Acta Societatis Botanicorum Poloniae

DOI: 10.5586/asbp.3593

Publication history
Received: 2018-03-08
Accepted: 2018-08-08
Published: 2018-10-01

Handling editor
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Authors' contributions
MK and JB: research designing; JB and MW: tree measurement, collection and preparation of needles for analyzes; KM and KG: genetic analysis; KM, JB, and KG: analysis of the data and drafting the manuscript

Funding
The research was performed as part of the EO-2717-24/12 project funded by the State Forests National Forest Holding and statutory activities of the Faculty of Forestry in Krakow (No. DS-3405) and Faculty of Agrobioengineering in Lublin (No. DS-4) funded by the Ministry of Science and Higher Education of Poland.

Competing interests
No competing interests have been declared.

Citation
Masternak K, Banach J, Głębocka K, Wajdzik M. Genetic variability in pitch pine (*Pinus rigida* Mill.) growing in the Niepołomice Forest as determined by ISSR markers. Acta Soc Bot Pol. 2018;87(3):3593. https://doi.org/10.5586/asbp.3593

ORIGINAL RESEARCH PAPER

Genetic variability in pitch pine (*Pinus rigida* Mill.) growing in the Niepołomice Forest as determined by ISSR markers

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Abstract
The study aimed to determine the genetic variability in pitch pine (*Pinus rigida* Mill.) growing in the Niepołomice Forest (southern Poland). In the late nineteenth and early twentieth century, Adolf Cieślar of the Department of Forestry Research in Mariabrunn near Vienna, Austria established the experimental crops of pitch pine. During the study, 227 trees that grew in seven subunits were considered; an analysis of genetic polymorphism using the intersimple sequence repeats (ISSR) technique revealed that pitch pine is genetically variable. The average number of alleles at a given locus for all the pine trees was 1.649, while the effective number of alleles at the loci was 1.435. The value of expected heterozygosity was 0.254, while the percentage of polymorphic loci was 75.30%. The average genetic distance between the examined pines was 0.082. Principal coordinate analysis (PCoA) divided the examined pines into three groups, which was also confirmed by the structure-analysis results of the software STRUCTURE. The resulting division was mainly generated by the SR70 primer, which was indicated to be the primer that differentiated the examined populations of pitch pine. Affiliation of particular trees to selected groups was based on their occurrence in individual crops. This suggests a different origin of the seeds used to establish the research plots of pitch pine in the Niepołomice Forest.

Keywords
experimental plot; marker; introduced species; interpopulation differences

Introduction

Pitch pine (*Pinus rigida* Mill.) is found in North America, from central Maine to New York and Southeastern Ontario, toward the south in Virginia and Southern Ohio, in the mountains of East Tennessee, Northern Georgia, and western South Carolina. As it mainly grows on poor soils, its scope is very fragmented; it mostly grows on shallow and sandy soils in the northeast (Pennsylvania). The species occurs in humid climates with an average annual precipitation ratio of 940–1,420 mm that is evenly distributed throughout the year. It is characterized by a high tolerance to extreme temperatures with tolerance down to −40°C in the winter in the northern part of the range of areas in which it occurs and up to +38°C in the summer throughout most of the occurrence area [1].

Pitch pine was introduced to the forests of Europe in 1743, while in Poland, it appeared before 1818 [2]. At the turn of the nineteenth and twentieth centuries, there was a period of intensive research concerning an evaluation of the effects of different subpopulations of species moving in the area of Europe. This was a period of high

activity of the Department of Forestry Research (DFR) in Mariabrunnnear Vienna (Austria), which founded more than 700 research plots, including those in Galicia (now the southern part of Poland and Western Ukraine), as well as in many other regions of the former Austro-Hungarian Monarchy. Experimental plots established in the years 1884–1905 by the DFR are valuable and unique research facilities of special cognitive value. These plots were founded at various heights (from 300 to 730 m a.s.l.) and can be described as testing grounds for the adaptation of many species of foreign origin to the conditions of the widely-understood Central Europe. About 750,000 seedlings of 32 species from North America and Asia were planted in the experimental plots [3]. Unfortunately, measurements and observations were not continued because of the two world wars and the changes that took place during border formations of the present countries in Central Europe; hence, there is a lack of broader research results.

Differentiation in pitch pine features is greater than that in other woody species [4]. However, not many studies have been conducted so far in this regard, and most studies investigate the variability in growth [5,6], morphological features [7,8], and cultivation suitability [9] of this species. Some studies refer to the comparison of dwarf forms of pine trees with stands of normal shape growing in New Jersey, Long Island, and the Shawangunk Mountains [10]. Fires [11,12], low soil richness [13], toxic concentrations of aluminum, and high wind speed [14] are significant environmental conditions leading to the formation of dwarf forms. The study conducted by Ledig et al. [7] highlighted the genetic background of dwarfism. Ledig and Little [15], in turn, found slight differences in the allele frequencies of the isoenzymatic loci with large phenotypic differences.

Studies on the genetic variability in *P. rigida* mainly focus on the analysis of isoenzymes [16–18]. Few articles described the variability of the species at the DNA level [19] as well as genes encoding ribosomal units [20]. With these results, relatively little knowledge about the genetic variability of that species was obtained. Estimating the genetic polymorphism is becoming increasingly common in forestry. Genetic variability helps determine the stability of stands in the face of possible changes in external conditions and is a source of information for improvement in breeding programs, along with increased breeding profits by choosing the most adaptable provenances [21]. In addition, a great inter- and intraspecies diversity in forest trees is essential for food security and sustainable forestry development [22]. Therefore, the aim of the present study was to evaluate the genetic diversity of the pitch pine population growing in the Niepołomice Forest on several research plots established in the late nineteenth century by the DFR in Mariabrunn. The analysis involved the intersimple sequence repeats (ISSR) markers, which combine the simplicity and low cost of the RAPD technique with the advantages of SSR and AFLP techniques. In addition, the study was also designed to evaluate as to how much of the parent population may have contributed in the origin of the seeds used to establish the research crops and to indicate the marker differentiating these populations.

Material and methods

Plant material

The study began in 2012 following the determination of the location of experimental plots established in 1887–1889 by the Department of Forestry Research in Mariabrunn in the area of the Niepołomice Primeval Forest. Based on Dubiel's [23] report, analysis of data contained in the forest management documentation for the Niepołomice Forest District and field examination became possible, which indicated seven plots on which *Pinus rigida* trees were grown (Fig. 1).

Experimental plots were located in the habitat of fresh mixed forest and humid mixed forest, as well as moist mixed forest. The number of trees at each location determined in 2014 differed considerably. Most pines were found in the tree crops located in Sitowiec (subunit 299-l) and Baczków forest ranges (subunit 203-g), while the least in the units 299-i, 299-k (Sitowiec forest range), and 46-f (Dziewin forest range) (Tab. 1). The available documentation for the experiments published by DFR lacks the data concerning the crops established in the Niepołomice Forest District. The only available information

Fig. 1 Locations of the experimental trials with *Pinus rigida* (red circle) established by DFR in the Niepołomice Primeval Forest (south Poland).

Tab. 1 Location of experimental crops of *Pinus rigida* in the Niepołomice Forest (Niepołomice Forest District) and the characteristics of growing trees.

No.	Forest range	Subunit	Geographical coordinates		Type of forest site*	Number of trees in 2014
			Longitude (E)	Latitude (N)		
1	Sitowiec	299-i	20°15′05″	50°00′40″	MDF(f)	11
2		299-k	20°15′22″	50°00′33″	MDF(f)	13
3		299-l	20°15′14″	50°00′27″	MDF(f)	82
4		301-l	20°14′30″	50°00′35″	MDF(w)	18
5	Baczków	203-g	20°25′56″	50°01′12″	MCF(w)	72
6	Dziewin	10-d	20°24′55″	50°04′08″	MCF(w)	20
7		46-f	20°24′22″	50°03′22″	MCF(w)	11

* MDF(f) – mixed deciduous forest (fresh variant); MDF(w) – mixed deciduous forest (wet variant); MCF(w) – mixed coniferous forest (wet variant).

on pitch pine is presented in Lesiński and Grabowski's [24] publication from the 1960s. Two research plots with an area of 0.25 and 0.20 ha were established at that time in the current Sitowiec forest range, where more than 80 *P. rigida* trees were inventoried. The results obtained by these authors showed a slower growth of the pitch pine till the age of 45 years as compared to the native Scots pine.

DNA extraction

In June 2014, needles were obtained from pitch pines shooting away from a twig in the crown of the tree for analysis of genetic variability. Rogers and Bendich's [25] modified

method was used to perform the extraction of genomic DNA. Biological material was collected in the form of 150 mg of needles from each tree; it was powdered in liquid nitrogen and then placed in 0.7 mL of Extraction Buffer I (4% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, and 1% PVP) with an addition of 0.1% β-mercaptoethanol. After a 50-min incubation at 65°C, all contents were centrifuged for 5 min at 13,000 rpm; the resulting supernatant was transferred into a mixture of chloroform and isoamyl alcohol (24:1), vigorously shaken for 5 min, and then centrifuged for 10 min at 13,000 rpm. The supernatant was transferred into an extraction buffer II (10% CTAB, 0.7 M NaCl) and stirred; the mixture of chloroform and isoamyl alcohol was added again and centrifuged for 5 min at 28,341.3 *g*. In the next stage, DNA was precipitated with isopropanol at −20°C and the solution was then centrifuged at 28,341.3 *g* for 10 min. The supernatant was discarded and the precipitate was washed in 70% ethanol. The resulting precipitate was dissolved in 50 µL of sterile water and stored at −20°C.

Tab. 2 The sequence of applied ISSR primers.

No.	Primer code	Primer sequence (5'→3')	Fragment size range (bp)	Number of amplified loci
1	SR22	(CA)8G	310–1,300	17
2	SR23	(CA)8GC	590–1,190	13
3	SR70	(AC)8YG	790–1,600	15
4	SR47	(CA)8A	590–1,190	10
5	SR6	(GT)8C	520–2,000	7
6	SR16	(GA)8C	510–780	6
7	SR69	(AC)8G	590–1,100	11
8	SR34	(TC)8CC	380–1,400	8
9	SR43	(GT)8A	590–1,100	9
Total				96

DNA amplification

A 10-µL volume of the reaction mixture was used, consisting of water, 1× concentrated reaction buffer, 2.5 mM magnesium chloride, 0.2 mM dNTP, 0.5 µM primer, 0.46 U Taq polymerase, and 40 ng of genomic DNA. The reactions were carried out in the presence of nine primers, whose sequences are shown in Tab. 2. Each PCR consisted of 38 cycles of amplification, comprising denaturation (30 s at 95°C), primer attachment (45 seconds in the first three cycles at 54°C, 53°C in three successive ones, 52°C in others), and DNA replication (2 min at 72°C). These cycles were preceded by a 7-min initial denaturation at 95°C and were finished with a 7-min elongation of the products at 72°C. Electrophoresis on a 2% agarose gel supplemented with 0.05 µL mL^{-1} ethidium bromide was carried out after each reaction. The PCR product was visualized under UV light, photographed, and the pictures were analyzed for the presence (1) or absence (0) of product (band) in the gel as compared to the 1-kb DNA fragment of standard length (Thermo Scientific, GeneRuler 100 bp DNA Ladder Plus).

Data analysis

The percentages of polymorphic loci (P%), the frequency of each allele, and the average number of alleles at the locus (N_a) were established. The effective number of alleles at the locus (N_e) that describes which part of the allele will be passed to the next generation [26] and the expected heterozygosity (H_e), i.e., one that would be observed in the population in the state of Hardy–Weinberg equilibrium [27], were estimated. Based on the Shannon's index (I) [28], the level of intrapopulation differentiation was determined. The genetic distance (D) between the examined individuals was calculated [29] and a principal coordinate analysis (PCoA) was performed based on it; this PCoA was further used to determine which of the applied primers are the most useful in generating the differences between groups of pitch pine trees growing in particular subunits. Also, a molecular analysis of variance (AMOVA) was performed. The mentioned parameters of genetic variability were calculated using GeneAlex ver. 6.0 [30].

PopGene ver. 1.3 [31] was used to determine the value of total genetic diversity (H_T), intrapopulation genetic diversity (H_S), and the relative genetic diversity (G_{ST}).

STRUCTURE ver. 2.3.4 [32] was used to determine genetic clusters based on a Bayesian inference. The following operating parameters were applied: 100,000 "burn-in", 100,000 MCMC replications (Markov chain Monte Carlo) with admixture model, and correlated allele frequencies. The number of possible clusters (K) was tested in the range

of 2–10; 20 repetitions were performed for each value of *K*. Because of the difficulty in estimating the most likely number of clusters, Evanno et al.'s [33] program was used to develop the procedure on the basis of a second-order rate of change in the likelihood function (ΔK) in the Structure Harvester [34]. In order to obtain a consolidated matrix of the probability, the results obtained were analyzed in CLUMPP [35] and then elaborated graphically using DISTRUCT software [36].

Tab. 3 The values of genetic variability parameters of pitch pine.

Subunit	P%	N_a	N_e	H_e	I
299-l	91.67	1.896	1.527	0.309	0.464
203-g	93.75	1.938	1.510	0.302	0.456
301-l	64.58	1.500	1.366	0.219	0.330
299-k	73.96	1.656	1.452	0.260	0.387
299-i	62.50	1.469	1.370	0.217	0.325
46-f	68.75	1.510	1.410	0.237	0.354
10-d	71.88	1.573	1.408	0.237	0.356
Mean	75.30	1.649	1.435	0.254	0.382

Tab. 4 Molecular analysis of variance.

Source	*df*	*SS*	*MS*	Est. var.	%
Among populations	6	608.187	101.364	3.021	16
Within populations	220	3,522.831	16.013	16.013	84
Total	226	4,131.018		19.033	100

Est. var. – estimated variation.

Tab. 5 Genetic distance between pitch pine individuals from seven subunits.

	299-l	203-g	301-l	299-k	299-i	46-f	10-d
299-l	-						
203-g	0.022	-					
301-l	0.094	0.087	-				
299-k	0.052	0.060	0.089	-			
299-i	0.072	0.065	0.073	0.092	-		
46-f	0.056	0.064	0.121	0.101	0.134	-	
10-d	0.050	0.070	0.129	0.099	0.131	0.030	-

Results

The highest values for all parameters of genetic variability were found in the pines growing in subunits 299-l and 203-g. The lowest genetic variability was noted for the individuals from subunits 299-i. The percentage of polymorphic loci ranged from 62.50% to 91.67% with an average of 75.30%. The average and the effective number of alleles at the loci were from 1.469 and 1.366 to 1.938 and 1.527, respectively. The average expected heterozygosity was 0.254, while Shannon's index ranged from 0.325 to 0.464 (Tab. 3).

The total genetic diversity (H_T) determined for 96 intermicrosatellite loci amounted to 0.262 (±0.042), while the genetic diversity within the examined populations of pitch pine (H_S) was 0.254 (±0.019). The value of the G_{ST} parameter reached 0.0279, which means that 97.2% of the total variability accounted for the intrapopulation differences. The remaining 2.8% was due to the differences between the populations. Different results were obtained using AMOVA, which showed over fivefold higher value of variability, which was attributable to interpopulation differences (Tab. 4).

The average genetic distance was 0.082. The biggest similarity was observed between pine trees from subunits 203-g and 299-l (0.022). The trees growing in subunits 299-i and 46-f (0.134) were the most genetically distant, as well as those in subunits 299-i and 10-d (0.131) (Tab. 5).

The PCoA based on the calculation of genetic distances allowed the graphical representation of pitch pine variability in a two-dimensional model. For analysis performed separately for each of the nine applied primers, it was found that most of them did not group the examined individuals for their relationship (Fig. 2A–H). In turn, the PCoA graph obtained after the combined analysis of all primers revealed that the first two components explained 57.29% of the observed variability, and the examined trees were distinctly grouped into three separate clusters, which suggests that the seedlings used to establish the research crops could have been grown from the seeds collected from three different locations (Fig. 2J). The first group was formed by the individuals from subunits 10-d and 46-f and some trees from subunit 299-l. The second group consisted of the trees from subunit 203-g and some individuals grown in subunit 299-l. In turn, the third group was formed by the pines from subunits 301-l, 299-k, and 299-i. A similar

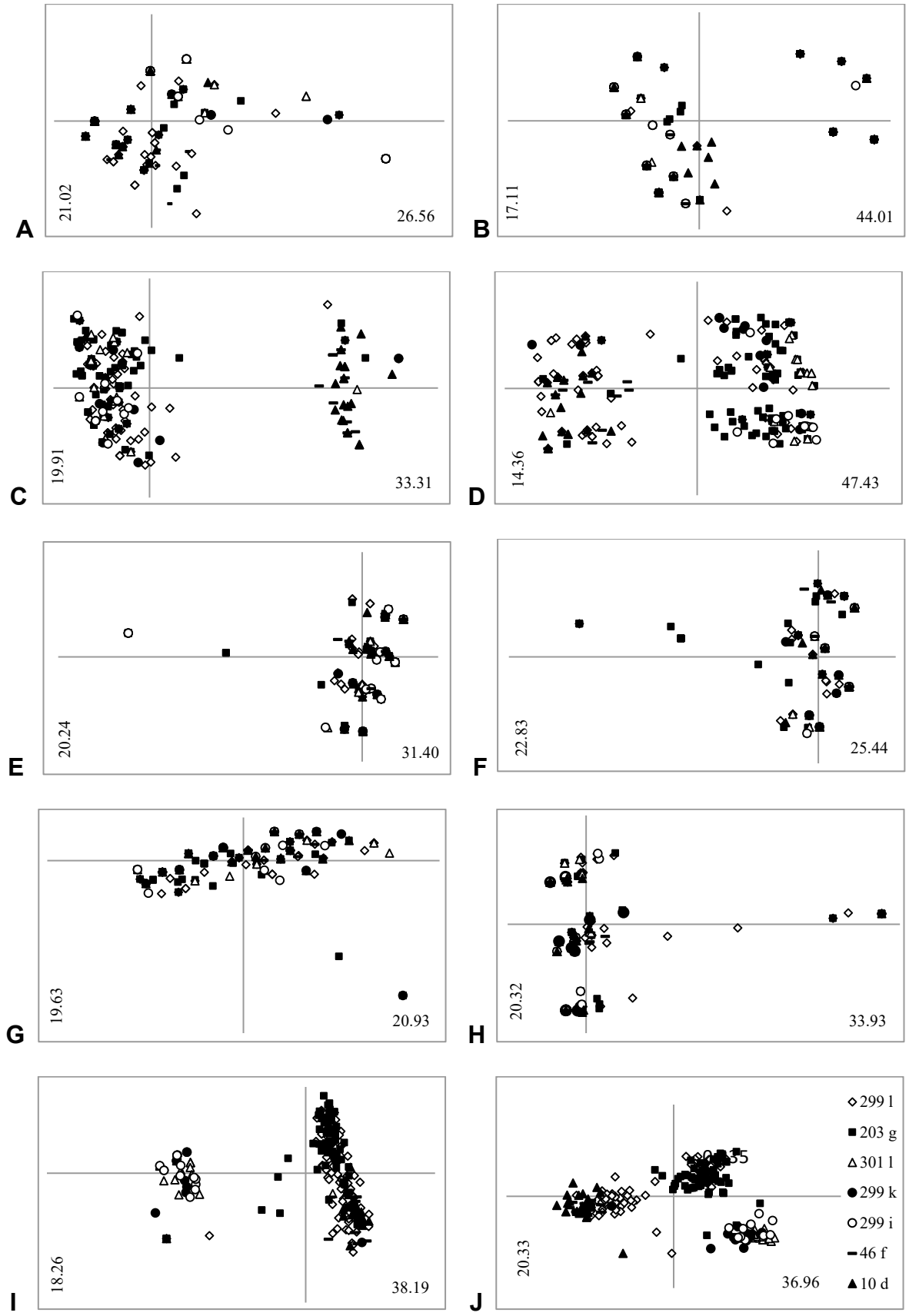

Fig. 2 The results of PCoA analysis performed based on genetic distance-based primers (**A**) SR6, (**B**) SR16, (**C**) SR22, (**D**) SR23, (**E**) SR34, (**F**) SR43, (**G**) SR47, (**H**) SR69, (**I**) SR70, (**J**) all primers.

distribution of the population was obtained using the primer SR70, as indicated in Fig. 2I. This primer may thus be regarded as a marker that differentiates the examined individuals of pitch pine.

The analyses performed using Structure and then Structure Harvester showed the presence of six genetic clusters ($K = 6$) (Fig. 3). Genetically, the most homogenous were the pines from subunit 301-l. The populations from subunits 299-k and 299-I contained many individuals of a mixed gene pool. However, the ones that shared genetic basis with individuals from the subunit 301-l were dominant. Individuals from these three subunits formed one cluster on the PCoA graph. In both the PCoA-based and Structure graphs, the pines from subunit 299-l were divided into two groups. The individuals from the first group showed a genetic similarity to the pines from subunit 203-g. In turn, in the other one, as in subunits 46-f and 10-d, it was not possible to indicate one dominant gene pool, but all individuals were characterized by similar cluster membership coefficient values.

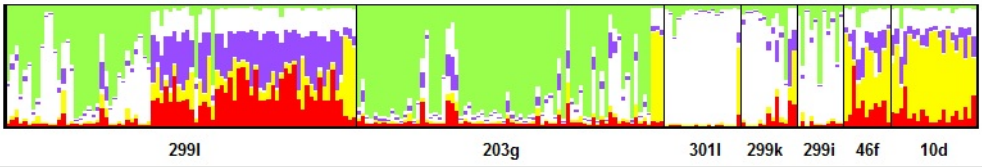

Fig. 3 Image of the STRUCTURE analysis for $K = 6$.

Discussion

The Niepołomice Forest is the largest forest complex located near Krakow, one of the main towns of the former Galicia. Therefore, it was easy to locate different types of experiments there. Probably, more than 20 experimental plots were established at the end of the nineteenth century within the area of the current Niepołomice Forest District, where *Quercus rubra*, *Juglans regia*, *Carya cordiformis*, *C. ovata*, *Pinus strobus*, *P. banksiana*, and *P. rigida* seedlings were planted. The determination of the location of the research crops was hampered because of the lack of historical data from the early twentieth century. However, Dubiel's [23] report on the occurrence of introduced species in the stands of Niepołomice Forest allowed us to find the majority of them. Using isoenzymatic markers, the inventory of trees and the analysis of the genetic variability in shagbark hickory has been performed so far [37]. The results presented in this paper are a continuation of the analysis of the research crops, as well as the introduced species, in the experimental region founded by the Department of Forestry Research in Mariabrunn in Central Europe.

In this study, the genetic variability in pitch pine was evaluated using, for the first time, the ISSR technique, which combines the simplicity of RAPD markers with several advantages of the AFLP or SSR techniques. The applied primers were composed of two or three nucleotide repeats. They also contained additional bases at the 3′ or 5′ end in some cases. As compared to the RAPD method, ISSR technique allows obtaining a greater number of products in the single reaction. Amplification involves the use of one primer that is complementary to the microsatellite sequence, which allows obtaining up to a few dozens of products flanked by microsatellites – a number that is usually higher than that from the reaction with random primers. Consequently, results that adequately reflect the degree of population variability are obtained in a shorter time and with lower consumption of reagents. Furthermore, ISSR primers are longer than the RAPD primers, which requires the use of a higher temperature for hybridization and makes them bind more specifically to the matrix. Using additional bases at the 5′ or 3′ end limits the number of nonspecific products formed in the reaction. The result is the greater reproducibility of this method. However, a disadvantage of this technique is the dominant character of the obtained products. Despite this disadvantage, the ISSR technique has been used to determine the level of genetic diversity in many forest tree species of the genera *Abies* [38], *Pinus* [4,39–41], *Picea* [42], *Pseudotsuga* [43], *Taxus* [44], *Fagus* [45], *Quercus* [46], and others. There are, however, no studies

on ISSR analysis in pitch pine. Using the above method, it was demonstrated in this study that the pitch pine species is genetically variable as 75.30% of the examined loci were polymorphic. This value is higher than those obtained for other pine species using ISSR technique, including values for *P. sylvestris* (P% = 42%) [39], *P. nigra* (P% = 51.04; H_e = 0.175) [47], *P. koraiensis* (P% = 60.70) [4], and *P. dalatensis* (P% = 50.53) [48]. Shannon's index (*I*) with a value of 0.382 indicated that the examined populations of pitch pine were characterized by more than threefold higher genetic variability as compared to the variability in *P. sylvestris* (*I* = 0.158) [39], while similar values were found for *P. tabulaeformis* (*I* = 0.3078) [49]. The value of total genetic diversity (H_T = 0.262) was lower than that in *P. koraiensis* (H_T = 0.348) [4] and *P. tabulaeformis* (H_T = 0.415) [41], as well as the alpine populations of *P. sylvestris* (H_T = 0.310), but it was similar to the values obtained for the populations of Scots pine derived from the Apennines (H_T = 0.217) [40].

Due to the lack of information on the polymorphism of intermicrosatellite loci, it is not possible to compare the results obtained here with previously reported ones by other authors. However, the observed genetic variation was slightly higher (H_e = 0.254) than that observed using isoenzymatic markers. Hawley et al. [50] obtained an average value of 0.226 for the expected heterozygosity (central populations, H_e = 0.234; marginal ones, H_e = 0.201) using the starch gel electrophoresis technique to evaluate the genetic variability in a small population of pitch pine (approx. 300 individuals) derived from a population of Camp Johnson (Vermont, USA). Misenti and DeHayes [18] examined a similar issue to obtain an H_e value of 0.246 for the central populations, while 0.209 for the marginal ones. A study on isoenzymatic variability was also conducted by Guries and Ledig [16], who determined the genetic variability in 11 populations of pitch pine growing in the eastern part of the USA and Canada. Based on the analysis of 21 isoenzymatic loci, they found the expected heterozygosity (H_e = 0.138) to be twofold lesser than what was observed in the present study.

The genetic variability within and between the examined populations of pitch pine obtained using AMOVA amounted to 84% and 16%, respectively, while on the basis of relative genetic diversity index, it was determined that only 2.8% of the variability is attributable to interpopulation differences. A similarly low level of genetic diversity within stands was reported by Wachowiak [51] in eight Scots pine populations that were assessed with nuclear DNA markers, while results obtained by Nowakowska [52] were higher by an order of magnitude (G_{ST} = 0.215). Although the results obtained are different, they indicated that, as observed in other anemophilous species, most of the observed variability for pitch pine is because of the differences within the populations. This is confirmed by a study by Cui et al. [49], who established the interpopulation diversity for *P. tabulaeformis* to be on the level of 13.6%. In turn, significantly higher values of this parameter were observed by Feng et al. [4] and Li et al. [39] in *P. koraiensis* (27.0%) and *P. sylvestris* (39.65%), respectively. The aforementioned variability in the G_{ST} parameter probably depends on the analyzed tree species. While studying the seedlings of pitch pine of different provenance in the nursery, Ledig et al. [7] stated that the distribution of variability within and between the examined populations also depends on the feature being analyzed. For pitch pine growth, almost all observed variability resulted from intrapopulation differences. Ledig and Clark [53] demonstrated that the variability in physiological traits in *Pinus rigida*, such as photosynthesis or respiration rate, equally depended on intra- and interpopulation differences.

Using the ISSR technique, a slightly lower value of the genetic distance (0.082) than the values reported for other species of pine studied was obtained. The analysis of eight populations of black pine in southern Spain and northern Morocco by ISSR markers showed that the genetic distance between the examined provenances is *D* = 0.150 [47]. A much higher genetic distance, in the range of 0.0856–0.3223, was obtained after applying this technique on the populations of *Pinus koraiensis* [4], *P. dalatensis* (*D* = 0.286) [48], and *P. tabulaeformis* (*D* = 0.3078) [49]. Similar results as described in this paper were obtained for *P. roxburghii* (*D* = 0.082) [54]. It can be assumed that ISSR method generates a higher coefficient of genetic distance than other techniques, such as the isoenzymatic technique, that allows to obtain its significantly lower value (*D* = 0.005) between pitch pine populations in the eastern part of the USA and Canada [16]. Also, genetic studies conducted on central and marginal populations showed a lower coefficient of genetic distance on a level of *D* = 0.024 [18].

Conclusions

The analyzed parameters of genetic variability in pitch pine growing in the area of Niepołomice Forest did not differ significantly from the values reported in the literature for *P. rigida* and other tree species of the genus *Pinus*. The applied clustering methods indicated the presence of three groups of pines with a different genetic background; the resulting division was associated with the location of trees on particular crops in the area of Niepołomice Forest. The resulting distribution was generated by one of the primers (SR70), which can be used as a marker that can help differentiate the origins of *P. rigida*. The results obtained suggest that the seedlings used to establish the experimental plots of pitch pine in the Niepołomice Forest could have originated from the seeds collected from various stands.

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