Analysis of the genetic diversity and population structures of black locust (*Robinia pseudoacacia* L.) stands in Poland based on simple sequence repeat markers

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ABSTRACT

Black locust (*Robinia pseudoacacia* L.) was introduced in Poland over 200 years ago, and its distribution area now covers the whole country, with the highest concentration of occurrence in the western part. Breeding of this species has been started in Poland for about 20 years, albeit on a limited scale, and two selected seed stands, 34 plus trees and two seed orchards have been registered. So far, selection efforts have not been coupled with the recognition of variation in the genetic structure of local populations. To fill the knowledge gap on genetic diversity, we selected the seven qualitatively best populations of *R. pseudoacacia* for our study. To clarify the origin of the Polish population of *R. pseudoacacia*, a sample from a seed stand in eastern Germany and from a seed orchard with Hungarian clones from the Oborniki Śląskie Forest District was added. In this study, three microsatellites were used to evaluate genotypes: Rops15, Rops16 and Rops18, with the Rops15 locus (motif AG) showing hypermutability. Moderate or high variability was observed in all microsatellite loci examined, with a total of 27 alleles identified. In all stands, the average observed number of alleles per locus was higher than the average effective number of alleles per locus. Three main clusters have been identified in the genetic structure of the population, one of which is represented by the population from the Regional Directorate of State Forests in Zielona Góra. The second group comprised the populations from Mieszkowice, the populations Pińczów and Wołów, and the population from Germany. The last one included population from Strzelce and Oborniki Śląskie, where the population from Hungary is represented. This may be of great importance for practice. Perhaps it is worth postulating the separation of two seed regions. Under most scenarios, climate change is projected to change the distribution of forest types and tree species in all biomes. It can be assumed that the importance of black locust in times of climate change is likely to increase.

KEY WORDS

Robinia pseudoacacia, non-native tree species, secondary distribution, genetic diversity, microsatellite

INTRODUCTION

Climate change is having a significant impact on the geographical distribution of plant species around the world. Most of these changes are related to a warming climate and a decrease in precipitation during the growing season (IPCC 2021). Evidence exists that the viability of European tree species is decreasing and mortality is increasing (Schuldt et al. 2020). In the future, these changes will lead to shifts in the ranges of native and introduced tree species in Europe (Puchałka et al. 2020, 2023; Dyderski et al. 2018). An example of such a species is the black locust (*Robinia pseudoacacia* L.), whose natural range is North America: Appalachian Mountains, Ozark Highlands and Ouachita Mountains. *R. pseudoacacia* is considered a drought-adapted tree species in native and secondary distribution that exhibits high morphological and physiological plasticity and also occurs in regions with annual precipitation below 600 mm a^{-1} (Mantovani et al. 2014; Klisz et al. 2021).

Black locust is one of the most widely cultivated tree species in the world (DeGomez and Wagner 2001). It occurs in at least 35 countries worldwide (Guoqing et al. 2014), most commonly in Europe, except Lithuania, Latvia, Estonia, Denmark, Norway, Finland and the Balkan countries (Dimitrova et al. 2022; Cierjacks et al. 2013; DAISIE 2006), as well as in the Americas, Africa (South Africa, Nigeria), Australia and Indonesia, and Asia. In Europe, black locust has been growing for more than 200 years and its largest distribution area is Hungary, where it currently occupies more than 23% of the forest area (Rédei 2013). Historical records indicate that black locust was introduced to Europe from a limited number of native populations in the northeastern Appalachians (Liesebach and Schneck 2012; Bouteiller et al. 2019). In the 17th century, *Robinia* seeds cultivated in Paris were spread throughout Europe. The seed orchards that were established sold seeds from trees that were already growing on the continent (Cierjacks et al. 2013). The black locust was introduced to Poland in 1806 (Tokarska-Guzik 2012). It has been planted in forests since 1860. Currently, six selected seed stands are registered, including three selected seed trees, 44 selected trees and two seed orchards (Wojda 2015). Seed stands of this species are projected to continue to increase until the end of 2035 (Program... 2011). Due to its negative impact on biodiversity and forest ecosystem functioning (Langmaier and Lapin 2020), there are no plans to increase its role in forests or expand the range of this species. Black locust is expected to provide material for fast-growing plantations on former agricultural land (Kowalewski 2013).

In recent years, several stands of black locust not covered by the selection programme have been found in Poland, characterised by a unique straight trunk shape. In fact, it is possible to distinguish (at least) two basic stem forms, referred to as the 'typical' and the 'straight stem form' (shipmast stem form). By far, the most common and predominant form is the 'typical' form, which is used to describe a short tree (up to 5 m tall) that often grows twisted and at an angle, with its crown sitting fairly close to the ground. Trees growing in connected stands may also be twisted in different directions (Zajączkowski 2013). It is presumed that trees with straight trunks, in turn, have a genetically determined ability to form straight trunks that have a much greater height above the ground, even when growing in isolation. Under these circumstances, crowns are more regular, branches are less stout and twigs are thinner and less thorny (Zajączkowski 2013). This tall-stemmed form was previously described as a distinct cultivar, namely, *R. pseudoacacia* L. var. *rectissima* Raber, although it is now considered only an ecotype. Apart from its visible anatomical characteristics, this ecotype is more resistant to damage by the black locust borer *Megacyllene robiniae* Forst. and to stem rot caused by *Fomes rimosus* (Berk.). On the other hand, it produces few seeds, and is therefore usually propagated vegetatively.

Because *R. pseudoacacia* is a pioneer species, it is usually used to colonise degraded environments with disturbed soil cover structure relatively easily (Ashby et al. 1980). This species spontaneously colonises open wastelands, mining areas, abandoned fields and pastures, or fire-damaged sites (Cierjacks et al. 2013 and Maringer et al. 2012), thus confirming its high invasive potential. Black locust is a valued species in arboriculture (Grünewald 2009 and Böhm 2011) and is used for production of fuelwood, quality wood, fodder, poles and honey (Keresztesi 1988 and Rédei et al. 2002). It has numerous useful properties, including a high relative growth rate and the production of a large biomass of high-density wood that is easy to dry and process and burns well. Its wood properties make it suitable for timber production in fast-growing tree plantations or

energy plantations (Halupa and Redei 2013; McKendry 2001). In Hungary, black locust accounts for about 19% of annual timber production (Malvoti 2015).

However, it is also a species that is currently considered invasive in Europe and Asia (Bartha et al. 2008; Nasir et al. 2005; Osada 1997; Raju 199; Everitt et al. 2007; Richardson and Rejmanek 2011) and is one of the most problematic invasive species in many European countries (Kleinbauer et al. 2010). Black locust has characteristics that are interpreted as negative in relation to native species and have implications for reducing biodiversity (Benesperii et al. 2012; Trentanovi et al. 2013). It forms dense patches with only one species relatively easily and displaces other species. The impact of black locust on the diversity of the flora is already well known and is considered to be clearly negative. Many authors (Taniguchi et al. 2007; Vítková and Kolbek 2010; Von Holle et al. 2006; Vítková et al. 2017) report that black locust forms specific communities in which the herbaceous layer differs significantly from that in stands with native tree species. Black locust significantly increases nitrogen levels in the soil (Rice et al. 2004) and increase in nitrogen levels is among the greatest threats to natural vegetation (Hicks et al. 2011). Conditions under the canopy are becoming more favourable for shade-tolerant and nitrophilous species (Dzwonko and Loster 1997; Hruška 1991; Vítková and Kolbek 2010), while most typical oligotrophic and acidotrophic species are disappearing (Benesperi et al. 2012). This species frequently invades drylands and poses a major threat to xerothermic communities (Vítková and Kolbek 2010; Vítková et al. 2017; Hegedusova and Senko 2011; Tokarska-Guzik et al. 2012).

Genetic variation exists within every species and forms the basis for selection and evolution. The analysis of the distribution of genetic diversity in a species provides useful information for conservation programmes and management at the species level (Dąbrowska et al. 2006). Various molecular markers such as randomly amplified polymorphic DNAs (RAPDs) (Dąbrowska et al. 2006, 2021; Mariette et al. 2001), amplified fragment length polymorphisms (AFLPs) (Mariette et al. 2002; Jump et al. 2007) and simple sequence repeats (SSRs) (Mariette et al. 2002; Gonzalez-Martinez et al. 2004; Arif et al. 2010) have been tested for genetic diversity assessment of forest trees. Till now, such studies on genetic diversity of *R. pseudoacacia* in Poland are not known. In particular, the level of genetic diversity among *R. pseudoacacia* populations and the nature of available genetic resources were unclear. Microsatellite markers are ideal tools for identifying individuals and studying genetic diversity due to their ubiquity, repeatability, high degree of polymorphism and codominant mode of inheritance (Carletti et al. 2019; Guan et al. 2019; Nasim et al. 2020). Therefore, SSRs are used to study genetic diversity, genetic linkage mapping and 'fingerprinting' of many tree species, including *R. pseudoacacia* (Guo et al. 2017, 2018; Dong et al. 2019). Knowledge of genetic diversity and variation in tree populations is important because its reduction can reduce a species' plasticity and resilience to biotic and abiotic stresses (Machida-Hirano 2015). Identification, selection and maintenance of genetic diversity within forest tree species is of particular importance due to their long lifespan, which requires adaptation to different environments and climates. Data obtained from genetic studies can be successfully used in breeding programmes for individual tree species (Sethuraman 2018; Hall 2020). In this study, three nuclear microsatellite markers (Rops15, Rops16 and Rops18) were used to analyse the genetic diversity and population structure of seven high-quality populations of black locust in Poland. These markers were used as a molecular tool to infer the origin of a Polish stand by comparing material with plants from seed orchards with clones of Hungarian origin in the Oborniki Śląskie Forest District (PN) and a seed stand in eastern Germany.

MATERIAL AND METHODS

Planting material

Plant material was collected from a total of nine locations (Tab. 1). Young leaves were collected from seven selected and previously described (Wojda et al. 2015) straight-stemmed stands in Poland (PL), one seed stand (also a straight-stemmed stand) in Buckow (eastern Germany) and a seed orchard in Oborniki Śląskie Forest District. The last one includes clones from 34 straight-stemmed plus trees from Hungary and was established in 2004 in Poland (Tab. 1). Plant material was from 50 trees in each stand and from 25 Hungarian clones. Collected leaves were stored in a cold room at –70°C until the start of analyses.

Table 1. Forest stands where plant material was collected for genetic analyses. The origin of the plant material is indicated in brackets

No.	Forestry division	Area (ha)	Tree age (years)	Geographical coordinates
1	Cybinka (PL)	1.05	72	N 52 7 23.7 E 14 56 43.5
2	Krosno 232 (PL)	3.18	92	N 52 8 24.1 E 14 55 14.3
3	Krosno 90 (PL)	1.14	39	N 52 5 40.2 E 14 58 13.7
4	Mieszkowice (PL)	1.31	50	N 52 51 31.5 E 14 11 40.7
5	Pińczów (PL)	3.19	38	N 50 15 53.0 E 20 42 7.2
6	Strzelce (PL)	1.36	40	N 50 29 37.7 E 18 2 54.2
7	Wołów (PL)	2.86	46	N 51 25 12.5
8	Oborniki Śląskie (HU)	1.09	15	N 51 22 39.5 E 16 53 23.7
9	Buckow (DE)	1.10	58	N 52 33 32.8

DNA isolation and SSR analysis

Total DNA was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's manual. Qualitative and quantitative evaluation of the extracted DNA was performed by electrophoretic separation in 0.8% agarose gel and spectrophotometric measurement of absorbance at 260 and 280 nm using Nano-Drop ND-1000. High-quality DNA was used for further analysis. Analysis of microsatellite regions was performed using multiplex polymerase chain reaction (PCR) according to the previously described procedure (Szyp-Borowska et al. 2016). PCR was performed with the following primers: Rops15, Rops16 and Rops18, as described by Lian (2004) (Tab. 2). The three microsatellites were amplified in a single multiplex PCR. Concentrations of the primer pairs in the primer premix were 0.2–0.3 μM of each designed primer pair. The cycling conditions for the multiplex were as follows: an initial step at 95°C for 15 min; then 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; and a final incubation at 70°C for 10 min. The reaction products were fractionated using a CEQ 8800 (Beckman-Coulter) sequencer.

Analysis of genetic diversity

To describe the genetic diversity of *R. pseudoacacia* populations, the number of alleles per locus (Na), the number of effective alleles per locus (Ne), the Shannon's information index (I), the observed heterozygosity (Ho), unbiased genetic diversity index (uHe) and the expected heterozygosity (He) were calculated using GeneAlEx 6.5 (Yeh et al. 1999) software. We examined the distribution of genetic variation within and among populations by analysis of molecular variance (AMOVA) using GenAlEx 6.5. software; the procedure is based on hierarchical variance of gene frequencies. F-statistics, including the inbreeding coefficient within individuals (FIS) and genetic differentiation between populations (FST), were calculated using GenAlEx version 6.5.

Results

Characteristics of the Microsatellite Loci

The total allele number amplified by the three loci was 45, of which 23 were rare alleles occurring below the frequency of <0.05. The number of alleles per locus varied from six (at locus Rops18) to 20 (at locus Rops15).

Table 2. Nuclear microsatellite loci used in the analysis of genetic diversity of *R. pseudoacacia*

Locus	Repeat	Primer sequence (5′–3′)	Ta. (°C)	Size range (bp)	No. of alleles	GenBank NCBI accession no.
Rops15	(CT)20	GCCCATTTTCAAGAATCCATATATTGG TCATCCTTGTTTTGGACAATC	54	112–254	43	AB120731
Rops16	(CT)13	AACCCTAAAAGCCTCGTTATC TGGCATTTTTTGGAAGACACC	56	195–223	15	AB120732
Rops18	(AC)8	AGATAAGATCAAGTGCAAGAGTGTAAG TAATCCTCGAGGGAACAATAC	54	135–219	13	AB120733

The locus Rops18 was the least polymorphic with the lowest expected and observed heterozygosity and allele diversity indexes. The lowest uHe indicated prevalence of several dominant alleles at these loci. The other two loci were highly polymorphic, with the uHe values exceeding 0.5 (Tab. 3).

Table 3. The genetic diversity indexes for each locus

Locus	Na	Ho	uHe	FIS
Rops15	20	0.968	0.716	−0.378
Rops16	19	0.677	0.555	−0.252
Rops18	6	0.051	0.063	0.121

Na – number of alleles; Ho – observed heterozygosity; uHe – unbiased genetic diversity; FIS – inbreeding coefficient.

Genetic Differentiation and Structure

Analysis of microsatellite loci revealed an exceptionally high level of variation between populations (FST = 0.412, p = 0.001) and high variation within populations. Moderate to high variability was detected at all microsatellite DNA loci examined, with a total of 45 alleles diagnosed. In the studied populations, the average number of alleles per locus (NA) ranged from 2.66 (Mieszkowice and Krosno 90) to 8.00 (Krosno 232). In the whole sample, it reached a value of 4.74. Ho in individual populations was high, ranging from 0.67 to 0.45 (Mieszkowice and Cybinka, respectively; Tab. 4). The sampled stands were characterised by a high degree of genetic variability. In all stands, Na was higher than Ne. The values of Shannon's index of genetic diversity and

the high degree of heterozygosity calculated according to the Nei formula indicate high polymorphism of the DNA loci studied in the sampled stands. The mean value of Ho was lower than He in the Pińczów stocks, indicating a lack of heterozygous genotypes relative to the expected value in a population in Hardy–Weinberg equilibrium.

Table 4. Genetic diversity of populations of *R. pseudoacacia* revealed by microsatellite loci

Forest stands	Ho	He	H	F
Cybinka	0.445	0.291	0.295	−0.310
Krosno232	0.600	0.469	0.473	0.076
Krosno90	0.620	0.353	0.358	−0.757
Miechów	0.67	0.373	0.380	−0.820
Pińczów	0.552	0.632	0.663	0.068
Strzelce	0.472	0.446	0.453	−0.060
Wołów	0.623	0.456	0.472	−0.306
Oborniki Śl.	0.44	0.49	0.501	0.145
Buckow	0.67	0.40	0.406	−0.663
Total	0.56	0.433	0.445	−0.260

He – average expected heterozygosity; H – heterozygosity; Ho – observed heterozygosity; F – fixation index

For all polymorphic loci, the most frequent alleles and rare alleles were determined for the studied populations (Fig. 1). The Krosno 232 population had the highest number of distinct and private alleles among all populations, while the Krosno 90 and Miechów populations had no private alleles. The degree of genetic
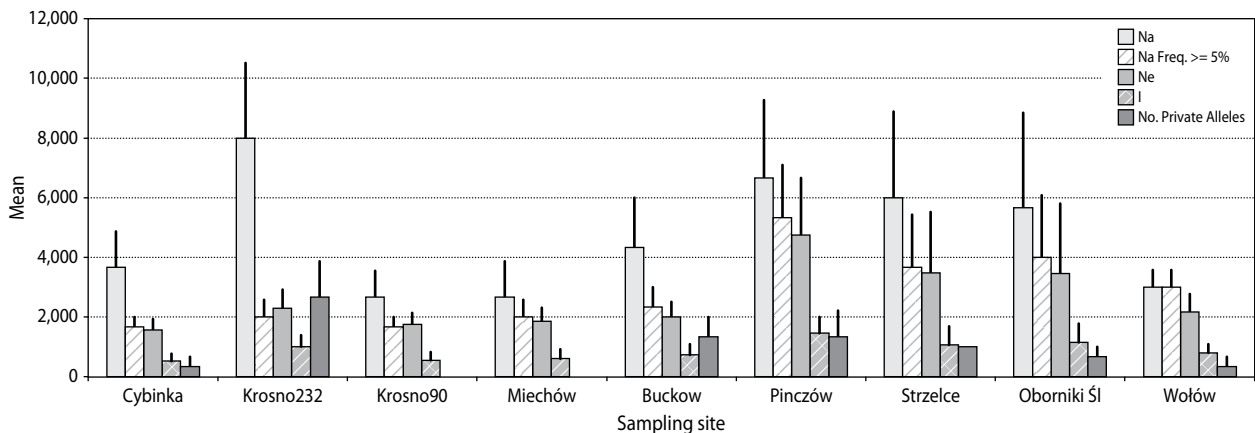
Figure 1. Allelic patterns for *R. pseudoacacia* populations. Na – number of alleles per locus, Ne – number of effective alleles, I – Shannon's information index

variability of these populations was the lowest among the studied populations. Their observed heterozygosity ranged from 0.620 to 0.670 (Tab. 4).

The highest values for I (1.452) and H (0.659), were found in the Pińczów population, indicating high genetic diversity. The population with the second highest diversity was Oborniki Śląskie, with values of I – 1.146 and H – 0.501. Ho was lower than He in these populations and F was positive, ranging from 0.145 (Oborniki Śląskie) to 0.068 (Pińczów) (Tab. 3). The fixation index (F) of the other populations of *R. pseudoacacia* was negative and ranged from -0.060 to -0.820, indicating the presence of an excess of heterozygotes.

FIS and FIT were used as indicators to evaluate the degree of purity of the population. FST served as an indicator of the degree of genetic differentiation of populations. The presence of low gene flow (Nm <1) between populations favours the occurrence of genetic drift, which reduces the degree of genetic differentiation of some populations, and genetic differentiation between populations increases FST (0.412). The mean FIS and FIT values for *R. pseudoacacia* were -0.170 and 0.246, respectively, indicating an increase in heterozygosity among populations (Tab. 5).

Table 5. The mean FIS and FIT values for population of *R. pseudoacacia*

F-statistics	Value	*P*
FST	0.412	0.001
FIS	−0.170	0.649
FIT	0.246	0.001
Nm	0.771	

FIS per locus varied from −0.377 to 0.120 with a mean of −0.170, and these results indicated an excess of heterozygosity. Furthermore, FIT ranged from −0.166 to 0.881, with an average of 0245. Moreover, FST ranged from 0.865 to 0.153 with a mean of 0.412, which indicated moderate differentiation among populations (Tab. 4). These results indicate that genetic variation mainly occurred within populations, accounting for 63% of the total variation, whereas the genetic variation among populations was only 37% (Tab. 6).

Model-based clustering generated three distinct subpopulations (Fig. 2). The first group consisted of populations from Zielona Góra Forest District. The

second group included the populations from Mieszkowice in the Szczecin Forest District, from Pińczów and Wołów as well as the German population. The third group included populations from Strzelce and Oborniki Śląskie, where the population from Hungary was represented.

Table. 6. Analysis of molecular variance (AMOVA) of genetic diversity of *R. pseudoacacia* populations

Source	df	SS	MS	Est. var.	%
Among pops	8	88.163	11.020	0.335	37
Within pops	275	157.488	0.573	0.573	63
Total	283	245.651		0.908	100

df – degrees of freedom; SS – sum of squares; MS – mean square; est. var. – estimated variance.
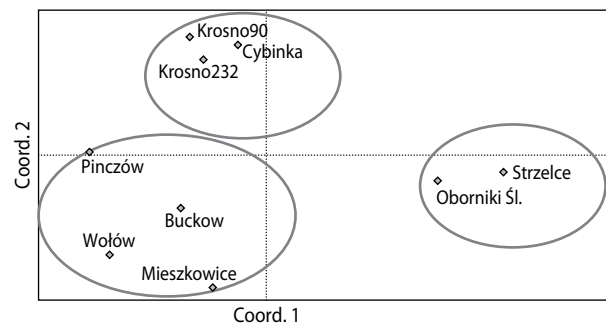
Figure 2. Relationships among the populations of *Robinia pseudoacacia*. A principal coordinate analysis (PCoA) based on pairwise genetic distance estimates for all populations. Percentages of total variance explained by coordinates 1 and 2 accounting for 68.99 and 14.23%, respectively

Discussion

Due to the adverse effects of climate change and the energy crisis, the role and importance of black locust have increased in many countries in recent years (Abri 2021). Because of its resilience to water deficits, it is considered a 'winner' species in the face of projected climate warming (Dyderski et al. 2018). Many European countries (e.g. Hungary, Germany, Greece, Poland and Turkey) and Asian countries (e.g. India, China and South Korea) have initiated their own breeding programmes for this species (Dunlun et al. 1995; Dini-Papanastasi and Panetsos 2000; Liesebach et al. 2004; Sharma and

Puneet 2006; Lee et al. 2007; Böhm et al. 2011; Kraszkiewicz 2013; Szyp-Borowska et al. 2015, 2020). To apply effective methods in a species management strategy, it is necessary to assess genetic differences among provenances and describe patterns of adaptive geographical variation (Sethurman 2018; Alizoti et al. 2022; Guo 2022). Most forest tree species introduced into Europe exhibit high variation within their natural range, and in contrast, the performance and survival of different provenances can vary substantially when planted outside their natural range (Eilmann 2013; Chakraborty et al. 2016; Merceron 2016).

The primary sources for selection and subsequent improvement of desirable traits are genetic resources for breeders. So, their knowledge, evaluation and use are of great importance for further breeding process (Boczkowska et al. 2016; Cuevas and Prom 2020).

The results obtained in this study provide a first insight into the genetic resources of *Robinia pseudoacacia* for the management of this species in Poland. In Poland, there are plans to further increase the seed base of this species (Programme... 2011), and it is expected that *Robinia* will provide material for fast-growing plantations on former agricultural land. Due to its usefulness, black locust, when grown on non-forested land (plantations, former farmland, degraded land, woodlots), can be an important source of wood raw material.

European black locust populations are characterised by low genetic diversity (Liesebach and Schneck 2012; Bouteiller et al. 2019; Alizoti et al. 2022), and populations introduced into foreign areas are often subject to founder effects, leading to an additional reduction in genetic diversity (Rijal et al. 2015; Bouteiller et al. 2021). In the present study, the mean Shannon index value was 0.873, which was lower than that reported by Guo et. al. (2022) for the 36 neutral SSR markers (I = 1.302) or those reported by Huo et al. (2009) and Sun et al. (2009) for the AFLP and ISSR (Inter Simple Sequence Repeat) markers, respectively. The level of genetic variability of the populations we studied can be considered relatively low (Ho = 0.28, He = 0.37). These values are lower than those of Guo et al. (2022), where the mean values of Ho and He were 0.551 and 0.608, respectively, or of Lian et al. (2002) (Ho = 0.615, He = 0.773) and Mishima et al. (2009) (Ho = 0.661, He = 0.739). The mean value of Ho for the three loci was higher than He in most populations. Only the population from Pińczów is charac-

terised by a lack of heterozygous genotypes relative to the expected value. The studied populations are characterised by low richness of rare alleles and unique alleles. Moderate population differences among the studied populations were indicated by FST values (0.412). The observed variability was mainly due to differences within populations; AMOVA showed that the genetic difference within populations was up to 63%.

Under most scenarios, climate change is projected to change the distribution of forest types and tree species in all biomes. It can be assumed that the importance of black locust in times of climate change is likely to increase, both due to rising temperatures and the lengthening of the growing season and in terms of the global environmental policy of countries. In some countries, such as Hungary, a breeding programme for this species has existed since the beginning of the 20th century (Keresztesi 1983) and the strong influence of the stand management system has affected the genetic variability of the progeny (Liesebach and Evald 2012).

Structural analysis revealed specific, extremely interesting correlations between Polish populations of *R. pseudoacacia*. The studied populations were clearly divided into three clusters; but two of them showed similarity to populations from neighbouring countries. A separate group is formed by the population from Strzelce and a seed orchard from Oborniki Śląskie, in which the population from Hungary is represented, and the second group with the population from Mieszkowice in the Szczecin Forest District, the Pińczów and Wołów populations and the population from Germany. This may be of great importance for practice. Perhaps it is worth postulating the separation of two seed regions, southern (similar to Hungarian) and western (similar to German). There is no universally applicable measure for adapting forests to climate change. Forest managers should, therefore, have sufficient flexibility to deploy the adaptation measures most appropriate for their local situations. The knowledge-based use and transfer of well-documented and characterised forest reproductive material can be an effective tool.

In the present study, the first assessment of *Robinia pseudoacacia* gene resources in Poland was conducted and provided important information for the breeding of *Robinia pseudoacacia*.

1. We found that these populations are characterised by relatively low genetic variability.

2. We observed that most of the populations are characterised by an excess of heterozygotes. Only in one population of Pińczów, we found a deficiency of heterozygotes.

3. We found that the studied populations are clearly divided into two clusters.

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