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Genetic identification of alien larch taxa – the case of the Tatra National Park

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Abstract: The natural consequences of introducing alien species can be significant. This is particularly a concern where the taxa have an invasive nature of spreading or in those that freely crossbreed with native species. The hybridization process may lead to impoverishment or even loss of the native gene pool. This is especially dangerous in unique areas that stand out due to their special natural characteristics, such as the Tatra National Park. The determination of the scale of occurrence of alien larch species in the national park and the evaluation of the genetic diversity of the native population is crucial for the conservation of genetic resources and strictly adheres to the latest conservation genetics trends.

We evaluated the possibility of effective use of molecular markers for taxonomic identification of the native European larch (*Larix decidua* Mill.), as well as the alien Japanese larch (*Larix kaempferi* [Lambert] Carriere) and the hybrid form (*Larix* × *eurolepis* Henry). Microsatellite markers were used to analyse the genetic diversity of individuals identified as European larch from natural refuges and artificial plantings.

Of the 148 trees analysed, 105 were identified as the European larch, 38 as Japanese larch, and five as hybrids. The analysis of the molecular variability of two European larch groups of indigenous and artificial origin showed comparable level of diversity.

This study confirmed the effectiveness of the use of selected molecular markers in identification of larch species, which is difficult based on morphological traits. The results indicate the possibility for the effective use of genetic tools in the creation of protection programmes, especially for naturally valuable sites, based on genetic taxonomic identification and richness verification of protected gene pools.

Keywords: taxonomic identification, *Larix decidua*, *Larix kaempferi*, *Larix* × *eurolepis*, alien species, conservation genetics

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Introduction

The natural consequences that result from the introduction of an alien species are always difficult to predict. A significant negative effect of the introduction is apparent, especially in cases where the taxa have an invasive nature of spreading or those that freely crossbreed with native species (Allendorf et al., 2001; Pyšek et al., 2004). In instances where distinguishing the native species from an alien or hybrid species on the basis of morphological features is very difficult or even impossible, the scale of the problem becomes even greater. This applies to the European larch (*Larix decidua* Mill.), whose gene pool has been contaminated by the Japanese larch (*Larix kaempferi* [Lambert] Carriere) and hybrid forms of these two species (Wagner, 2013).

In Europe, the European larch is endemic and is characterized by a relatively small and strongly divided range (Fig. 1), which is associated with areas that have continental climatic characteristics (Pâques et al., 2013). The largest and most compact fragment is found in the Alps, but the larch is found in the Western Carpathians (Tatra), Sudetes, Eastern Carpathians, and Świętokrzyskie Mountains (Madeyski, 1974; Hultén & Fries, 1986). Isolation of individual patches of the range resulted in the creation of local and unique ecotypes, described as independent taxa or subspecies. One such variant is *L. decidua* subsp. *decidua* var. *adenocarpa* Borbas from the Tatras, which is mainly found in the upper parts of the upper subalpine forest, along the upper border of the forest. The European larch variety present in the Tatras is one of the many examples of the natural uniqueness of this area (Mirek, 1996; Mráz et al., 2016a, 2016b). The geomorphological separation and specificity of the Tatra climate contributed to the creation of habitats and plant communities that were characteristic only for this region. The Tatra Mountains are the only massif in the Carpathians with a typical alpine habitat, which is closely related to the richness of flora and fauna and the presence of endemic as well as relict species (Zięba et al., 2018). These factors decided that the initial concept of a protective area for the Tatras in the form of a national park appeared as early as the 1880s, following the example of the first North American national park in Yellowstone. Eventually, the idea for the creation of the Tatra National Park (TNP) came to fruition in 1954. Currently, the area of the park extends to more than 20,000 ha, which makes it one of the largest national parks in Poland (Mirek, 1996).

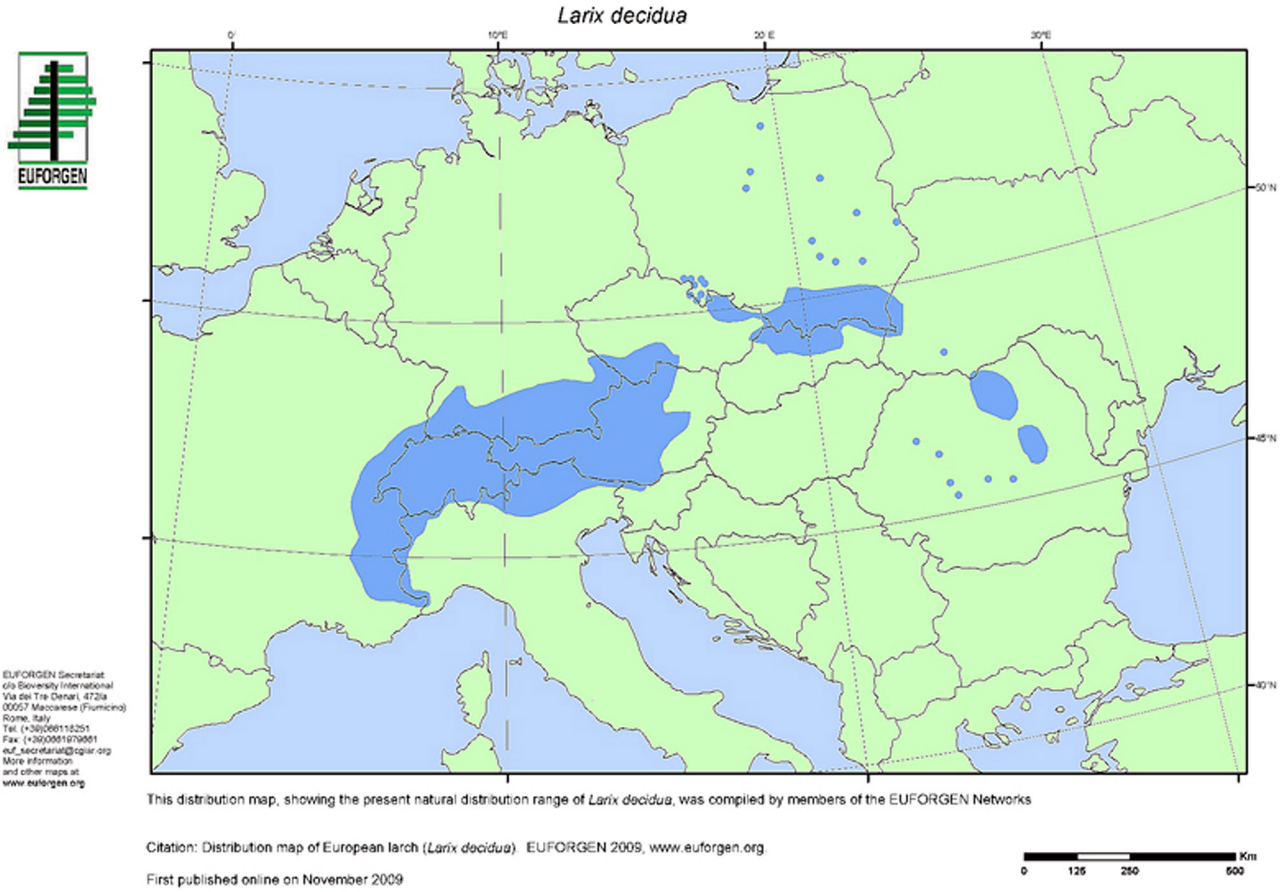

Fig. 1. Distribution range of European larch (*Larix decidua* Mill.) in Europe (distribution map courtesy by EUFORGEN)

The history of the presence of the larch in the Tatra forests is turbulent, reflecting the economic trends of the past years. This species, due to the characteristics of the wood, was a valuable material for the metallurgy, construction, and mining industries that developed in the 18th and 19th centuries. As a consequence of these factors, the larch was almost completely removed from the areas accessible to man, and natural populations were preserved only in higher locations in the mountains, in isolated and hard-to-reach places (Madeyski, 1974; Ralska-Jasiewiczowa, 1983). The massive, and often uncontrolled, introduction of spruce, fir, and larch, frequently of unknown origin, to the Tatra forests, began in the 20th century (Sokołowski, 1936; Madeyski, 1974). These activities resulted in the introduction of alien species to the TNP, which were not native flora of the Tatra Mountains or even Poland.

Since the 19th century, efforts to increase the productivity of forest stands in Europe by introducing alien species have been common. One of these species was the Japanese larch (Larsson-Stern, 2012). It quickly spread out of control (Filipak & Pilarek, 2003) because of its morphological similarity to the European larch (Schmidt, 2012) and the ease of crossbreeding. The Japanese larch, originating from an oceanic climate, has adapted particularly well in places with high humidity (Filipiak & Napierała-Filipiak, 2008), especially in coastal or mountain areas (Schmidt, 2012) in European forests. In addition, the hybrid larch (*Larix* × *eurolepis* Henry) was extensively introduced into forests, especially in central Europe (Sander & Läänelaid, 2007; Da Ronch et al., 2016). It is difficult to distinguish the hybrid larch from its parental species, as well as the parents between themselves. There are no clear morphological differences between the European and Japanese larch (Schmidt, 2012). The morphological features of the hybrid may be characterized by indirect parental traits or may manifest the dominance of the features of one of the parent species (Vidakovic, 1991).

Reports in the literature (Madeyski, 1974) indicate that, probably, the Japanese larch was introduced in the Tatra Mountains within the afforestation region of the lower subalpine forest in the first half of the 20th century. Presumably, this introduction was accidental and related to the difficulty of identification of these two species, which is not uncommon in the history of introductions (Danielewicz & Wiatrowska, 2012). Currently, it is necessary to distinguish tree species to properly implement conservation tasks and strive to preserve the uniqueness and genetic identity of subpopulations such as the Tatra larch. At present, the best available tool for taxonomic identification is DNA sequence polymorphisms analysis (Armenise et al., 2012; Laiou et al., 2013; Coissac et al., 2016), which is the basis for the creation of effective programmes for the protection of genetic resources. Molecular markers have become powerful tools to determine the extent of hybridization processes and to obtain knowledge that is necessary to implement conservation genetics programmes (Coart et al., 2003; DeSalle & Amato, 2004; Smulders et al., 2008).

The main goal of this study was the taxonomic identification of a selected group of trees using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to verify the occurrence of alien larch taxa in the TNP. In addition, to determine the genetic diversity of European larch from natural origins and artificial plantings, nuclear simple sequence repeat markers (*n*SSRs) were used. This study was undertaken to determine the scale of occurrence of alien larch species in the national park and to analyse whether artificial regeneration modified the species' gene pool in TNP. Such knowledge is crucial for conservation and preservation of existing genetic resources of the European larch in TNP and strictly adheres to the latest trends in the conservation genetics.

Methods

Population sampling

Shoots with needles were collected from 148 trees; of these, 48 trees, due to their location and age, constituted a group of individuals recognized as larches of natural origin. These trees grew in the area of the upper forest border and in the upper subalpine forest in the entire TNP, and their age was most often estimated to be at least 100 years. The remaining 100 trees were collected from the Strążyska Valley, Jaworzynka Valley, Uplaz Kalacki, and Kopieniec

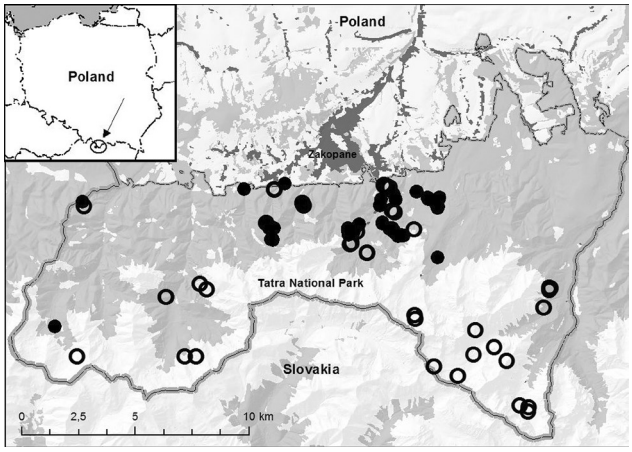

Fig. 2. Geographical distribution of the studied trees from the Tatra National Park. The black circles indicate the location of the trees from artificial plantings and empty circles from natural refuges

(approximately 25 samples per location). These are regions where we suspected the presence of individuals who belonged to alien taxa and were introduced as artificial plantings in the mid-20th century. Fig. 2 shows the location of trees from which DNA samples were collected.

Molecular methods

According to the methodology specified by the manufacturer, the EURx DNA isolation kit was used to conduct DNA extraction. Length polymorphism of the *rbcL* gene was determined using primers available in the literature (Petit et al., 1998). The *Taq*I restriction enzyme (Thermo Fisher Scientific) and the so-called chloroplast marker ll-*Taq*I (Petit et al., 1998; Acheré et al., 2004; Jagielska, 2008) were used to identify the European, Japanese, and hybrid larch species. DNA amplification included the following steps: initial denaturation at 94°C for 6 min., 35 cycles of specific amplification (45 sec. at 94°C, 45 sec. at 55°C, 3 min. and 30 sec. at 70°C), and final elongation at 72°C for 10 minutes. The PCR mixture comprised 10 μL of RedTaq Ready Mix (Sigma-Aldrich), primer mix (0.25 μM each of primers) and 20 ng of the genomic DNA. The amplified DNA fragments were purified and subjected to digestion with the restriction enzyme *Taq*I (Thermo Fisher Scientific). Five units of the enzyme *Taq*I was used for digestion of 20 ng of the PCR product in 60 min. at 65°C. The DNA fragments obtained after digestion were electrophoretically separated on 1.5% agarose gel stained with ethidium bromide (0.5 mg/mL). Fig. 3 presents the interpretation of the obtained electrophoresis images, which were consistent with the methodology for the genetic identification of larch (Acheré et al., 2004; Jagielska, 2008). A sample of Japanese larch from the Arboretum Wirty in the Kaliska Forest Inspectorate was used as a reference sample in the analyses.

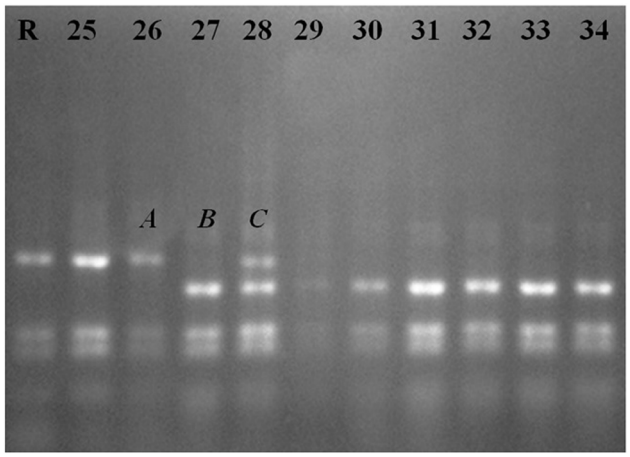

Fig. 3. Picture of electrophoretic separation of PCR products after DNA digestion by restricted enzyme. Trees' numbers 25-34, sample A – Japanese larch (fragment of length 601bp), B – European larch (fragment of length 481bp), C – hybrid larch, R – reference sample

The polymorphism and genetic structure of the European larch individuals identified using the ll-*Taq*I marker were determined with 11 *n*SSRs (Table 1) developed for *Larix decidua* (Isoda & Watanabe 2006; Wagner et al., 2012; Wagner, 2013). The amplification was carried out with two PCR-multiplex reactions (Appendix 1, Supplementary materials). DNA amplification included pre-denaturation (95°C, 15

Table 1. Characteristics of microsatellite DNA markers used to study genetic diversity of European larch

Locus	Primer sequence (5'–3')	Fluorescent dyes	Source
LD42	F: TCGTATGCATTGTCCAAATTTCC R: TCCAAGTGAGGTCACACGAG	FAM	Wagner 2012
LD101	F: ACACCAAGGACTCTCTGACTAC R: GGTGATTCCAGAAGCAGGTG	VIC	Wagner 2012
LD31	F: TTGAACTAGGGAGATCCGGC R: AATAAAATAGCATTCCATGTGTAGC	FAM	Wagner 2012
LD45	F: TGTGGGAGGTATAGCTTGGC R: AGTAGGATGGAATGATGGAAACAC	FAM	Wagner 2012
LD50	F: GAAGGCGACTTTACATGCCC R: TCCATCTTTATGTCTCTTCCATGC	PET	Wagner 2012
LD56	F: AGCCATCGTGGTTCTTCTTTG R: CTTGTAACTGTGCACCCACC	PET	Wagner 2012
bcLK189	F: ACCATACGCATACCCAATAGA R: AGTTTTCCTTTCCCACACAAT	NED	Isoda and Watanabe 2006
bcLK211	F: CCATTCTCCATAGGTTCATTG R: ATGCTCCTTACTAAGTCAGATACAC	NED	Isoda and Watanabe 2006
bcLK228	F: CCCTAACCCTAGAATCCAATAA R: GAGGAAGGCGACAAGTCATT	PET	Isoda and Watanabe 2006
bcLK229	F: ATGCCCAAAAACGAAAAAGT R: TTTGCACTGCCAGATTCAGA	PET	Isoda and Watanabe 2006
bcLK263	F: CGATTGGTATAGTGGTCATTGT R: CCATCATACCTTCTTGAAGAG	NED	Isoda and Watanabe 2006

minutes), 32 cycles of specific amplification (94°C for 30 sec., 56°C for 1 min. and 72°C for 1 min.) and final elongation at 60°C for 30 min. The PCR products were separated in the ABI 3130XL automatic sequencer (Applied Biosystems, Foster City, CA, USA) with the LIZ600 size standard. Genotypes were scored using GENOTYPER 3.7 software.

Statistical methods

Using the GenAlEx v.6 software (Peakall & Smouse, 2006, 2012), the genetic intra-population parameters were calculated as follows: N_A, the mean number of alleles in the locus; N_E, the effective number of alleles in the locus; H_O and H_E, the observed and expected heterozygosity, respectively, and F_{IS}, Wright's inbreeding coefficient. The web server of the software Genepop v. 4.0 (Raymond & Rousset, 1995; Rousset, 2008) was used to test the deviations from Hardy-Weinberg Equilibrium (HWE) with the exact test (Guo & Thompson, 1992) based on a Markov Chain Monte Carlo simulation (MCMC) with default setting, as suggested by the author. To compare the rates of genetic diversity as well as assess allelic richness (AR), a rarefaction procedure using HP-Rare 1.0 was applied to distinguished larch groups, despite the different sample sizes (Kalinowski, 2005). Using the Kruskal–Wallis nonparametric test in the Statistica 12 package, the significance of the observed differences in the mean values of genetic parameters between the two larch groups was verified. Using the MicroChecker program 2.2.3 (Van Oosterhout et al., 2004, 2006), the presence of null alleles that may result in excess homozygotes in these populations was determined, and the data were corrected. The analysis of molecular variance (AMOVA) was conducted in the GenAlEx to determine the hierarchical distribution of molecular variability, both within and between the two studied larch groups. The permutation procedure (999 permutations) was used to estimate the significance of the individual components to the variance. Furthermore, we used principal coordinate analysis (PCoA) in the GenAlEx to show the genetic similarity between individuals from natural and artificial sites based on pairwise F_{ST} values (Hartl & Clark, 1997).

Results

Species identification using the RFLP of the ll-*Taq*I marker showed that, for a total of 148 samples collected from the territory of TNP, 105 were European larches, 38 were Japanese larches, and five were hybrids (Fig. 4).

Within the group of 48 larches that were assumed in the field as being native, the analysis showed the presence of a single Japanese larch located near Lake Morskie Oko (sample no. 123). This study indicated the presence of alien taxa in the Tatra Mountains in the regions of Strążyska Valley, Jaworzynka Valley, Uplaz Kalacki, and Kopieniec. In the group of 100 larches that were considered to have originated from artificial plantings, 58 European, 37 Japanese, and five hybrid larches were found. Individuals of hybrid origin (*Larix* × *eurolepis*) mainly occurred in the Strążyska Valley (three trees) and Jaworzynka Valley (two trees). Most, as many as 13, Japanese larches were identified in the Kopieniec area. Nine individuals of this species were recorded in the Strążyska Valley and the surrounding subalpine forests. The remaining individuals of this species were observed as follows: seven in the Jaworzynka Valley, five in Nosal and Kuźnice, two in Uplaz Kalacki, and one in the area of the High Tatras.

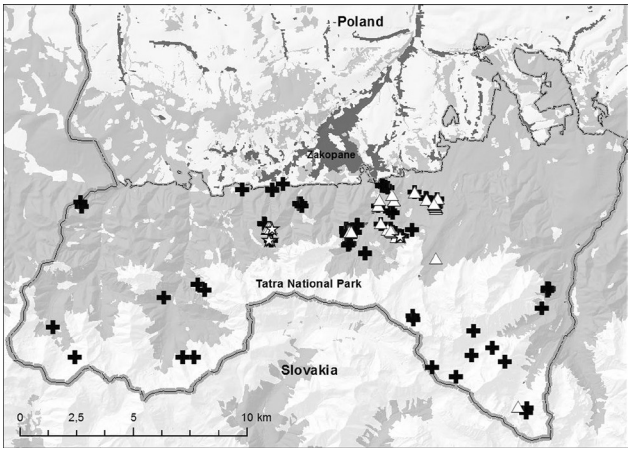

Fig. 4. Location of identified larch taxa in the Tatra National Park. The crosses indicate the location of European larches (*Larix decidua* Mill.), the stars Japanese larches (*Larix kaempferi* (Lambert) Carriere), and the triangles Euro-Japanese larches (*Larix* × *eurolepis* Henry)

The analysis with using 11 *n*SSRs was conducted for 105 trees identified as the European larch. In this group, 46 trees were individuals of probable indigenous origin (natural), whereas the remaining 59 were planted. All tested loci were polymorphic, and a total of 166 alleles were identified. The analysis of the genetic variability of individuals of indigenous and artificial origin (Table 2) showed a high similarity between the analysed groups. The average number of alleles (N_a) ranged from 7 to 20 in the group of indigenous larches and from 7 to 22 in the group of larches of artificial origin, with mean values for the groups of 12.91 and 13.27, respectively. Slightly higher values of the mean effective number of alleles (N_e), observed (H_o) and expected heterozygosity (H_e) were observed in the group of individuals of artificial origin. The average allelic richness (AR) was 12.608. In the group of individuals indicated as indigenous, the AR was slightly higher

Table 2. Genetic diversity estimates for natural and artificial group of European larch. Na – mean number of alleles in the locus; Ne – effective number of alleles in the locus; AR – allelic richness (based on 85 genes); Ho and He – observed and expected heterozygosity; F_{IS} – Wright's inbreeding coefficient (the coefficient denoted with asterisk (*) is significantly different from zero at the level of $p < 0.001$)

Group	Locus	Na	Ne	AR	Ho	He	F_{IS}
natural	LD42	7.00	4.03	7.000	0.718	0.752	0.045
	LD101	7.00	1.85	6.914	0.413	0.461	0.103
	LD31	17.00	6.16	16.594	0.826	0.838	0.014
	LD45	9.00	5.99	8.843	0.739	0.833	0.113
	LD50	14.00	6.69	13.894	0.762	0.851	0.104*
	LD56	10.00	6.95	9.848	0.783	0.856	0.086
	LK189	12.00	7.94	11.767	0.804	0.874	0.080
	LK211	17.00	4.84	16.599	0.826	0.793	–0.041
	LK228	18.00	12.71	17.619	0.848	0.921	0.080
	LK229	11.00	4.98	10.762	0.717	0.799	0.102
	LK263	20.00	12.21	19.818	0.773	0.918	0.158
	Mean	12.91	6.76	12.696	0.746	0.809	0.077
artificial	LD42	8.00	4.16	7.522	0.714	0.759	0.060
	LD101	7.00	2.17	6.435	0.559	0.540	–0.036
	LD31	15.00	8.95	14.143	0.786	0.888	0.115
	LD45	8.00	5.77	7.720	0.746	0.827	0.098
	LD50	14.00	5.86	12.975	0.763	0.830	0.081
	LD56	12.00	7.90	11.362	0.800	0.873	0.084
	LK189	11.00	7.26	10.637	0.847	0.862	0.017
	LK211	20.00	5.02	18.743	0.797	0.801	0.005
	LK228	16.00	11.51	15.516	0.847	0.913	0.072
	LK229	13.00	5.18	12.170	0.763	0.807	0.055
	LK263	22.00	12.19	20.489	0.810	0.918	0.117
	Mean	13.27	6.91	12.519	0.767	0.820	0.061
	Mean	13.09	6.83	12.608	0.756	0.814	0.069

(12.696) than in individuals from artificial plantings (12.519). However, these small differences that were observed were not statistically significant. The values of the inbreeding coefficients (F_{IS}) were positive in most cases, that indicates the excess of homozygotes. However, significant F_{IS} values were observed only for one locus, in the group of natural larches (LD50). The AMOVA showed no differentiation between the groups of individuals with natural and artificial origins. Approximately 8% of the total variance was the variability between individuals, and 92% was intra-individual variability (Table 3). In PCoA (Fig. 5), the first two axes explained 12.92% of the variation. Individuals from both subpopulations formed a homogeneous group with no tendency towards separation.

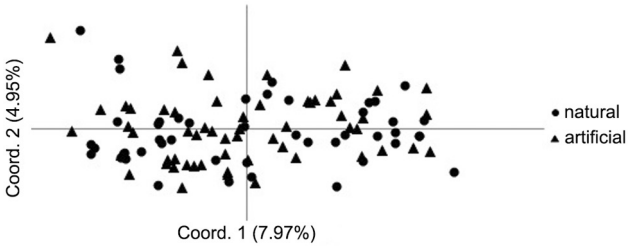

Fig. 5. Principal coordinate analysis (PCoA) based on genetic distances between individuals from two studied groups of European larch

Discussion

The lack of clear morphological traits that allow error-free identification of larch taxa (Pâques et al., 2006) prompted to seek a solution to the problem by using biochemical markers (Bergmann & Ruetz, 1987; Haecker & Bergmann, 1991; Ennos & Qian, 1994). Currently, species verification is based on DNA sequence polymorphism analysis (Scheepers et al., 2000; Eriksson, 2017). A set of mitochondrial and chloroplast DNA markers with interspecific polymorphic patterns, which can be used to identify species, have been developed for larches (Acheré et al., 2004; Gros-Louis et al., 2005; Philippe et al., 2016). This was confirmed by the present study, wherein the use of the chloroplast DNA marker ll-*Taq*I allowed us to outline the problem of contamination of the native European larch gene pool in the sample of trees studied in the stands of the TNP. The presence of alien flora species of the Tatra Mountains, such as the Japanese larch (*Larix kaempferi*) and hybrid larch (*Larix* × *eurolepis*), was found in the analysed group of larches. The results obtained confirm earlier suspicions of the possibility that foreign taxa may be introduced to the TNP during artificial plantings conducted in the mid-20th century, particularly in the regions of Strążyska Valley, Jaworzynka Valley, Uplaz Kalacki, and Kopieniec.

The introduction of plants always involves risks and unpredictable consequences, mainly related to the possible invasion of an alien species (Olaczek, 2000), which depends on the phenotypic plasticity and/or genetic differentiation to achieve the required levels of fitness (Richardson & Pyšek, 2006). The visible effects of the introduction of alien species may be revealed with a delay of several dozen or hundreds of years (Szwagrzyk, 2000). The time of

Table 3. Hierarchical analysis of molecular variance (AMOVA) based on pairwise FST values

Source	df	SS	MS	Variance	% total	p
Among groups	1	4.520	4.520	0.000	0%	ns
Among individuals	103	503.546	4.889	0.342	8%	0.001
Within individuals	105	441.500	4.205	4.205	92%	0.001

df – degree of freedom, SS – Sum of squares, MS – Mean squares.

the delay between the start of the invasion and the typical phase of exponential growth, called the lag phase (Richardson et al., 2011), is important from a practical point of view (Richardson & Pyšek, 2006). Knowledge of the lag phase duration can help predict, mitigate, or avoid the possible effects of an invasion. Preventive activities should take into consideration all introduced species, including high-risk species and 'sleeper weeds' (sensu Groves et al., 2003). The key aspects in the discussion about the possibility of plant invasions in a given area are the origin, residence and invasion status of an alien species (Pyšek et al., 2004). The threats of introduction involve the formation of secondary populations of alien species, the ability to control and transform phytocoenoses while displacing native dendroflora and transformation of the species composition of communities (Danielewicz & Wiatrowska, 2012), as well as the transformation of soil conditions (Vanderhoeven et al., 2005).

In Poland, the Japanese larch is considered a species that can pose a threat to biodiversity (Tokarska-Guzik et al., 2011). It is not strongly invasive, according to the definition by Pyšek et al. (2004), in occupying new ecological niches. The greatest threat lies in its ability to hybridize with the native species. The potential for hybridization is observed in many species of plants, including trees of the genera *Larix*, *Sorbus*, *Populus*, and *Salix*, and this generates anxiety for many biologists and conservation geneticists. However, there is currently no clear agreement on how to treat products of hybridization involving alien species (Pyšek et al., 2004). Hybridization has been shown to be an important mechanism for the evolution of invasive species (Ellstrand & Schierenbeck, 2000) and poses a much greater threat to the native gene pool than the physical presence of alien species. The process may have different evolutionary consequences for the taxa involved (Ellstrand & Schierenbeck, 2000; Baack & Rieseberg, 2007). The resulting hybrids may be better adapted to the given environmental conditions if they are favoured by natural selection (Rieseberg et al., 2007) and may cause the impoverishment of the native gene pool or lead to the elimination of parental forms. This particularly applies to protected areas, such as the TNP, where the loss of genetic purity poses a great threat to the natural character of the ecosystem. The Tatras are the most important natural origin for European larch in Poland (Holeksa & Szwagrzyk, 2004). Therefore, in protected areas or in large natural forest complexes, the introduction of alien species for breeding has not been recommended (Barzdajn, 2006).

In Europe, in the case of larch, it is difficult to present the origin and the scale of contamination of the native gene pool or to assess the actual impact of introductions on current populations (Jansen & Geburek, 2016). As in the case of TNP, the knowledge is often based only on historical sources because there is no information about the source of seeds and origins of seedlings. This study conducted on a selected group of individuals showed that approximately 26% of the examined trees represent the alien larch species. It is surprising that as many as 25 individuals, out of 38 identified Japanese larches, grow in the upper subalpine forest – that is, between 1200 and 1550 m a.s.l. These are individuals whose age is estimated to be 15–30 years and are likely of the spontaneous recruitment. This observation confirms previous suggestions (Schmidt, 2012) that the Japanese larch, despite difficult high-mountain conditions, performs well and can successfully compete with the native species to occupy a few sufficiently sunlit gaps. The presence of alien species found in the study at locations higher than 1200 m a.s.l. is very worrying and provoke the question of a possible reduction of the native gene pool. The most pessimistic scenario assumes the total disappearance of genotypes preserved by thousands of years of presence in the area that would be an irreparable loss.

The taxonomic identification showed only five hybrid individuals. Single one was located in the lower subalpine forest area (900–1200 m a.s.l.) and the others grow in the upper subalpine forest area. Their ages ranged 45–60 years, which indicates that they were probably planted as part of the afforestation activity conducted in this area. The lack of hybrids in the group of younger trees that, due to their age are suspected to have originated from natural regeneration, may be optimistic, as it indicates the lack of hybridization. The reason for this may be difficult climatic conditions that are unfavourable for seed formation and germination or the spatial distribution of larches and pollen dispersion limitation. Larch is a species that does not form dense forest complexes, and the higher the elevation above sea level is, the lower the density of trees, which causes the average pollen production per square metre to decrease (Lewandowski & Burczyk, 2000). However, this study was conducted on a subjectively chosen group of trees, and this observation should not be generalized for the entire Tatra region.

The range of variability of the indigenous larch for TNP is comparable to the variability of individuals from artificial plantings. This confirmed previous reports that species of forest trees are characterized by a stable genetic structure and can recover. Therefore, there is no need to preserve every single genotype to maintain the proper level of genetic variation (Savolainen, 2000). The maintenance of the appropriate level of genetic diversity is essential for the future sustainability and adaptability of forest ecosystems (Alberto et al., 2013). In TNP, we found a high level of genetic diversity in both natural and artificial

groups with He = 0.809 and He = 0.820, respectively. In general, the genetic diversity of the investigated groups was similar to populations of *Larix decidua* from the French Alps (He = 0.761) (Nardin et al., 2015), the Swiss Alps (He =0.750) (Pluess, 2011), the Romanian Carpathians (He = 0.738) (Gramazio et al., 2018) and the Alps and the Western Carpathians (He = 0.686) (Dostálek et al., 2018).

One of the assumptions of conservation genetics is the creation of protective plans based on taxonomic verification and the identification of the risk of the alien species to spread (Tokarska-Guzik et al., 2012). However, the problem of plant introduction is very complex. It concerns long-term and global impacts on forest ecosystems, unexplained continuity of changes and the nature of evolutionary processes (Faliński, 2004). It is necessary to take appropriate countermeasures to adequately forecast and counteract the negative effects of these processes. One of the basic tasks of genetic resource conservation is the taxonomic identification of the species, subspecies, or races, providing the basis for active protection of indigenous populations, which counteracts their extinction.

In the case of the larch, only the use of molecular markers (Jagielska, 2008) allows for taxonomic verification and determination of the degree of hybridization, which was confirmed in the obtained results. In the TNP, there is a need to take active measures to eliminate individuals of alien taxa that threaten the native larch genetic resources in the Tatras. The presence of alien species is dangerous, especially in protected areas where relict species and endemics are of particular concern. Therefore, their gradual and systematic elimination from national parks and nature reserves, with a complete ban on re-introduction to these areas, is recommended (Szwagrzyk, 2000). Furthermore, this study showed that the use of afforestation in the studied locations in the TNP did not affect the level of genetic variability of the larch.

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