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Molecular studies did not support the distinctiveness of *Malva alcea* and *M. excisa* (Malvaceae) in Central and Eastern Europe

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Abstract: Relics of *Malva alcea* are found in Central and Western Europe. A similar taxon, *M. excisa,* is native to the eastern parts of Europe. According to selected sources, the geographical range boundary of the above taxa intersects Poland. Taxonomic research relying on key morphological features (the depth of corolla petal incisions and the type of hairs covering the stem) did not clearly validate the distinctness of those species.
Genetic variation between *Malva alcea* and *M. excisa* was analyzed using ISSR and ISJ markers. The performed analysis did not reveal statistically significant differences at the level of genetic diversity between *M. alcea* and *M. excisa* populations. The obtained genetic identity values ($I = 0.985$) do not support the identification of eastern populations as a distinct biological species of *M. excisa.* The applied DNA markers did not reveal species-specific bands supporting molecular identification of those taxa. The obtained genetic identity values were verified by neighbor-joining grouping which showed that *M. alcea* and *M. excisa* did not form corresponding clusters, thus pointing to an absence of significant differences between the analyzed taxa. Differences between the above species were not revealed by an analysis of the sequences of chloroplast regions *trn*H-*psb*A and *rpo*C1, either.

Key words: *Malva alcea*; *Malva excise*; genetic diversity; molecular markers

Introduction

The genus *Malva* L. includes about 40 species occurring in Europe, Africa and temperate regions of Asia (Mabberley 1997). In European flora, depending on the literature source, from 11 to 13 species of *Malva* have been recognized (Dalby 1968; Mosyakin & Fedoronchuk 1999; Mirek et al. 2002; Rothmaler et al. 2005). In central and eastern Europe, majority of them are alien species, brought in the Middle Ages (e.g., *Malva alcea* L., *M. neglecta* Wallr. and *M. sylvestris* L.), or species which run wild from the present-day cultivation, like, among others, *M. verticillata* L. (Zając 1979; Olyanitskaya, Tzvelev 1996; Mirek et al. 2002; Rothmaler et al. 2005; Didukh 2010).

The *Malva* species have been used by humans since the Middle Ages. For example, *Malva sylvestris* is a species that was recommended for cultivation already in the early Medieval sources, such as, among others, Capitulare de Villis (c 800 CE), Leechbook (c 940 CE) and Lacnunga (c 950 CE) (wyrtig.com). The descriptions of medicinal properties and use of the other *Malva* species can also be found in many old literature sources, for example, Marcin of Urzędów (1595, after Furmanowa et al. 1959), Syreniusz (1613, after www.zielnik-syrenniusa.art.pl), Jundziłł (1791) and Kluk (1805–1811). Also, the present literature on the subject provides information about the use values of these plants (Ożarowski 1982; Nowiński 1983; Rakhmetov 2000; Didukh 2010).

For many years, species of the genus *Malva* have been the object of scientific interest throughout the world. Numerous studies have been carried out to date to investigate their biology, ecology and taxonomy (Bates 1968; Ray 1995, 1998; El Naggar 2001; Tate et. al. 2005). However, the taxonomic status of some members of the genus, including *M. excisa* Rchb., remains uncertain.

M. excisa is widely distributed in Central and Eastern Europe and in southern Sweden, where it grows on dry hills, in the forest edge zone and in roadsides (Ilĭn 1974; Walas 1959). It is closely related to another member of the genus, *M. alcea,* a species common in Western Europe. Both species show high morphological similarity. The key features which allow to discriminate between *M. excisa* and *M. alcea* are based solely

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on the size and type of stem hairs and on the shape of petal apices (Walas 1959). In the case of *M. excisa*, stem hairs are small and stellate and corolla petals are deeply incised or doubly incised, while *M. alcea* is characterized by large, single or trifurcate hairs and slightly incised petals. At present, *M. excisa* is either treated as a separate species (Walas 1959; Ilĭn 1974; Olyanitskaya 1999; Majorov 2006) or is considered to be a subspecies of *M. alcea* (Olyanitskaya 1999; Dalby 1968; Rutkowski 2004).

In Poland, *M. excisa* is very rarely reported (Ćwikliński & Głowacki 2000), while the floristic monographs compiled in the territories situated east of the Polish border, including in Lithuania, Belarus and the Ukraine, almost exclusively report *M. excisa*, but do not mention (except in very rare cases) *M. alcea* (e.g. Ilĭn 1974; Tzvelev 2000). In contrast, the floras of the countries located west of the Odra River, do not contain *M. excisa* (De Langhe 1978; Rothmaler et al. 2005). The unclear taxonomic status of both species and the problems with their classification result primarily from their high morphological similarity. Another difficulty in their classification may be posed by populations from east Poland, displaying intermediate features between the two species, which suggests their hybrid character. There are no sufficient data available to determine whether *M. excisa* should be regarded as a separate species or as a variety or subspecies of *M. alcea*.

Methods that rely on DNA polymorphism have been increasingly applied to determine fixed interspecific differences and to identify species difficult to distinguish based on their anatomical and morphological characteristics. Numerous taxonomic studies using various molecular marker classes have shown that true biological species differ in both qualitative and quantitative terms at the molecular level (Cronberg 1996; Sawicki et al. 2006; Szczecińska et al. 2006). They have fixed species-specific alleles in individual loci, which permit their easy molecular identification, and they often differ with respect to allele frequencies.

Due to some taxonomic uncertainty concerning species delimitations (Celka et al. 2006; 2007; 2010a, 2010b), we aimed to determine whether the two investigated taxa are genetically distinct. The taxonomic status of the species was verified with the use of both genotyping and sequencing methods to support an unambiguous evaluation of the taxonomic position of *M. excisa*. Semi-specific ISJ (Intron-Exon Splice Junction) markers are based on the sequences which are commonly found in plants and which are indispensable for post-transcription DNA processing (Weining & Langridge 1991). ISJ primers are partly complementary to the sequences on the exon-intron boundary. Those markers had been successfully used in taxonomic studies of the genera *Polygonatum* Mill. (Szczecińska et al. 2006), *Orthotrichum* Hedw. (Plášek & Sawicki 2010; Sawicki et al. 2010), *Aneura* Dumort. (Bączkiewicz et al. 2008), as well as in studies investigating the population genetics of *Uncinula necator* (Schwein.) Burrill (Stummer et al. 2000), *Chamaedaphne calyculata* (L.) Moench (Szczecińska et al. 2009). ISSR (Inter Simple Sequence Repeat) markers (Zietkiewicz et al. 1994) are the second category of markers applied in this study. Owing to a high degree of diversity at both the population and the interspecific level, ISSR markers are widely applied in taxonomic studies (Vanderpoorten et al. 2003; Dogan et al. 2007) as well as in studies investigating genetic variation at the species level (Gunnarsson et al. 2005; Liu et al. 2007). The application of the two types of genotyping markers makes it possible to estimate the variability of the studied species both in the non-coding (SSR markers) and coding (ISJ markers) regions of the genome. Two chloroplast regions, noncodnig *trn*H-*psb*A and coding *rpo*C1, which are candidate regions for plant bar-coding, characterized by relatively high variability (Newmaster et al. 2008; Erickson et al. 2008), were also used in the present study.

Material and methods

Species and sampling

Malva alcea is found in Central and Western Europe, parts of Southern Europe as well as in the southern parts of Sweden (Hultén & Fries 1986). It is a perennial plant which propagates by seeds. Vegetative reproduction may be achieved by splitting of the main root which undergoes internal necrosis over time (Łukasiewicz 1962). *M. alcea* is found predominantly in the areas of Medieval settlement, such as towns, castle ruins and villages. In some regions, it has a tendency to propagate from former cultivations. Outside those habitats, the species is encountered in Europe in dry and warm locations on calcareous, nitrogen-rich soils (Hegi 1925; Heß et al. 1970). It is observed in forest and shrub margins, in ruderal plant communities, in dry and warm habitats, on sunny slopes, along roads and on field boundary strips (Ascherson & Graebner 1898–1899; Hegi 1925; Hermann 1956; Walas 1959; Garcke 1972; De Langhe et al. 1978; Dostál 1989; Adler et al. 1994; Mossberg et al. 1995). *M. alcea* is associated with nitrophilous plant communities of the classes *Artemisietea* and *Chenopodietea* in ruderal habitats (Ellenberg 1978; Dostál 1989; Matuszkiewicz 2001; Zarzycki et al. 2002; Rothmaler et al. 2005). It is also known to accompany xerothermic grasslands and stands of the classes *Festuco-Brometea, Rhamno-Prunetea* and *Trifolio-Geranietea sanguini* (Fijałkowski 1994).

Malva excisa is found in Eastern Europe. According to Walas (1959), the western range limit of the species intersects Poland. It occurs in similar habitats to *M. alcea*, including thickets, meadows, forest margins, orchards, gardens, along roads and households as a ruderal plant (Ilĭn 1974; Olyanitskaya 1999; Zubkevich 1999; Majorov 2006).

Population abundance was estimated at each locality. Genets consisting of several to between ten and twenty ramets were regarded as an individual. Experimental material was sampled from 20 localities (13 localities of *M. alcea* and 7 of *M. excisa*) in 2007 (Fig. 1, Table 1). Samples were collected from localities representing the entire habitat spectrum (settlements, roadsides, roadside ditches, cemeteries, thickets) where mallow is populously represented. To avoid repeated sampling of the same clone, samples were collected from individuals that were 3 m apart (in small populations) or 10 m apart (in large populations). Material sampled from 10 individuals in each population was placed in silica gel (Chase & Hillis 1991).

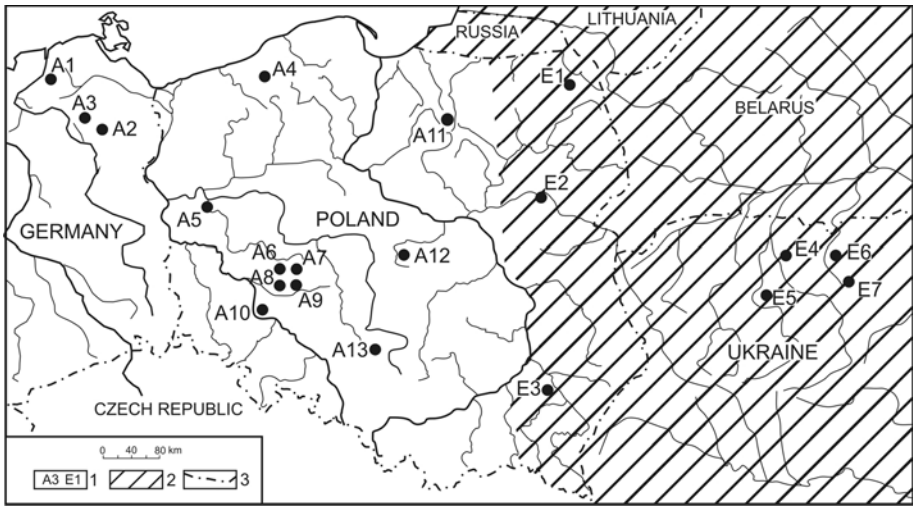

Fig. 1. Distribution of sites of sample collection and the hypothetical distribution range of *Malva excisa* determined on the basis of literature. 1 – sites of sample collection (A – *Malva alcea*, E – *Malva excisa*); 2 – hypothetical range of *M. excisa*; 3 – borders of countries.

Table 1. Studied populations of *Malva* and their GenBank accession numbers for *rpo*C1 and *trn*H-*psb*A regions: A – *M. alcea*, E – *M. excisa*, N – number of plant sampled.

Symbol	Population	Geographic locality	Population size	*N*	rpoC1, trnH-psbA
A-1	Teterow (Meclenburg-Vorpommern, Germany)	N53°47′22.6″ E12°35′52.3″	50	7	HM214515, HM214494
A-2	Feldberg (Meclenburg-Vorpommern, Germany)	N53°20′34.1″ E13°27′29.0″	4	4	HM214516, HM214495
A-3	Fürstenwerder (Meclenburg-Vorpommern, Germany)	N53°22′36.4″ E13°34′06.5″	50	6	HM214517, HM214496
A-4	Sławsko (Zachodniopomorskie region, Poland)	N54°23′13.9″ E16°42′50.6″	30	10	HM214518, HM214497
A-5	Skwierzyna (Lubuskie region, Poland)	N52°35′36.5″ E15°31′37.3″	15	7	HM214519, HM214498
A-6	Daleszyn 1 (Wielkopolskie region, Poland)	N51°56′00.4″ E17°00′05.1″	100	10	HM214520, HM214499
A-7	Daleszyn 2 (Wielkopolskie region, Poland)	N51°55′46.1″ E17°00′05.2″	3	3	HM214521, HM214500
A-8	Gostyń (Wielkopolskie region, Poland)	N51°55′10.8″ E17°00′51.7″	15	5	HM214522, HM214493
A-9	Dusina (Wielkopolskie region, Poland)	N51°55′17.7″ E17°01′15.2″	40	10	HM214523, HM214501
A-10	Mojęcice (Śląskie region, Poland)	N51°17′28.0″ E16°35′41.4″	40	10	HM214524, HM214502
A-11	Rapaty (Warmińsko-Mazurski region, Poland)	N53°43′53.6″ E20°09′29.3″	40	10	HM214525, HM214503
A-12	Tum (Łódzkie region, Poland)	N52°03′22.5″ E19°13′57.9″	50	10	HM214526, HM214504
A-13	Pątnów (Łódzkie region, Poland)	N51°08′06.3″ E18°36′30.7″	15	9	HM214527, HM214505
E-1	Dowspuda (Podlaskie region, Poland)	N53°57′31.6″ E22°49′20.5″	150	10	HM214528, HM214506
E-2	Wirów (Podlaskie region, Poland)	N52°26′35.7″ E22°32′12.3″	25	10	HM214529, HM214507
E-3	Nowosielec (Podkarpackie region, Poland)	N50°25′23.5″ E22°07′54.8″	15	7	HM214530, HM214508
E-4	Rokytne (Rivne region, Ukraine)	N51°16′59.4″ E27°12′29.2″	20	7	HM214531, HM214509
E-5	Gubkiv (Rivne region, Ukraine)	N50°49′34.1″ E27°02′42.2″	15	5	HM214532, HM214510
E-6	Olevsk (Zhytomyr region, Ukraine)	N51°12′26.0″ E27°39′41.0″	20	7	HM214533, HM214511
E-7	Pidluby (Zhytomyr region, Ukraine)	N50°55′14.6″ E27°45′08.3″	20	7	HM214534, HM214512

Plants were counted and leaves were sampled at each locality (Table 1). Additional, two samples of *M. moschata* L. were used as an outgroup based on previous phylogenetic studies (Garcia et al. 2009). ISSR and ISJ analyses were performed on every collected individual, and the sequencing of chloroplast regions was carried out on a single individual from each population. GenBank accession numbers are given in Table 1.

Molecular analysis

DNA was extracted from 40 mg dry leaf tissue using the DNeasy Plant extraction kit (Qiagen). The isolated DNA was dissolved in water and stored at −20°C. Two marker categories were used in the analysis of genetic variation: microsatellite ISSR (Inter Simple Sequence Repeat) markers developed by Zietkiewicz et al. (1994), and ISJ markers (Weining & Langridge 1991). Similarly to RAPD and AFLP markers, the target sequence of ISSR and ISJ markers does not require prior identification which makes those markers suitable for studying species for which species-specific primers amplifying microsatellite loci (SSR-Simple Sequence Repeat) have not yet been developed. Yet contrary to SSR markers, ISSR primers are complementary to repeated sequences rather than to fragments flanking those sequences. More details on ISJ-markers can be found in Sawicki & Szczecińska (2007). The sequences of ISSR and ISJ primers used for DNA amplification in this study are given in Table 2.

ISSR-PCR and ISJ-PCR reactions were performed in 20 μL of a reaction mixture containing 40 ng genomic DNA, 1.0 μM of primer, 1.5 mM $MgCl_2$, 200 μM each dATP, dGTP, dCTP, dTTP, 1× PCR buffer (Sigma, supplied with polymerase), 1 μL BSA and 1 U Genomic Red Taq polymerase (Sigma). ISSR marker reactions were performed under the following thermal conditions: (1) initial denaturation – 5 min at a temperature of 94°C, (2) denaturation – 1 min at 94°C, (3) annealing – 1 min at 50°C, (4) elonga-

Table 2. Sequence of 8 primers successfully used in the ISSR and ISJ analysis and the number of amplified bands per primer.

Primer	Sequence (5'-3')	Number of the amplified bands	Number of polymorphic bands
IS807	$(AG)_8T$	15	13
IS810	$(GA)_8T$	16	13
IS813	$(CT)_8T$	16	13
IS822	$(TC)_8A$	7	6
IS825	$(AT)_8G$	19	17
IS828	$(TG)_8A$	17	15
IS831	$(ACC)_6$	16	0
IS834	$AT(GAT)_5G$	18	15
IS840	ACTTCCCCACAGGTTAACACA	15	15
IS843	CATGGTGTTGGTCATTGTTCCA	17	16
IS846	$GGGT(GGGGT)_2G$	19	19
ISJ 2	ACTTACCTGAGGCGCCAC	14	13
ISJ 4	GTCGGCGGACAGGTAAGT	17	14
ISJ 5	CAGGGTCCCACCTGCA	16	15
ISJ 6	ACTTACCTGAGCCAGCGA	14	12
Total		236	196

tion – 1.5 min at 72°C, final elongation – 7 min at 72°C. Stages 2–4 were repeated 34 times. The following reaction conditions were applied to ISJ primers: (1) initial denaturation – 3 min at 94°C, (2) denaturation – 1 min at 94°C, (3) annealing – 1 min at 48°C, (4) elongation – 1.5 min at 72°C, final elongation – 5 min at 72°C. Stages 2–4 were repeated 39 times. The products of the ISSR-PCR reaction were separated on 2% agarose gel, and the products of the ISJ-PCR reaction – on 1.5% agarose gel, followed by DNA staining with ethidium bromide. After rinsing in deionised water, agarose gel was analyzed in a transilluminator under UV light at a wavelength of 302 nm with the application of the Felix 1010 system.

The reproducibility of the ISJ and ISSR markers was checked by randomly selecting 10 samples and amplifying the extracted DNA twice. The error rate was calculated as the ratio between all differences and all band comparisons in ten duplicated ISJ-ISSR profiles (Bonin et al. 2004). The calculation of the error rate of ISSR and ISJ bands resulted in seven differences in 1921 comparisons, giving an error rate of 0.36%.

For the amplification and sequencing of *trn*H-*psb*A and *rpo*C1 we used the primers of Sang et al. 1997 and from Royal Botanical Garden in Kew website. The chloroplast regions were amplified in a volume of 25 μL containing 20 mM $(NH_4)SO_4$, 50 mM Tris-HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 1 μL BSA, 200 μM each, dATP, dGTP, dCTP, dTTP, 1.0 μM of each primer, one unit of Taq polymerase (Qiagen) and 1 mL of the DNA solution. The reaction was processed at 94°C for 1 min followed by 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min, with a final extension step of 72°C for 5 min. Finally 5 μL of the amplification products were visualized on 1.5% agarose gel with ethidium bromide staining. Purified PCR products were sequenced in both directions using the ABI BigDye 3.1 Terminator Cycle Kit (Applied Biosystems) and were then visualized using an ABI Prism 3130 Automated DNA Sequencer (Applied Biosystems).

Data analysis

Statistical analysis was based on 236 loci. The analyses of genetic data were performed using PopGene v. 1.32 (Yeh & Boyle 1997) and Arlequin v. 3.01 (Excoffier et al. 2006). The following parameters were used to estimate genetic diversity at the population level and at the species level: the percentage of polymorphic loci (P %), the number of alleles per locus (A_E), and genetic diversity (H_E). Allele frequency in locus was identified in view of band presence or absence. It was assumed that every observed band resulted from the amplification of a single locus, therefore, the number of observed bands corresponded to the number of investigated loci. Only allele "1" (band present) or allele "0" (band absent) were observed in every locus. Differences in allele frequencies between the investigated populations were determined with the use of Statistica 7 (Statsoft, USA). Nei's unbiased genetic identity (I) and genetic distance (D) were estimated for each population pair (Nei 1972). Genetic differentiation among populations was estimated by Nei's gene diversity statistics (Nei 1973). The amount of gene flow among those populations was estimated at $N_m = (1/Gst - 1)/5$ (Slatkin 1987). The analysis of molecular variance (AMOVA) was used to partition genetic variance within and among species. AMOVA analyses were carried out using ARLEQUIN v. 3.0 (Excoffier et al. 2006).

The correlation between the genetic (D) and geographical distance (in km) separating the studied populations was determined by Mantel's test (Mantel 1967) using ARLEQUIN v 3.01 (Excoffier et al. 2006)

The neighbor-joining dendrogram was constructed based on the matrix of Nei & Li (1979) genetic distances among individuals using PAUP 4 (Swofford 2003). Clade support for the phenogram was estimated by a bootstrap analysis with 1000 replicates (Felsenstein 1985). Incongruence between the ISJ and ISSR datasets was assessed by comparing clade support on the consensus tree. For example, if population A was included in clade A with significant bootstrap support based on ISJ markers, but resolved as a member of clade B with significant support based on ISSR markers, the trees based on these markers were considered incongruent.

The significance of differences between the studied populations with respect to coefficient values was estimated by an analysis of variance and the LSD test. Principal coordinate analysis (PCO) was performed on the binary data matrix using Genalex 6.0. (Peakall & Smouse 2006) to assess the dimensionality of data and to describe the major patterns of variation within and among populations of analyzed species. Electropherograms were edited and assembled using Sequencher 4.5 (Genecodes Inc.). The assembled sequences were aligned manually with BioEdit 7 (Hall 1999).

Table 3. Genetic diversity of analyzed populations of *Malva*. Total genetic diversity of *M. alcea* and *M. excisa* at the species level; *N* – the number of amplified bands per population, P is the percentage of polymorphic loci, A_E – the effective number of alleles, H_E – Nei's (1973) gene diversity.

Species	Population	*N*	*P*	A_E	H_E
M. alcea	Teterow	194	37.8	1.233	0.132
	Feldberg	160	32.8	1.263	0.142
	Fürstenwerder	196	32.8	1.201	0.117
	Sławsko	196	47.9	1.300	0.174
	Skwierzyna	176	29.4	1.227	0.124
	Daleszyn 1	174	42.0	1.280	0.159
	Daleszyn 2	116	16.8	1.111	0.065
	Gostyń	144	32.8	1.178	0.107
	Dusina	186	42.0	1.261	0.151
	Mojęcice	202	48.7	1.307	0.175
	Rapaty	196	34.4	1.220	0.125
	Tum	196	47.9	1.298	0.174
	Pątnów	182	47.9	1.282	0.170
Species level		232	75.3	1.432	0.246
M. excisa	Dowspuda	182	52.1	1.311	0.180
	Wirów	196	59.6	1.293	0.170
	Nowosielec	168	18.5	1.124	0.071
	Rokytne	184	20.2	1.137	0.076
	Gubkiv	188	23.5	1.167	0.093
	Olevsk	160	22.7	1.150	0.084
	Pidluby	190	41.2	1.272	0.153
Species level		234	82.4	1.471	0.270
Total			83.2	1.450	0.259

Table 4. Hierarchical analysis of molecular variance (AMOVA) for populations of *Malva alcea* and *M. excise*.

Source of variance	d.f.	Sum of squares	Variance component	Total variation (%)	*P*-value[a]
Among species	1	48.48	0.00	0.00	0.001
Among populations	18	982.2	5.93	39.00	0.001
Within populations	134	1248.2	9.315	61.00	0.001

[a]Significance tests after 1,000 permutations

Gaps were excluded from all phylogenetic analyses. Maximum parsimony (MP) analyses of the sequenced regions were conducted using PAUP 4.0b10 (Swofford 2003), employing heuristic searches with 1000 random addition replicates and TBR branch swapping. The statistical significance of clades within inferred trees was evaluated using the bootstrap method (Felsenstein 1985) with 1000 replicates.

Results

Genetic differentiation between M. alcea and M. excisa
The performed analysis did not reveal statistically significant differences at the level of genetic diversity between *M. alcea* and *M. excisa* populations. Both species were characterized by a very similar number of amplified loci which reached 232 and 234, respectively (Table 3). The average value of genetic identity between *M. alcea* populations and between *M. excisa* populations was 0.830 and 0.860, respectively. Genetic identity between the above species reached $I = 0.985$. The applied DNA markers did not permit the molecular identification of the studied species. None of the 236 amplified bands proved to be species-specific. *M. alcea* was characterized by 4 private alleles (IS825-17, IS825-21, IS828-8 and ISJ5-4), and *M. excisa* – by 2 private alleles (IS825-1 and IS825-5), but the occurrence frequency of those alleles was very low, within the range of 0.032 to 0.063. Bands occurring only within a given species but showing polymorphism at the intra-specific level were considered to be private allels.

The investigated taxa also showed minor differences as regards their genetic variation. *M. alcea* proved to be slightly less genetically diverse than *M. excisa*. Genetic variation parameters for *M. alcea* and *M. excisa* reached $P = 75.3$, $A_E = 1.432$, $H_E = 0.246$ and $P = 82.4$, $A_E = 1.471$, $H_E = 0.270$, respectively. The above findings can be attributed to minor differences in allele frequencies observed between populations of each species. In addition to private alleles, statistically significant differences in allele frequencies were reported in only 18 loci.

The results of the AMOVA analysis demonstrated that the greatest variability coincided with intra-population variability (61%), while there were no differences between the studied taxa (Table 4). The absence of differences between *M. alcea* and *M. excisa* was also confirmed by the neighbor-joining grouping method (Fig. 2), based on the Nei's genetic distance. Populations representing both taxa did not generate two separate clusters. The results of the neighbor-joining anal-

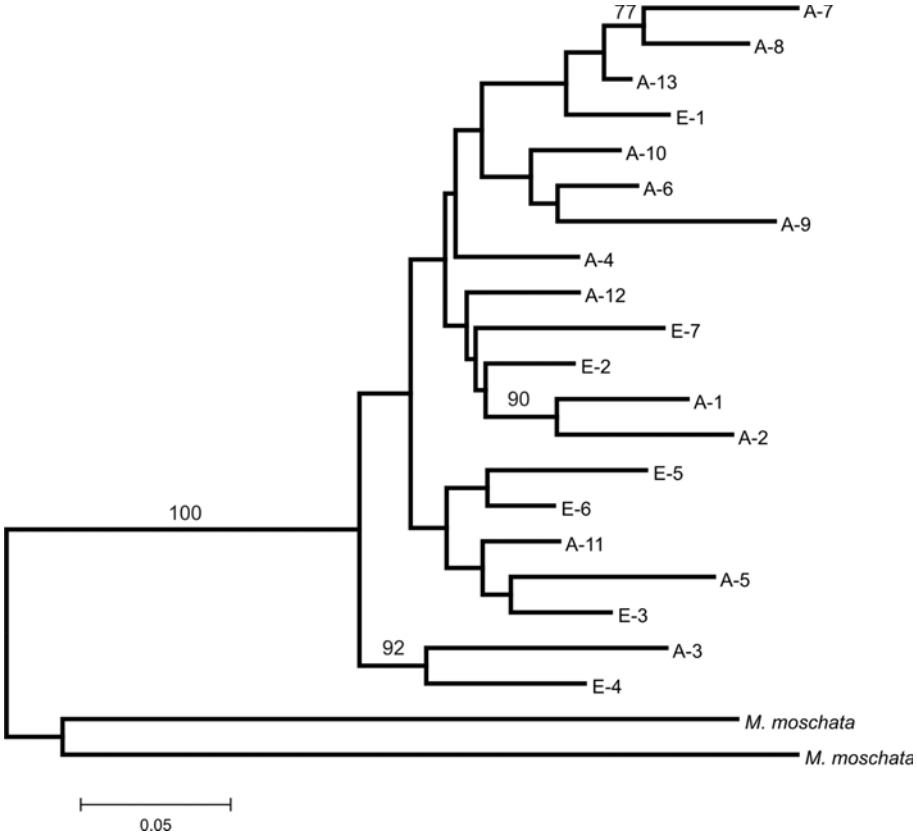

Fig. 2. Phenogram based on Nei's (1972) genetic distance for the analysed populations of *Malva alcea* and *M. excisa,* based on ISSR and ISJ markers. Bootstrap support values are given above branches.

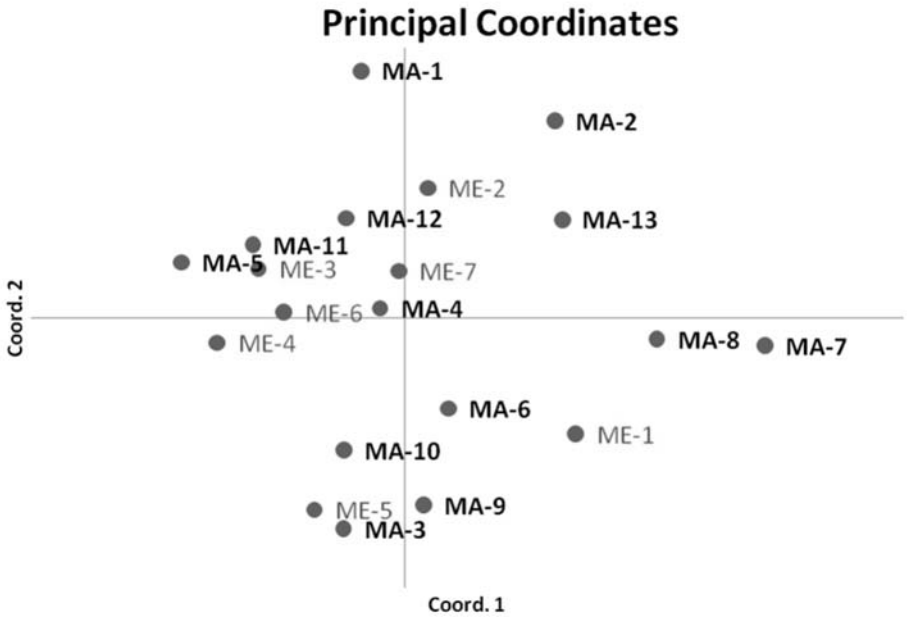

Fig. 3. Principal coordinates analysis of *Malva alcea* and *M. excisa* populations.

ysis were complementary with the data obtained in the PCO analysis (Fig. 3).

Molecular diversity within populations

An analysis of the genetic diversity of 15 *M. alcea* and 7 *M. excisa* populations performed with the application of ISSR and ISJ markers revealed a total of 236 loci, producing an average of 15.7 bands per primer. The highest effectiveness in terms of the number of revealed loci was reported in respect of two ISSR primers: IS825 and IS846, which revealed 19 loci each, while the smallest number of loci (7) was amplified by primer IS822 (Table 2).

The applied markers proved to be relatively poly-

morphic in reference to the investigated species. 83% of 236 amplified loci were polymorphic. However, the studied populations differed significantly as regards the number of revealed loci and their polymorphism. The Mojęcice population was characterized by the largest number of bands (202), while the smallest number of bands (116) was amplified for the Daleszyn 2 population. The highest number of polymorphic loci in the studied *M. alcea* and *M. excisa* populations was revealed in the Wirów population (59.6%), while the lowest polymorphism values (18.6%) were noted in the Daleszyn 2 population. Molecular diversity calculated based on polymorphic ISSR and ISJ bands was relatively high at $A_E = 1.45$, $H_E = 0.259$ (Table 3). The analyzed populations differed significantly as regards the value of genetic diversity parameters. The Daleszyn 2 population and the Rokytne and Nowosielec populations were characterized by a low level of genetic diversity ($A_E = 1.11$, $H_E = 0.064$) and the following genetic diversity parameters were reported: $A_E = 1.13$, $H_E = 0.076$; $A_E = 1.24$, $H_E = 0.712$, respectively. The highest level of genetic diversity was observed in respect of the Dowspuda population ($A_E = 1.311$, $H_E = 0.180$). The genetic diversity level determined for each studied population was correlated with population size ($r = 0.75$, $P > 0.05$)

Molecular differentiation between populations
An analysis of the genetic diversity of *M. alcea* and *M. excisa* populations has shown that 61% of the observed variation accounts for inter-population diversity and 39% for intra-population diversity. The number of migrants calculated based on the G_{ST} coefficient was low, at Nm = 0.409. The high proportion of inter-population diversity in the investigated species is due mostly to differences in allele frequency noted between particular populations. Statistically significant differences were reported in respect of around 80% of the analyzed alleles. The analyzed populations were characterized by a low share of high-frequency alleles which were found in 50% and more of the studied populations. The highest share of the above alleles was observed in the Gubkiv and Fürstenwerder populations (32 alleles), while only 2 high-frequency alleles were found in the Olevsk and Daleszyn 2 populations. The remaining populations had from 6 to 20 such alleles. The experiment revealed practically no alleles which were specific to a given population and were absent from other populations. Only 6 private bands were noted in the total number of 236 revealed loci. Two specific bands were characteristic of the Wirów population, while the Mojęcice and Feldberg populations had two private bands each.

Genetic differences between the analyzed populations were also validated by the average genetic identity index which reached $I = 0.828$. The lowest degree of genetic identity ($I = 0.729$) was noted between the Daleszyn 2 population and the Skwierzyna population, while the Feldberg and Pątnów populations proved to be most similar ($I = 0.959$).

A statistical analysis performed with the use of Mantel's test did not reveal any correlations ($P < 0.05$ $r = -0.204$) between the geographical and genetic distance separating the studied populations. The highest genetic distance (0.316) was observed between the samples from Skwierzyna and the Daleszyn 2 population which were not separated by the longest geographic distance. The genetic distance between two most geographically isolated populations was 0.241.

Grouping of individuals
Based on the NJ analysis, the majority of individuals were grouped according to their population classification (Fig. 4). Only populations A-7 and A-8 formed a common, undeveloped clade. Several populations could be easily divided into subpopulations. This was particularly noticeable in population A-13 whose representatives formed two different groups. A less clear-cult division into subpopulations was noted in populations A-9 and E-2. Similarly as in the analysis at the population level, *M. alcea* and *M. excisa* individuals did not form separate groups.

Sequence data of chloroplast regions
The length of the *trn*H-*psb*A region ranged from 498 bp in *M. alcea* and *M. excisa* to 508 bp in *M. moschata*. Except for one sample of *M. excisa* from Gubkiv (one deletion of T), no variation was found among the analyzed individuals of *M. alcea* and *M. excisa*. Samples of those species differ from the *M. moschata* outgroup with respect to 10 substitutions and 17 indels. The length of the *rpo*C1 gene ranged from 672 to 674 bp. Two indel positions were found in *M. alcea* and *M. excisa*, but they did not correspond to their taxonomical affiliation. Only one substitution was found in a sample of *M. excisa* from Rokytne (Ukraine), and *M. moschata* differed from *M. alcea* and *M. excisa* in terms of one fixed substitution. Since no parsimony-informative sites were found in *M. alcea* and *M. excisa* populations, the Maximum Parsimony phylogenetic tree is not presented.

Discussion

The results of this experiment do not validate the taxonomic distinctness of *M. alcea* and *M. excisa*. The obtained genetic identity values ($I = 0.985$) do not support the identification of eastern populations as a distinct biological species. Similar results were reported by Dodd & Helernum (2006) for two subspecies of *Delphinium variegatum* Torrey & A. Gray.: ssp*thornei* Munz and ssp. *kinkiense* (Munz) M. J. Warnock ($I = 0.997$), and by Godt & Hamrick (1999) for two subspecies of *Saracenia rubra*: ssp. *rubra* Walter and ssp. *alabamensis* (Case & Case) Schnell ($I = 0.900$). The genetic identity index, calculated based on DNA markers, should be less than 0.7 for distinct biological species (Zieliński & Polok 2005). Subject to the applied DNA markers, genetic identity values for *Polygonatum* Mill. (Solomon seal) species of the section *Odorata* ranged from 0.25 to 0.72, with an average of 0.57 (Szczecińska et al. 2006). Similar results were reported

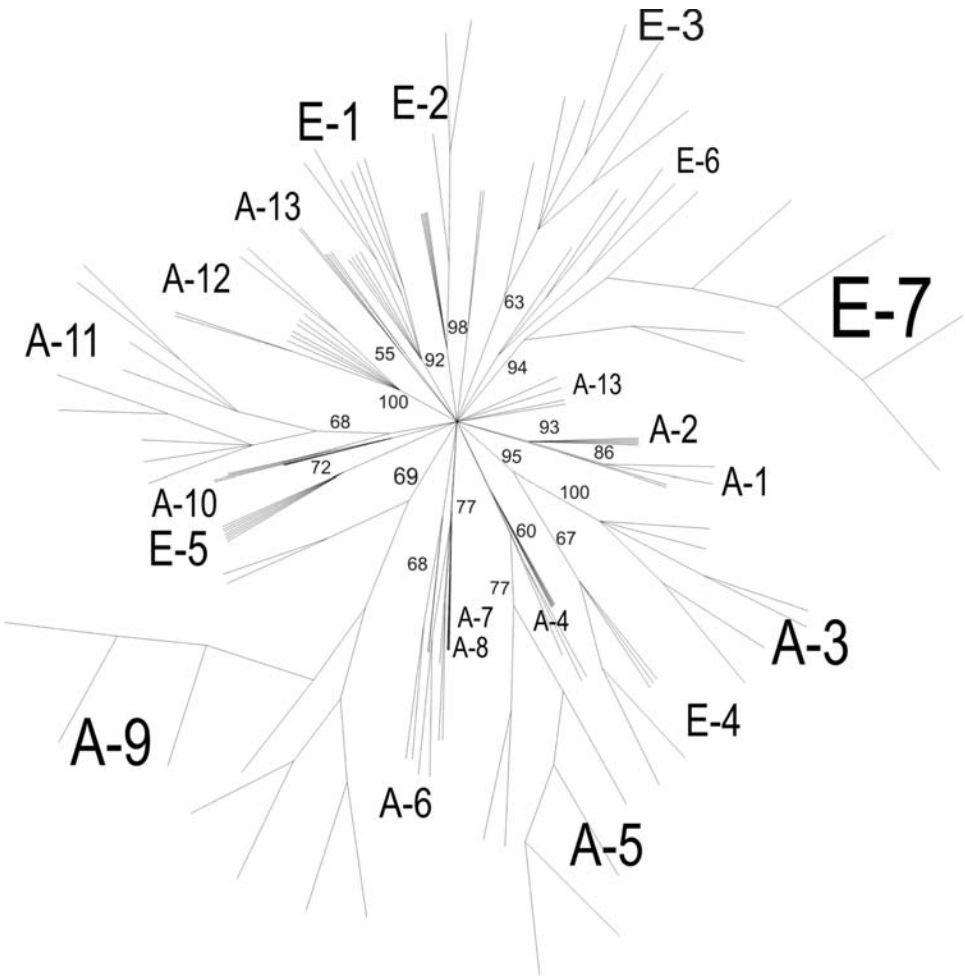

Fig. 4. Neighbor-joining phenogram of the ISJ-ISSR phenotypes of individuals from populations of *Malva alcea* and *M. excisa*. Bootstrap values are given above supported branches.

in an analysis of species of the genus *Pistacia* L. performed with the use of AFLP markers, where genetic identity between species pairs ranged from 0.34 to 0.76 (Kafkas 2006). Species of the genus *Orobanche* L., analyzed with the application of RAPD markers, showed genetic identity values in the range of 0.39 to 0.92, but species-specific bands were determined even in the most closely related species of *O. aegyptiaca* Pers. and *O. crenata* Forssk. (Paran et al. 1997).

The observed genetic identity parameters were also validated by neighbor-joining grouping where *M. alcea* and *M. excisa* populations did not form corresponding clusters, thus pointing to an absence of significant differences between the two taxa.

Even in very closely related species of the genus *Sphagnum* L., which were characterized by a very high degree of genetic identity (Cronberg 1996; Cronberg 1998; Sawicki & Zieliński 2008) and where no differences in the analyzed DNA sequences were found (Shaw & Cox 2005), populations of particular species were identified by UPGMA grouping (Sawicki & Zieliński 2008).

True taxonomic species usually have specific molecular markers that allow their molecular identification. Species-specific markers supporting the molecular identification of *M. alcea* and *M. excisa* were not found, which testifies to the absence of significant differences between those taxa. In most cases, even closely related species have marker alleles. As regards *P. multiflorum* (L.) All and *P. odoratum* (Mill.) Druce, closely related species of the genus *Polygonatum*, the number of species-specific markers ranged, subject to the applied marker class, from 12 (ISJ markers) to 30 (RAPD markers), accounting for more than 22% of all amplified loci (Szczecińska et al. 2006). An even higher number of marker bands were determined in an analysis of sibling species of the liverwort*Aneura pinguis* (L.) Dumort., where as many as 41 of the 112 analyzed bands supported molecular identification of each cryptic species (Bączkiewicz et al. 2008). As regards the analyzed mallow species, only 6 private alleles were found, but the frequency of their occurrence in particular populations were low. Molecular markers which enable identification of a given taxon are often found also for taxonomic ranks below the species level.

Species at an early stage of divergence often do not have specific alleles. The frequency of particular alleles may differ in taxa at an early stage of speciation. Despite a high degree of genetic identity and the absence of specific markers, the good botanical species of *Lolium perenne* L. and *L. multiflorum* Lam. differed

significantly with respect to both allele frequency in the analyzed loci and a significant number of private alleles (Polok 2005). The above requirement was not met by *M. alcea* and *M. excisa,* either. A statistical analysis revealed significant differences in the frequency of only 28 alleles, including private alleles, which raises serious doubts as regards the taxonomic status of *M. excisa*.

An analysis of the sequences of chloroplast regions *trn*H-*psb*A and *rpo*C1 did not enable the molecular identification of *M. alcea* and *M. excisa*, although the analyzed cpDNA regions are among the most variable in the plant world (Newmaster et al. 2008; Erickson et al. 2008) and have been widely used as plant barcodes (Edwards et al. 2008; Sawicki et. al. 2009). The *trn*H-*psb*A and *trn*L-F spacers were the most variables chloroplast sequences in phylogenetic studies of the genus *Malva*, which did not include *M. excisa* (Garcia et al. 2009). Despite the absence of differences between *M. alcea* and *M. excisa,* both analyzed regions supported the molecular identification of *M. moschata* which is closely related to *M. alcea* (Garcia et al. 2009). The present sequencing results correspond to those obtained using genome-scan markers, and provides further evidence that *M. alcea* and *M. excisa* share a common gene pool.

The results of this experiment are consistent with the findings of previous taxonomic studies investigating *M. alcea* and *M. excisa*. A mere analysis of variations in corolla petals and hairs does not support the identification of two distinct species of *M. alcea* and *M. excisa* in Central and Eastern Europe (Celka et al. 2006, 2007). The key characteristics that differentiate *M. alcea* and *M. excisa* are the depth of corolla petal incisions and the type of hairs covering the stem. The apex of corolla petals has a deep, single or double incision in *M. excisa*, while a shallow and gentle incision is characteristic of *M. alcea* (Walas 1959; Ilĭn 1974). Studies investigating variations in corolla petals of Central and East European mallows have shown that the investigated attributes (the length and width of petals, the length from the petal base to the incision point, the depth of incision, the ratio of petal length to incision depth) are complementary in those populations. Some East European mallow specimens and populations are more similar to Central European populations than to those found in the Ukraine. The ratio of petal length to incision depth, which should be the highest for the Ukrainian population (*M. excisa*), reached its maximum value in reference to the Central European mallow (Celka et al. 2007). The other key attribute differentiating the two species is the presence of hairs along the entire stem (Walas 1959; Ilĭn 1974) or only in its upper part (Tzvelev 2000). The stem of *M. excisa* is covered with only stellate hairs (small, with clinging branches and large, with protruding branches), while the stem of *M. alcea* features unifurcate, bifurcate and trifurcate hairs (with long branches) and tufted-stellate hairs. Studies investigating hair types (Celka et al. 2006) have shown that in accordance with the plant hair terminology proposed by Payne (1978), mallow plants may be characterized by simple (unibranched), bifurcate (2-branched) and stellate (multi-branched, multicellular) hairs. Stellate hairs vary in the number of branches (from three to ten). A hair analysis of 18 *M. alcea* populations from Central and Eastern Europe has shown that simple, bifurcate and stellate hairs cover the stem in all studied populations. Simple and bifurcate hairs were more frequently observed in the lower parts of the stem, while stellar hairs occurred mainly in the upper section of the stem (Celka, personal communication). The Principal Coordinate Analysis of hairs in the upper and lower parts of the stem has revealed that specimens from the majority of the investigated populations belong to a single, large group, while differences were noted in respect of several specimens from various populations. Two main groups were determined in the graphic representation of the Manhattan difference, computed by Ward's method, but specimens from different populations and various geographic regions were intermixed in each group (Celka, personal communication).

The performed molecular analyses and the results of previous morphological studies investigating the diversity of corolla petals and stem hairs did not reveal significant differences between *Malva alcea* and populations termed as *M. excisa*. *M. alcea* is considered a highly diverse species (Walas 1959; Dalby 1968; Hlavaček 1982; Slavík 1992), but its different forms are not geographically or ecologically correlated. The above can be attributed to the fact that *M. alcea* was a cultivated species in the past, with a tendency to propagate to anthropogenic habitats in both Medieval and contemporary times. The conducted genetic and morphological analyses clearly indicate that the name *M. excisa* should be treated as a synonym of *M. alcea*, and not as a separate taxonomic unit.

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