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Comparison of two methods for identifying alien genotypes in clonal seed orchards and consequences of misidentification

Ein Vergleich zweier Methoden zur Identifizierung fremder Genotypen und die Konsequenzen deren Fehlerkennung

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Abstract

Genetic gains in forestry are often implemented by producing improved forest seeds in seed orchards. However, unwanted alien genotypes are often accidentally introduced into seed orchards, or genotypes are planted in incorrect locations, both of which can reduce genetic gains. Such errors can be detected using markers, mainly isoenzymatic proteins and microsatellite DNA. These markers differ in their sensitivity, meaning that they can yield different assessments of seed orchard genetic material even when plant material is identical. The main objective of this paper was to compare these two verification methods and their consequences for genetic improvement. Two uneven-aged Scots pine clonal seed orchards were analysed using sets of isoenzymatic and microsatellite loci identified in other studies. The statistical analysis allowed comparison of the actual architecture of seed orchards to the planned layout. The number of clones was also compared to the effective number of clones.

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The results of microsatellite DNA analysis indicate that misplaced ramets are present from 12.29% to 30.89% of the time. Errors had an impact on breeding efficiency by reducing the relative effective number of clones. Isoenzyme and microsatellite methods had different discriminatory powers, which affected the results of the study. This study indicates that seed orchards can contain large numbers of incorrectly identified individuals. Microsatellite DNA analysis is recommended over isoenzyme analysis for detecting such errors, as the former is a more sensitive analytical method.

Zusammenfassung

Der Züchtungsfortschritt im Forstwesen wird meistens durch die Verwendung von verbessertem Material aus Samenplantagen erreicht. Züchtungsprogramme der Forstbäume führen in der Praxis oft zum Auftreten fremder Genotypen in Samenplantagen, welche den Erfolg des Züchtungsfortschritts reduzieren. Molekulare Marker wie Isoenzyme und DNA-Mikrostelliten eignen sich, um solche Fehler festzustellen. Diese Methoden unterscheiden sich hinsichtlich ihrer Genauigkeit des Ergebnisses. Daher ist es möglich, für identisches Pflanzenmaterial zu unterschiedlichen Ergebnissen zu kommen. Der Vergleich dieser Überprüfungsmethoden und die Konsequenzen der getroffenen Entscheidung ist das Ziel dieser Arbeit. Die Analysen wurden auf zwei nicht-gleichaltrigen Klonsamenplantagen unter Anwendung von Isoenzym- sowie Mikrosatellitenmarkersets durchgeführt. Eine statistische Analyse ist erfolgt. Sie ermöglichte sowohl den Vergleich zwischen dem Plan und der tatsächlichen Anordnung der Klone als auch eine Schätzung der effektiven Anzahl der Klone. Die Ergebnisse basierend auf Mikrosatellitenmarkern beweisen das Auftreten von fremden Genotypen in einer Häufigkeit zwischen 12.29 % und 30.89 %. Die Fehler beeinflussten die Züchtungseffizienz negativ dadurch, dass die effektive Anzahl der Klone reduziert war. Unterschiede bei der Aussagekraft der beiden Ansätze hatten einen Einfluss auf die Ergebnisse. Die vorliegende Studie unterstreicht den Nutzen von Klonidentifikationsanalysen in Samenplantagen. Es empfiehlt sich, auf Mikrosatelliten-DNS als die sensitivere analytische Methode zurückzugreifen.

Introduction

Modern forest management can benefit from advances in scientific knowledge, everywhere from seed production to felling technology. In many countries, seed production is based on forest tree breeding. In practice, this means that seeds obtained from clonal seed orchards account for an increased share of the seeds used in forest regeneration (Przybylski et. al. 2015). Gains from forest tree breeding are based on phenotypic selection of forest trees displaying desirable traits. Clones of selected individuals are then reproduced within seed orchards (Chałupka et al. 2011). For maximum genetic gains, it is necessary to avoid errors when establishing orchards, because mistakes cause undesirable genotypes to be present in an orchard's genetic pool (Kaya & Isik 2009). Genotypes not from a plus tree ramet may be accidentally introduced into clonal seed orchards, displacing a plus tree from the gene pool of

the orchard. This phenomenon was mentioned by Paule (1991), Burczyk et al. (2000), Gömöry et al. (2003) and Kaya & Isik (2009). Two categories of errors in Polish seed orchard establishment were described by Odrzykoski (2007). Category I errors are caused by mistakes in the spatial architecture of the seed orchard, due to planting ramets in the wrong place but using clones that are a part of the seed orchard design. Category II errors arise from misidentification, which place individuals that are not part of the breeding population into the orchard (Odrzykoski 2007).

Detection of alien genotypes in seed orchards can be done using molecular markers, which help identify planting errors. Markers can be divided into three groups: morphological markers, biochemical markers and DNA markers (Dzialuk and Burczyk, 2001). This paper focuses on the use of biochemical and DNA markers.

Until recently, the analysis of morphological features was the primary method of describing tree population genetic variability. However, plant phenotype is of limited value because morphological variability among genotypes can be low and the number of useful phenotypic features is limited. Therefore, the probability of committing an error using morphological markers is high. In addition, determining the inheritance of traits and environmental effects on overall morphological variability is not possible, and therefore morphological features are of limited value beyond the initial stages of clone identification (Dzialuk and Burczyk, 2001).

The application of biochemical marker analyses was a breakthrough in population genetics. These approaches were used by a number of researchers investigating graft errors in seed orchards, such as Breitenbach-Dorfer et al. (1992), Burczyk et al. (2000), Gömöry et al. (2003), and Przybylski et al. (2019). One of the newer techniques for identifying individuals is microsatellite DNA markers (Simple Sequence Repeats; SSR), used in criminal trials for alleged theft of wood (White et al. 2000). They are widely used in affinity relatedness and to obtain unique genetic profiles to identify individuals (Burczyk 2006; Dzialuk, 2009). Unfortunately, SSR markers have some disadvantages, such as the presence of null alleles and high costs of testing.

Comparative tests using molecular markers involve developing a set of markers that will provide the maximum chance to detect differences between genotypes, with minimal financial outlay. In the human population, the discriminatory power of an 11 SSR marker set reaches 0.949156 (94.91%), which is accepted for identification of individuals in cases of criminology and contested paternity (Konarzewska et al. 2006). The highest discriminatory power of DNA markers obtained in tests reach 0.99999 (99.99%) (Wysocka et al. 2008). Recent studies of plant populations have achieved high discriminatory power using SSR markers (de Barba et al. 2016).

A review of literature showed that two biochemical testing tools, isoenzymatic markers and microsatellite DNA, may be used to verify seed orchard identities. However, these methods differ in discriminatory power. The main objective of the present

study was, therefore, to evaluate the discriminatory power of systems based on isoenzymatic proteins and DNA and to evaluate the consequences of applying these methods on estimates of breeding efficiency.

Methods

Plant material

The study consisted of the molecular verification of individuals in two scots pine clonal seed orchards. The Susz District seed orchard (N53°45′21″, E19°12′16″) was established in 1977-1978 and Pniewy District seed orchard (N52°30′32″, E16°15′24″) was established in 1993 (Figure 1). These are first generation seed orchards, and as a result the genetic value (breeding value) of cloned plus trees planted in these orchards is unknown. In the Susz seed orchard, 9 838 ramets were planted from 50 plus tree clones, and in Pniewy, 1 733 ramets were planted from clones of 53 plus trees. Material from 138 ramets at Susz and 131 ramets at Pniewy was collected for molecular analyses. Samples were collected from two blocks within each analysed seed orchard from all trees that remained after the most recent systematic thinning.

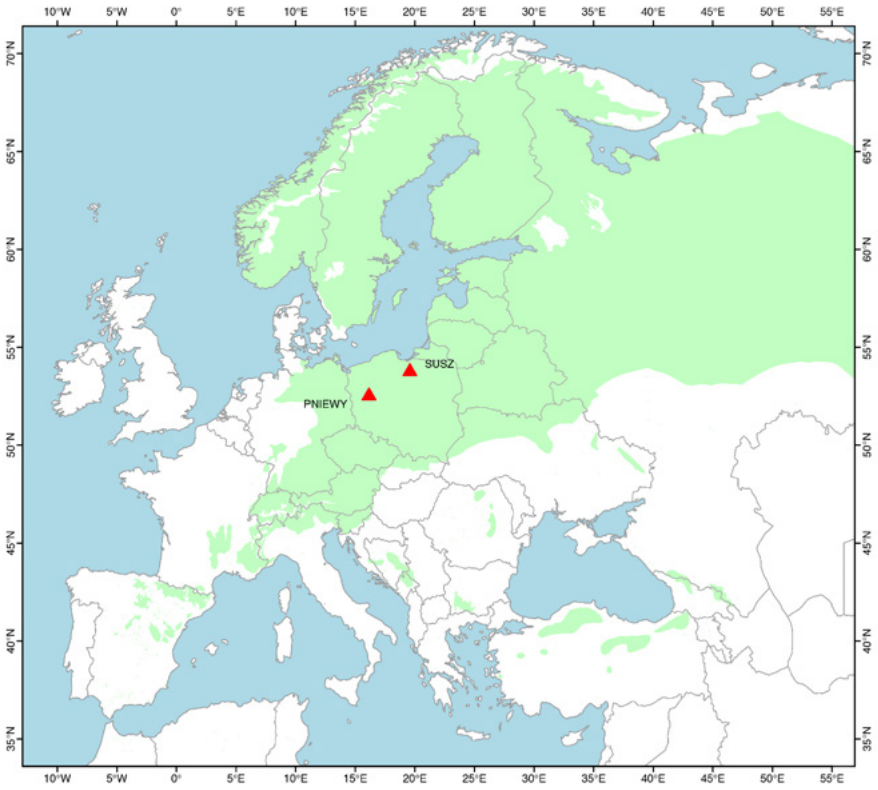

Figure 1: Pinus sylvestris (L) occurrence range (source IUFRO) and location analyzed seed orchards in Poland.

Abbildung 1: Vorkommensbereich von *Pinus sylvestris* (L.) (Quelle IUFRO) und Lage der untersuchten Samenplantagen in Polen.

The reference samples for the Susz seed orchards came from the original parental plus trees. When it was not possible to collect reference material in this way, because plus trees were dead, a repeated set of 4 ramets from the tested seed orchard constituted the reference sample. For the Pniewy orchard, the reference samples were obtained from a clonal archive outside of the seed orchard.

Genotype analysis

Isoenzymatic proteins (IZO)

Variation was examined in eight enzymatic proteins (Table 1) extracted separately

from winter buds. Protein extraction was conducted using a 150-µl extraction buffer (Odrzykoski, 2002). After filtering through Miracloth filter paper, the resulting solutions were used to moisten pieces of Whatman 3ET filter paper, which were subsequently placed onto electrophoresis gel. Using two buffer systems (Odrzykoski, 2007), the electrophoresis was conducted in 13% starch gel (Starch-Art). Following electrophoresis, gels were cut into 1.5-mm slices, which were individually used to visualise the phenotype of the analysed proteins. Location of individual bands was determined using the methods of Conkle et al. (1982), with a modification comprising the use of an overlay procedure with 2% agar, excluding EstB and Got. Identification of the loci and interpretation of the obtained zymograms were carried out using the approach described by Odrzykoski (2002).

Table 1: List of buffer systems (A, C), enzymes with European Community numbers (E.C.), and enzyme loci used in this study.

Tabelle 1: Liste der Puffersysteme (A, C), Enzyme (mit E.C. Nummern) und Enzymort, die in dieser Studie verwendet wurden.

System	Enzyme	No. E.C.	Locus
A	Esterase	3.1.1.2	*EstB*
	Glutamate-oxaloacetate transaminase	2.6.1.1	*GotA* *GotB* *GotC*
	NADH-diaphorase	1.8.1.4	*DiaC*
	Glutamate dehydrogenase	1.4.1.2	*Gdh*
C	6-phosphogluconate dehydrogenase	1.1.1.44	*PgdA*
	Shikimate dehydrogenase	1.1.1.25	*SdhA* *SdhB*
	NAD-dependent malate dehydrogenase	1.1.1.37	*MdhA* *MdhC*
	Alcohol dehydrogenase	1.1.1.1	*AdhA* *AdhB*

Microsatellite DNA (SSR)

DNA was isolated with a commercially available set of microsatellite markers (Marchel-Nagel – Nucleo Spin Plant II) after extraction from 3-4 needles from each graft. Needles were ground in a mortar using liquid nitrogen, until obtaining about 20 mg

of dry powdered mass. The yield of DNA extracted for each sample was evaluated by measuring absorbancy at wavelengths of 260 and 280 nm. (Quawell Q500). An average absorbancy for wavelengths of 260/280 nm was 1.80, at which the samples produced 80 ng DNA/µl, which was diluted to 20 ng DNA/µl. Four microsatellite loci were used in testing: Spac11.6, Spag7.14 (Soranzo et al. 1998), and PtTX3107, PtTX4001 (Elsik and Williams 2001), which differ in the type of repeating motif and the length of alleles. A multiplex PCR was used for loci Spac11.6, PtTX3107 and PtTX4001. A separate reaction was used for locus Spag7.14. In all cases, reactions were performed with 25µl of a solution composed of ultrapure water, Taq DNA polymerase (0.1 U/µl), $MgCl_2$ (4mM), dNTPs (0.5mM), primers and matrix DNA. The optimised thermal profile of the reaction is provided in Table 2. For locus Spac11.6, the touchdown PCR method was used (Balletti et al. 2012). The mixture of all PCR products was separated in a Beckman Coulter sequencer, in the polymer LPA I, using the Frag I length standard (as recommended by the manufacturer). The results obtained were analysed with CEQ™800 software, which provided chromatograms that then were used to determine the lengths of individual alleles.

Table 2: Thermal profile of PCR used in this study.

Tabelle 2: Thermisches Profil der verwendeten PCR.

Step	Temperature (°C)	Time (minutes)	
First denaturation	94	5	
Denaturation	92	1	40 cycle's
Annealing	58 - 60	1	
Elongation	72	1	
Finish elongation	70	7	

Deficiency of heterozygotes may prove the presence of null alleles, which may not be detected in the sample directly. Therefore, an analysis was carried out to determine their presence and frequency, using the methods of Chakraboty et al. (1992), Brookfield (1996) and van Oosterhout et al. (2004). Analyses were performed by Micro Checker v2.2.3. (van Oosterhout et al. 2004).

Statistical analyses

To determine the discrimination power of the clonal verification system, the cumulative probability of obtaining identical genotypes (P_{ID}) for two different clones was calculated as:

$$P_{ID} = 2\,((\sum p_i^2)^2 - \sum p_i^4)$$

where p_i = the mean allele frequency (i^{th}alleles) in the sample of grafts from the two tested seed orchards combined (Taberlet & Luikart, 1999). Calculations were performed using GeneAlEx 6.5 software (Peakall & Smouse, 2006). The genotypes obtained were used to estimate the rates of category I and II errors in seed orchards. In order to assess the impact of errors on genetic variability, the actual composition of genotypes and their spatial arrangement (W1) was compared with the originally designed seed orchard population (W0). For W0, it is assumed that no errors were made and all ramets were planted as specified in the planting plan. For both W0 and W1, the effective numbers of clones (*Nc*) was calculated based on Kang & Lindgren (1999) and Kang et al. (2001):

$$Nc = \frac{Ng}{(\frac{CV\%}{100})^2 + 1}$$

where: *Ng* = corrected census number of genotypes after molecular verification and *CV*% = coefficient of variation for the number of ramets. The relative effective number of clones was calculated as *Nr* = *Nc/N* (Kang et al. 2001).

Results

Null alleles were not relevant for the interpretation of the results. Based on the data obtained, null alleles were proven to be present for the locus Spac11.6 in Susz and for loci PtTX3107 and Spag7.14 in Pniewy. The null allele frequency ranged from 0.09 to 0.14 (Table 3).

Table 3: Seed orchards and loci, in which null alleles were detected using Micro Checker software. Allele frequency was estimated by four software methods: p1 – Chakraborty et al. (1992), p2 – Brookfield (1996), p3 – modified Brookfield method (1996), and p4 – Oosterhout et al. (2004).

Tabelle 3: Samenplantagen und Enzymort (Locus), in denen mit der Micro-Checker-Software Null-Allele nachgewiesen wurden. Die Allelfrequenz wurde mit vier Softwaremethoden geschätzt: p1 – Chaakraborty et al. (1992), p2 – Brookfield (1996), p3 – modifizierte Brookfield-Methode (1996) und p4 – Oosterhout et al. (2004).

Seed orchards	Locus	p1	p2	p3	p4	Means
Susz	Spac 11.6	0.11	0.09	0.07	0.09	0.09
Pniewy	PtTX3107	0.13	0.17	0.13	0.12	0.14
Pniewy	Spag7.14	0.09	0.10	0.07	0.08	0.09

The discriminatory power of markers varied. Using four microsatellite DNA loci, discriminatory power was $4x10^{-6}$, while the use of thirteen isoenzymatic loci resulted in a sensitivity of $8x10^{-5}$, which is one order of magnitude less (Table 4). It should be noted that any of the proposed marker systems can be used to verify identity in clonal seed orchards.

Table 4: Comparison of the individual and accumulated probability of obtaining identical genotypes (P_{ID}) for unrelated ramets in clones from two seed orchards combined. Loci were added in the order given in column one.

Tabelle 4: Vergleich der individuellen und kumulativen Wahrscheinlichkeit, um identische Genotypen (P_{ID}) für nicht verwandte Ramete in Klonen aus zwei kombinierten Samenplantagen zu erhalten. Loci wurden in der angegebenen Reihenfolge hinzugefügt.

Locus	Loci SSR	P_{ID} (by locus)	P_{ID} (locus combination)	Loci IZO 13	P_{ID} (by locus)	P_{ID} (locus combination)
1	Spac11.6	0.46	0.46	EstB	0.41	0.41
2	PtTX3107	0.03	0.01	PgdB	0.39	0.1637
3	PtTX4001	0.06	0.0009	SdhA	0.52	0.0858
4	Spac7.14	0.005	0.000004	SdhB	0.82	0.0708
5				MdhA	0.82	0.0581
6				MdhC	0.41	0.0238
7				Gdh	0.39	0.0093
8				DiaC	0.54	0.0051
9				GotA	0.91	0.0047
10				GotB	0.31	0.0014
11				GotC	0.37	0.005
12				AdhA	0.41	0.0002
13				AdhB	0.38	0.00008

The presence of category I and category II errors was proven in both seed orchards. The error rates differed depending on verification method. A category II error was found using SSR, while the IZO verification system for the same ramets either did not detect an error or indicated a category I error. The SSR and IZO methods gave

different results for 11 ramets from the Susz seed orchard and for 3 ramets from the Pniewy seed orchard. In addition, for SSR analyses, due to the poor quality of extracted DNA, genotype was not determined for 15 ramets from Susz and 9 ramets from Pniewy. Due to differences between analytical methods, the error rate in seed orchards is interpreted as a percentage ratio between false ramets and all ramets analysed in a seed orchard. A detailed summary of false ramets is given in Table 5. On the basis of IZO, the category I error rate ranged from 10.69% in the Pniewy orchard to 13.04% in Susz orchard. For SSR analyses, category I error rate was 5.69% in Susz and 8.20% in Pniewy. The number of category II errors for IZO ranged from 9.16% in the Pniewy orchard to 24.64% in Susz and for SSR it ranged from 12.29% in Pniewy to 30.89% in Susz.

Table 5: Summary information on the two seed orchards used in this study, and the number of ramets that were in correct and incorrect locations and that were alien genotypes.

Tabelle 5: Informationen zu den beiden Samenplantagen dieser Studie, die Anzahl der Rameten, die richtig bzw. falsch lokalisiert wurde sowie die fremden Genotypen.

	Susz	Pniewy
Year of establishment	1977-78	1993
Number of clones	50	53
Number of ramets in seed orchards	9 838	1 733
Number of studied ramets	138 IZO 123 SSR	131 IZO 122 SSR
Number of correctly located ramets	86 IZO 78 SSR	105 IZO 97 SSR
Number of incorrectly located ramets – Category I error	18 IZO 7 SSR	14 IZO 10 SSR
Number of ramets of alien genotypes – Category II error	34 IZO 38 SSR	12 IZO 15 SSR

Error rates affected seed orchard parameters. The results showed that the planned number of genotypes was lower than the actual number of genotypes in orchards, where the latter increased from 10 to 13 genotypes per orchard for SSR analyses (Table 6). This indicates an average increase in the number of orchard genotypes by 0.22. The SSR method allowed reporting of the increased number of additional genotypes, with 1 to 3 additional genotypes, depending on orchard (Table 6). Additional genotypes affected the effective and relative effective number of clones. In summary, in

each case, alien genotypes decreased the relative effective number of clones, from 0.14 for the Susz seed orchard to 0.04 for Pniewy using SSR analyses (Table 6).

Table 6: Corrected number of genotypes after molecular verification (Ng), the mean number of ramets per genotype (Nsg) and effective (Nc) and relative effective (Nr) number of clones in seed orchards. Results show in two variants analysis: W0, originally planned composition of the seed orchards; W1, both seed orchards after molecular verification.

Tabelle 6: Korrigierte Anzahl der Genotypen nach molekularer Identifikation (Ng), mittlere Anzahl der Rameten pro Genotyp (Nsg) sowie effektive (Nc) und relative effektive Anzahl (Nr) der Klone in den Samenplantagen: W0, ursprünglich geplante Zusammensetzung der Samenplantagen; W1, beide Samenplantagen nach molekularer Identifikation.

	Susz		Pniewy	
SSR	W0	W1	W0	W1
Ng	50	60	53	66
Nsg	2.46	2,05	2,30	1,85
Nc	38.12	43.28	42.88	51.42
Nr	0.86	0.72	0.81	0.77
IZO	W0	W1	W0	W1
Ng	50	57	53	65
Nsg	2.76	2.42	2.47	2.01
Nc	43.28	41.13	44.81	50.92
Nr	0.87	0.72	0.84	0.78

Discussion

In most clonal seed orchards that have been verified, errors involving incorrect graft assignment were identified (Burczyk et al. 2000, Dzialuk 2009, Gömöry et al. 2003). Alien genotypes are introduced when errors occur during the complex procedures of establishment and maintenance of seed orchards. Errors have various causes, but since they are all due to human mistakes, with care they can be eliminated. The main causes of errors include: misidentification when collecting plant material; errors made during work in a forest nursery; poor legibility of labels or label removal during ramet transport; errors when manually copying seed orchard designs; and cases where the rootstock outgrows the scion. It appears that errors during the establishment of orchards are not large (Suchowera and Chełmiński 2009). Regardless of the cause of an error, the impact of each type of error is different. Category I errors do not alter

the gene pool of a seed orchard. However, they can increase the risk of self-pollination. In contrast, Category II errors modify the gene pool by introducing alien pollen, which reduces breeding efficiency (Kaya et al. 2006). Alien genotypes may also modify the basic genetic characteristics of seed orchards, influencing heterozygosity and introducing unwanted alleles into the gene pool of seed orchard progeny (Stoehr et al. 2004).

The discriminatory power of a microsatellite DNA marker set can be evaluated by calculating the probability that two different individuals in a population have identical genotypes (P_{ID}) for the marker set. Dzialuk and Burczyk (2005) used P_{ID} to evaluate the discriminatory power of a set of 7 microsatellite loci (5 chloroplast loci and 2 nuclear loci), which can be used to identify stolen trees. A probability of P_{ID} from 10^{-3} to 10^{-4} is usually accepted as sufficient to identify individuals (Waits et al., 2001). Similar values could be adopted to identify Scots pine plus trees in Poland. The calculation of P_{ID} requires data on allele frequency in a reference population. Where ramets are identified in seed orchards, a reference population should consist of all Scots pine plus trees. Unfortunately, no such data exist for loci used in the present study. For the current analysis, material from 245 Scots pine plus trees was available. Polish populations of Scots pine are distinctive, with low interpopulation diversity, as determined using microsatellite loci, with $F_{ST} = 0.033$ (Nowakowska, 2007). This feature is common in various *Pinus* species (e.g. Marquardt and Epperson, 2004). Considering this, it can be assumed that allele frequencies in the 245 plus trees in this study provides a reliable estimate of allele frequency in the entire population of Scots pine plus trees in Poland.

An important result obtained here is that the discriminatory power of microsatellite markers is higher than that of isoenzymatic loci. This is consistent with other studies (Cieślewicz 2009). In the present study, a higher value of P_{ID} obtained for microsatellite DNA markers allowed category II errors to be identified, which has not been observed in isoenzymatic analyses. On the other hand, although isoenzymatic analyses are an older analytical technique than molecular biology, their effectiveness in verifying seed orchard identities remains high. The usability of isoenzymatic techniques is confirmed by the values of P_{ID} obtained using it. Przybylski et al. (2019) found PID of 0.0001, while Dzialuk & Burczyk (2005) report a P_{ID} of 0.004. Thus, given there are around 3,650 plus trees in Poland (unpublished data from the Forestry Research Institute), it is possible to assign an individual genotype to each ortet in seed orchards throughout Poland. It should be added that isoenzymatic tests are cheaper than microsatellite DNA analysis, the issue being that many steps in microsatellite cannot yet be automated.

Markers selected in this study were used previously for verification of ramets in seed orchards. Breitenbach-Dorfer et al. (1992), in studying a polymorphism of four enzymatic loci in *Abies alba*, demonstrated the usefulness of these loci for identifying individual clones. Burczyk et al. (2000), in analysing the variability of 14 isoenzymatic

loci, detected errors in the distribution of plus tree grafts in one plot of the Gniewkowo seed orchard. Gömöry et al. (2003) used 12 isoenzymatic loci to verify the identity of clones in three seed orchards, detecting errors of from 27% to 49% in graft assignment. In turn, Lewandowski (2006) describes using eleven isoenzymatic loci to identify grafts in a seed orchard. The analysis of 10 microsatellite loci from chloroplast DNA enabled the identification of erroneously distributed grafts in one plot of a seed orchard in the Gniewkowo forest district (Burczyk et al., 2000). Slavov et al. (2004), using three microsatellite loci, verified 152 ramets representing genotypes of 59 trees in a Douglas spruce orchard.

The presence of alien genotypes should be considered in terms of their effects on genetic gain in clonal seed orchards. Genetic gain refers to the capacity to pass genetic information on to the next generation (Trojankiewicz and Burczyk 2005). One important factor determining the high genetic gain of clonal seed orchards is the uniform distribution of unrelated clones in an orchard, which determines the maximum distance between ramets of the same clone. The lack of quantitative balance and the non-synchronised phenology of flowering between individual clones can lead to mating of related individuals (Burczyk 1998). This study has shown the negative effects of unwanted genotypes on the relative effective number of clones, which was reduced by 0.14. Moreover, errors in identification increased the number of genotypes by 0.22, thus reducing the efficiency of spatial arrangements in seed orchards.

Production seed orchards are created by planting a limited number of individuals. The main purpose of production orchards is to produce seeds that transfer desirable genetic traits to the progeny. For this reason, special attention should be paid to establishing an orchard correctly, arranging it with optimal clone spacing and ensuring a balanced representation of clones. Since errors in establishment of clonal orchards are common, clones in existing orchards should be verified. The best tool for verifying seed orchard clonal identities is microsatellite DNA markers, which possess high discriminatory power. The lower accuracy of isoenzymatic markers, as with morphological markers, makes them more appropriate for earlier stages of verification.

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