Zālītis 2007, Lībiete 2008). However, the genetic diversity and differentiation of even-aged spruce stands has not been investigated. The use of neutral DNA markers can reveal levels of genetic diversity and population structure. The origin of much of the reproductive material utilised in the establishment of even-aged spruce stands in the 1960s and 1970s is unknown. In addition, wind storms in 1967 and 1969 destroyed thousands of hectares of forest stands, particularly affecting spruce (Ērglis and Matuzānis 1973, Ērglis 1977). There is anecdotal evidence that as a result of the storms there was a deficit of spruce reproductive material for regeneration, and that spruce reproductive material was imported from other regions, e.g. Ukraine. However, there are no records that can confirm this, or provide information of the volume of material potentially introduced, or where it was deployed. Therefore, the aim of this study was to utilise DNA markers to analyse even-aged spruce stands with differing growth potential to determine if differences in the genetic diversity of differentiation could be identified between perspective and non-perspective even-aged spruce stands.

Materials and Methods

Within the State Research Programme project "Growth potential of even-aged spruce forests in fertile forest ecosystems", a repeated assessment of 283 pure even-aged Norway spruce stands was performed in 2015-2017 according to the methodology developed in 2002 (Libiete and Zalitis 2007). According to this methodology, the even-aged spruce stands are divided into three growth potential groups depending on the volume growth: 1) perspective - the volume growth equal to or above 10 m^3ha^{-1} a year; 2) increased risk - the recent volume difference positive yet less than 10 m^3ha^{-1} a year; 3) non-perspective - the recent volume difference negative or close to zero. The growth potential group of each individual stand is in practice determined according to the parameters of linear correlation between the tree diameter and total width of last five annual rings; as described previously (Lībiete and Zālītis 2007, Lībiete 2008). In order to determine possible influence of the genetic factors on the growth potential of spruce seven pure even-aged spruce stands from the assessed compartments were selected for the genetic diversity analyses. Three of the selected compartments belonged to the group of perspective stands and four ones to the group of non-perspective stands. The age of the analysed stands ranged from 39 to 48 years, mean height varied from 15 to 21 m, mean diameter from 15 to 19 cm, mean basal area from 21 to 37 m^2 ha^{-1} and mean standing volume from 171 to 386 m^3 ha^{-1} (Table 1).

Table 1. Main stand parameters of the analysed stands

Stand	Area, ha	Site type	Stand age in 2015 (years)	Mean height (m)	Mean diameter (cm)	Basal area (m^2ha^{-1})	Standing volume (m^3ha^{-1})	Growth potential group in 2017	Coordinates
604-290-1	1.00	*Myrtillosa mel.*	48	21	18	37	386	Non-perspective	56.742, 24.153
604-377-3	3.10	*Hylocomiosa*	42	15	15	26	215	Perspective	56.684, 24.126
610-236-8	0.90	*Myrtillosa turf. mel.*	43	16	15	23	201	Non-perspective	56.736, 24.020
610-256-8	1.40	*Myrtillosa turf. mel.*	44	15	16	20	171	Non-perspective	56.728, 24.065
611-53-16	2.50	*Mercurialiosa mel.*	40	21	19	32	315	Perspective	56.590, 23.749
705-43-3	0.70	*Hylocomiosa*	42	17	19	21	191	Perspective	57.381, 22.023
711-368-16	2.60	*Hylocomiosa*	39	16	15	31	264	Non-perspective	57.156, 22.799

Spruce needle samples were collected from the even aged spruce stands: 604-290-1 (25 individuals), 604-377-3 (24 individuals), 610-236-8 (21 individuals), 610-256-8 (15 individuals), 611-53-16 (31 individuals), 705-43-3 (31 individuals), 711-368-16 (35 individuals). In addition, 48 samples were analysed from two 'natural' spruce stands to compare to the even aged stand results. These stands

were the Rēzekne spruce forest genetic resource (FGR) stand (coordinates: 56.599, 27.414), and the Moricsala nature reserve (coordinates: 57.195, 22.147). Both of these stands were considered to be autochthonous and naturally established. The mean age of the Rēzekne FGR stand is over 100 years, and the Moricsala nature reserve is the oldest nature reserve in Latvia, established in 1912, and is a strictly protected area.

Table 2. Microsatellite loci utilised for genotyping spruce individuals

Locus	Repeat motif	Reference
SpAGC1	$(TC)_5TT(TC)_{10}$	Pfeiffer et al, 1997
SpAGC2	$(TA)_{11}(GA)_{20}$	Pfeiffer et al, 1997
SpAGG3	$(GA)_{23}$	Pfeiffer et al, 1997
UAPgCA91	$(CA)_{20}$	Hodgetts et al, 2001
UAPgTG25	$(TG)_{27}$	Hodgetts et al, 2001
UAPgAG150	$(AG)_{19}$	Hodgetts et al, 2001
WS0033.A18	$(TA)_{26}$	Rungis et al, 2004
WS0022.B15	$(AG)_{12}$	Rungis et al, 2004
WS0073.H08	$(AT)_{14}$	Rungis et al, 2004
PAAC17	$(AC)_{30}$	Scotti et al, 2000
WS0073cG10	$(GGC)_8$	Rungis, unpublished
paGB3	$(AT)_{11}$	Besnard et al, 2003
WS0081aA12	$(AT)_9$	Rungis, unpublished
EAC2C08	$(AC)_{25}$	Scotti et al 2002a
EATC1D02A	$(TCC)_4N_{15}(TCA)_{16}TCC(TCA)_5(TCC)_4(TCA)_4$	Scotti et al 2002b
EATC2B02	$(CAT)_8$	Scotti et al 2002b
EATC2G05	$(AAT)_5(CAT)_{16}CAA(CAT)_4$	Scotti et al 2002b
EATC1E03	$(CAT)_4CGT(CAT)_8CGT(CAT)_4CGT(CAT)_4CGT(CAT)_4$	Scotti et al 2002b
SpAC1F7	$(AC)_{12}$	Pfeiffer et al, 1997

DNA from spruce needles was isolated using a CTAB-based method (Porebski et al. 1997). Genotyping of the even-aged stands was done using 19 nuclear SSR markers (Table 2). Each forward primer was labelled with a different fluorophore (6-FAM, HEX or TMR) to facilitate visualisation using capillary electrophoresis. The PCR reactions for the nuclear SSR markers were carried out in a 20 µl solution containing a final concentration of 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.2µM of each primer, 1.5 µl DNA solution, 1× Taq buffer and 1U of recombinant Taq DNA polymerase (Thermo Scientific). PCR cycling conditions consisted of an initial denaturation step of 95°C for 4 min; 35 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 60 s; followed by a final extension step of 72°C for 10 min. All PCR reactions were carried out in an Eppendorf Mastercycler ep gradient thermal cycler. Amplification fragments were separated on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems) and visualized with GeneMapper 3.5. Genotype data was checked using the Micro-checker software (Van Oosterhout et al. 2004) to identify errors caused by the presence of null alleles and other factors. Analysis of nuclear SSR data was done using GenAlEx 6.5 (Peakall and Smouse 2012) and Fstat v.2.9.3.2 (Goudet 1995). Phylogenetic analysis was conducted using MEGA version 5.2 (Tamura et al. 2011).

Results

The mean age of the stands in both growth potential groups was similar, with the non-perspective stands slightly older (41 and 44 years, respectively). The standing volume in the non-perspective stands was on average higher, although no statistically significant differences were detected (Figure 1). This trend confirms the former high productivity of the non-perspective stands.

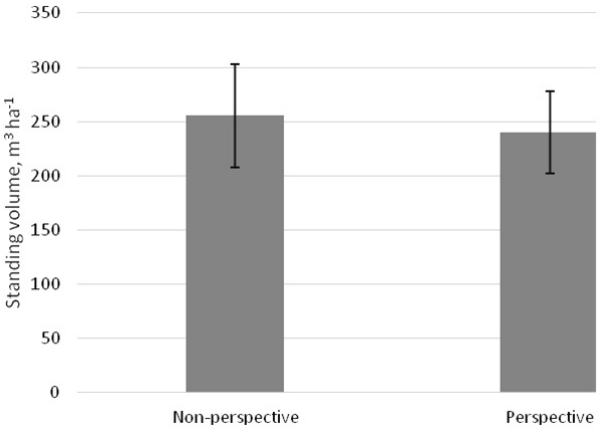

Figure 1. Mean standing volume in the analysed stands by growth potential group

A total of 19 SSR markers were utilised to genotype the seven even-aged spruce stands with differing growth potential. As reported previously (Hodgetts et al. 2001), marker UAPgAG150 amplified two loci, which were independently genotyped, therefore the stands were genotyped at a total of 20 loci. The number of loci amplified at each locus ranged from 4 (UAPgAG150A) to 23 (SpAGC1), with a mean of 12.45±1.30. The number of effective alleles ranged from 1.59 (EATC1E03) to 9.89 (EAC2C08), the mean value is 4.86±0.62. Shannon's information index (I) ranged from 0.78 (EATC1E03) to 2.48 (WS0022.B15), the mean value is 1.72±0.13. The mean observed heterozygosity (0.53±0.04) was lower than the expected heterozygosity (0.72±0.04), and the fixation index was above zero for all analysed loci, ranging from 0.04 (SpAGG3) to 0.69 (WS0033.A18), the mean value is 0.26±0.04. This indicates that there was an excess of homozygotes compared to the expected values assuming that the populations are in the Hardy-Weinberg equilibrium. When these genetic diversity parameters were calculated for each stand separately, a similar range of values were obtained. Most of the loci had lower observed heterozygosities than the expected values across all analysed stands (with the exception of the locus SpAGG3). As the loci with potential null alleles were at least partially overlapping among all analysed populations, all loci were retained for further analysis, with the caveat that the

level of genetic polymorphism might be underestimated at the loci with potential null alleles.

Table 3. Pairwise *Fst* values between analysed even-aged spruce stands. *Fst* values shown below diagonal. Probability, *P* (random >= data) based on 999 permutations is shown above diagonal

	711-368-16	610-236-8	604-290-1	610-256-8	705-43-3	611-53-16	604-377-3
711-368-16	0.000	0.001	0.001	0.001	0.105	0.001	0.001
610-236-8	0.024	0.000	0.001	0.001	0.001	0.002	0.202
604-290-1	0.084	0.052	0.000	0.001	0.001	0.001	0.001
610-256-8	0.073	0.052	0.026	0.000	0.001	0.001	0.001
705-43-3	0.003	0.020	0.078	0.070	0.000	0.001	0.001
611-53-16	0.036	0.011	0.051	0.058	0.032	0.000	0.100
604-377-3	0.028	0.003	0.054	0.055	0.031	0.004	0.000

The differentiation of the analysed stands was generally low, with an *Fst* value of 0.039 (p <0.001) (Table 3). Ten pairwise *Fst* values were above 0.05, with the highest differentiation being between 604-290-1 and 711-368-16 (*Fst* = 0.084).

Table 4. Genetic diversity parameters in even-aged spruce stand groups with differing growth potential assessments

	Non-perspective	Perspective
Na	11.250 (1.160)	10.950 (1.125)
Na Freq. ≥ 5%	5.000 (0.513)	5.350 (0.519)
Ne	4.702 (0.571)	4.868 (0.635)
I	1.692 (0.121)	1.689 (0.128)
Nu	1.500 (0.352)	1.200 (0.313)
H_e	0.715 (0.036)	0.713 (0.038)
H_o	0.538 (0.039)	0.528 (0.040)

Mean values over all loci shown, with standard errors in brackets. Na denotes the number of alleles, Na Freq. ≥ 5% denotes number of alleles with a frequency larger or equal to 5%, Ne denotes the number of effective alleles, I denotes Shannon's information index, Nu denotes the number of unique (private) alleles, H_e denotes expected heterozygosity, H_o denotes observed heterozygosity.

The analysed even aged spruce stands were divided into two groups according to their growth potential. The stands 711-368-16, 610-236-8, 604-290-1 and 610-256-8 were assessed as having a negative prognosis (non-perspective), while the stands 705-43-33, 611-53-16 and 604-377-3 were assessed with a positive prognosis (perspective). Genetic diversity parameters were similar between each of these growth potential groups (Table 4), with the average number of alleles over all analysed loci 11.250±1.160 in the non-perspective group and 10.950±1.125 in the perspective group. Similarly, the other genetic diversity parameters were similar between the negative and positive groups: observed heterozygosity (0.538±0.039 vs. 0.528±0.040, respectively) and expected heterozygosity (0.715±0.036 vs. 0.713±0.038, respectively). The genetic differentiation between the two groups was low (*Fst* – 0.01, p <0.001). Rarefaction analysis, implemented in the software program Fstat (Goudet 1995), taking into account the differing numbers of individuals analysed in each growth potential group did not detect any significant differences between the negative and positive groups in allelic richness, observed heterozygosity, genetic diversity or relatedness.

The analysed stands were planted in the 1970s, however the origin of the utilised reproductive material is not known. At that time, improved material from Norway spruce seed orchards was not available, and the most likely source of seeds used for forest renewal was from local stands collected during mast years. However, the location and number of individuals from which seeds were collected was not recorded. In addition, there are some anecdotal reports of reproductive material being imported from Ukraine and other regions in cases were the local seed collections were insufficient to ensure reforestation. Therefore, these even aged planted spruce stands were compared to Norway spruce individuals collected from the Moricsala nature reserve, which was established in 1912, and no forest management techniques have been utilised in this area since that time. In addition, Norway spruce individuals were sampled from the Rēzekne Norway spruce genetic resource stand, sampling mature individuals with an estimated age of over 100 years. Individuals collected from these two forest stands were genotyped with a subset of 14 of the previously described SSR markers (SpAGC1, SpAGC2, SpAGG3, UAPgCA91, UAPgTG25, UAPgAG150, WS0033.A18, WS0022.B15, WS0073.H08, PAAC17, WS0073cG10, paGB3, WS0081aA12, EAC2C08, EATC1D02A, EATC2B02, EATC2G05, EATC1E03, SpAC1F7), and compared to the seven previously analysed even-aged spruce stands.

The genetic diversity parameters in the negatively and positively assessed spruce stands were similar, as previously described. In addition, the natural spruce stands did not have significantly higher levels of genetic diversity (Table 5). Rarefaction analysis, implemented in the software program Fstat (Goudet 1995), taking into account the differing numbers of individuals analysed in each growth potential group and the natural populations did not detect any significant differences between the even-aged spruce stands and the natural stands in allelic richness, observed heterozygosity, genetic diversity or relatedness.

The analysed stands were not highly differentiated (*Fst* 0.035, p <0.001), but the principal coordinates analysis based on pairwise Nei genetic distances separated the stands. The even aged spruce stands were clustered according to their geographic location, with the two stands from the western region of Kurzeme more differentiated, and the remaining even aged stands, from the central region, less differentiated (Figure 2). The two

Table 5. Genetic diversity parameters in even-aged spruce stands, and the Rēzekne genetic resource stand and the Moricsala nature reserve stand. Mean values over all loci shown, with standard errors in brackets

	711-368-16	610-236-8	604-290-1	610-256-8	705-43-33	611-53-16	604-377-3	Rēzekne	Moricsala
Na	10.000 (1.144)	7.714 (0.848)	8.571 (0.875)	7.786 (0.967)	9.857 (1.148)	8.786 (1.017)	8.929 (0.822)	11.000 (1.351)	10.929 (1.282)
Na Freq. ≥ 5%	4.786 (0.482)	4.286 (0.474)	4.500 (0.653)	4.714 (0.474)	5.286 (0.615)	4.500 (0.635)	4.929 (0.579)	5.071 (0.549)	5.286 (0.588)
Ne	4.710 (0.684)	3.775 (0.509)	4.323 (0.795)	4.438 (0.745)	5.185 (0.885)	4.218 (0.782)	4.419 (0.707)	5.027 (0.820)	5.404 (0.895)
I	1.704 (0.147)	1.481 (0.141)	1.526 (0.162)	1.557 (0.155)	1.729 (0.159)	1.511 (0.167)	1.617 (0.137)	1.693 (0.180)	1.772 (0.154)
Nu	0.357 (0.169)	0.000 (0.000)	0.214 (0.114)	0.214 (0.114)	0.286 (0.163)	0.143 (0.097)	0.143 (0.097)	0.500 (0.203)	0.571 (0.202)
H_e	0.720 (0.039)	0.661 (0.047)	0.649 (0.058)	0.684 (0.049)	0.719 (0.044)	0.645 (0.058)	0.689 (0.044)	0.693 (0.060)	0.730 (0.045)
H_o	0.595 (0.054)	0.497 (0.069)	0.563 (0.058)	0.539 (0.052)	0.559 (0.053)	0.517 (0.067)	0.564 (0.054)	0.501 (0.063)	0.540 (0.069)

Mean values over all loci shown, with standard errors in brackets. Na denotes the number of alleles, Na Freq. ≥ 5% denotes number of alleles with a frequency larger or equal to 5%, Ne denotes the number of effective alleles, I denotes Shannon's information index, Nu denotes the number of unique (private) alleles, H_e denotes expected heterozygosity, H_o denotes observed heterozygosity.

natural stands are not geographically close; however, they were genetically similar. These stands are naturally established, old (>100 years old), and given the almost continuous distribution of Norway spruce within Latvia, and the large pollen dispersal distances, no structuring of sub-populations or isolation by distance is expected. The differentiation and clustering of the even aged Nor-

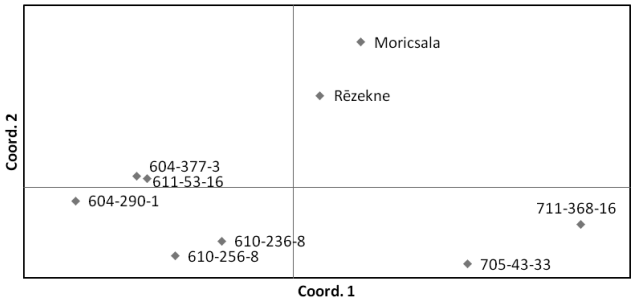

Figure 2. Principal coordinate analysis of pairwise genetic distances between the analysed spruce stands. Percentage of variation explained by axis 1 – 56.72%, axis 2 – 24.20

way spruce stands is probably due to the reproductive material utilised when establishing these stands. While there are no records of the origin of the material utilised, at the time these stands were established, improved seed material from the Latvian Norway spruce breeding programme was not available, and the stands were probably established using seed collected from local stands. The seeds may have been collected from a limited number of mother trees close by, which could result in the higher geographic differentiation of the even aged stands compared to the natural stands. However, the levels of genetic diversity were not lower, which could be explained by the high genetic diversity within stands and individuals, and by the high level of pollen flow.

Discussion

In Latvia, the management regime of the Norway spruce is contradictory to the ecological demands of this tree species. The Norway spruce is a shade-tolerant tree species, and natural formation of even-aged pure stands is not characteristic. According to the results of a survey performed by A. Zviedris (1960), in 205 clearcut areas, where Norway spruce was the dominant species in the previous generation, none of these areas contained even-aged stands of regenerated spruce. Historically, in the second half of previous century, the Norway spruce was also often planted on sites more suitable for Scots pine, due to reduced browsing damage and lower thinning costs in spruce stands compared to pine stands. The planting density of spruce was high (exceeding 4,000 trees ha^{-1}) but in stands initially established for intense cultivation of spruce for pulpwood the number of planted trees often exceeded 7,000 trees ha^{-1}. However, due to the collapse of pulp industry in the beginning of the 21st century, the intended reduced rotation age (40 years) was not applied to these plantations, thinning of young stands was often delayed and trees were subject to increased competition. Results of former and ongoing studies suggest that in order to ensure favourable stand development, mutual competition among the trees must be reduced as early as possible, preferably before the stand has reached the mean height of 5 m (Zālītis and Lībiete 2003, Zālītis and Jansons 2009, Zālītis et al. 2017).

The genetic analysis and comparison of the perspective and non-perspective even-aged spruce stands indicated that the genetic diversity was not decreased in the non-perspective stands, and that genetic differentiation between stands and groups with differing growth potential assessments was low. Geographically close even-aged stands were also genetically similar, particularly the two stands from the western region of

Kurzeme. The similarity between geographical and genetic distances was not as pronounced for the two 'natural' stands analysed, the Rēzekne genetic resource stand and the Moricsala nature reserve. However, the overall genetic diversity parameters were not significantly lower in the even-aged stands. While the origin of the reproductive material utilised to establish the even-aged stands is not known, seeds were collected from local stands within one forestry district, and were often deployed in the same district. The genetic relatedness of the analysed even-aged stands maybe a reflection of this practice, and the limited number of trees that seeds were collected from. In contrast, the genetic diversity parameters, including relatedness were not significantly lower in the even-aged stands. This result is not unexpected, even if seeds were collected from a limited number of trees, as the open-pollinated nature of Norway spruce, together with the high levels of genetic polymorphism and heterozygosity, and long pollen dispersal distances, can ensure a high level of genetic diversity in open-pollinated progeny derived from a small number of mother trees.

Considering this, the results obtained in this study are not surprising and suggest that the growth potential of even-aged Norway spruce stands is more dependent on the influence of environmental factors and management regime than genetic factors. This is a positive message, as in this case the conditions may be changed by the application of suitable management regime. Repeated assessment of the growth potential of even-aged spruce monocultures has demonstrated their further decline over ten years, posing a challenge to spruce forest management in the future. This challenge should be addressed by increasing the flexibility of the management regime and regulations that would allow for reduced rotation age in problem situations (instead of the currently utilised 81 years). Another option is an uneven-aged management model for spruce forests that potentially may be more suitable for private forest owners.

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