

# Genetic relationships between some of *Malva* species as determined with ISSR and ISJ markers

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**Abstract:** Two categories of DNA markers were used to determine genetic relationships among eight *Malva* taxa. A maximum parsimony analysis validated the division of the genus *Malva* into the sections *Bismalva* and *Malva*. The species classified into those sections formed separate clusters. *M. moschata* was a distinctive species in the section *Bismalva*, as confirmed by previous genetic research based on ITS and cpDNA sequence analyses. The applied markers revealed a very high level of genetic identity between *M. alcea* and *M. excisa* and enabled molecular identification of *M. alcea* var. *fastigiata*. Species-specific markers were determined for the majority of the analyzed species, permitting their molecular identification. A specific marker supporting the differentiation of *M. alcea* and *M. excisa* was not found.

**Key words:** *Malva*, genetic similarity, molecular markers, ISJ, ISSR

## 1. Introduction

The genus *Malva* comprises around 40 species world-wide (Mabberley 1987), including 13 species occurring in Europe (Dalby 1968). In Central and Eastern Europe, they are mostly alien species (Olyanitskaya & Tzvelev 1996; Mosyakin 1999; Mirek *et al.* 2002; Rothmaler *et al.* 2005). Some of them, including *Malva alcea*, *M. neglecta*, *M. pusilla* and *M. sylvestris*, were introduced to Europe already in the Middle Ages (Zajac 1979; Rothmaler *et al.* 2005), while others, such as *M. verticillata*, are encountered only as cultivated forms or, in rare instances, as escapees from cultivation (Dostál 1989; Mosyakin 1999; Rutkowski 2004). Some species of the genus *Malva* have been used for medicinal, ornamental, consumption and grazing purposes for centuries. Mallow owes its functional character to the presence of mucilage and tannins, a large number of ornamental flowers and leaves and edible fruits. Information on the properties of mallow plants was quoted by numerous historical sources (including Marcin of Urzędowa 1595, as cited in Furmanowa *et al.* 1959; Syreniusz 1613, as cited at [www.zielnik-syrenniusa.art.pl](http://www.zielnik-syrenniusa.art.pl); Jundziłł 1791; Kluk 1808).

Species of the genus *Malva* receive wide coverage in scientific papers investigating variations in their seeds, seed coats (Celka *et al.* 2006a; Kumar & Dalbir Singh 1991), pollen grains (El Naggat 2004) and stem hairs (Inamdar & Chohan 1969; Inamdar *et al.* 1983; Celka *et al.* 2006b), as well as the morphology of corolla petals (Celka *et al.* 2007), the ecology of individual specimens and the entire population (Celka *et al.* 2008) and the taxonomy of the entire genus (Ray 1995, 1998). Based on flower structure characteristics, the European species of the genus *Malva* have been divided into two sections: *Bismalva* and *Malva* (Dalby 1968). The section *Bismalva* comprises species with solitary flowers in the leaf axils or in a congested, terminal raceme, while the species of the section *Malva* have two or more flowers in each leaf axil. The monographers of the East European flora (Olyanitskaya & Tzvelev 1996) additionally distinguished subsections *Planocentrae* and *Conocentrae* within the section *Malva*, which group species characterized by smaller flowers and narrower epicalyx bracts. A different division, based on an ITS sequence analysis, was proposed by Ray (1995, 1998) who differentiated two groups: Malvoid and Lavateroid, and placed the species of the section *Bismalva* (excluding

*M. moschata*) in one group with the members of the genus *Lavatera*.

The objective of the present study was to determine the genetic relationships among eight mallow taxa found in Europe and to find DNA markers enabling their molecular identification. Several analyses were conducted to verify whether the genetic similarity of the studied taxa supports the internal division of the genus *Malva* into groups and sections. Two types of DNA markers were applied. Semi-specific intron-exon splice junction (ISJ) markers are based on sequences that are commonly found in plants and 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 have been successfully used in previous taxonomic studies of the genera *Polygonatum* (Szczecińska *et al.* 2006), *Sphagnum* (Sawicki & Zieliński 2008) and *Aneura* (Bączkiewicz *et al.* 2008), revealing a high number of species-specific bands. The other category of markers applied in this study were ISSR (inter simple sequence repeat) markers (Zietkiewicz *et al.* 1994). Owing to high variation in both the population and the interspecific level, ISSR markers are widely applied in taxonomic studies (e.g. Vanderpoorten *et al.* 2003; Dogan *et al.* 2007) as well as in studies investigating genetic diversity at the species level (e.g. Gunnarsson *et al.* 2005; Liu *et al.* 2007; Szczecińska *et al.* 2009). 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 repeats) have not yet been developed. However, 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 and Szczecińska (2007).

## 2. Material and methods

### 2.1. Species studied

The studied material represented the following taxa: *Malva alcea* L., *M. alcea* L. var. *fastigiata* (Cav.) K. Koch, *M. excisa* Rchb., *M. moschata* L., *M. neglecta* Wallr., *M. pusilla* Sm., *M. sylvestris* L. and *M. vericillata* L. Two samples of *Alcea rosea* were additionally included as an outgroup taxon, based on results of a previous phylogenetic analysis (Ray 1995, 1998; Escoba Garcia *et al.* 2009). The localities of the collected samples are given in the Appendix.

DNA was extracted from 40 mg of dry leaf tissue, using the DNeasy Plant extraction kit (Qiagen). The isolated DNA was eluted with water and stored at  $-20^{\circ}\text{C}$ . The sequences of ISSR and ISJ primers used for DNA amplification in this study are given in Table 1.

PCR reactions were performed in 20  $\mu\text{l}$  of a reaction mixture containing 40 ng genomic DNA, 1  $\mu\text{M}$  primer, 1.5 mM  $\text{MgCl}_2$ , 200  $\mu\text{M}$  dNTP (dATP, dGTP, dCTP, dTTP), 1x PCR buffer (Sigma, supplied with polymerase), 1  $\mu\text{l}$  BSA and 1 U Genomic Red Taq polymerase (Sigma). ISSR marker reactions were performed under the following thermal conditions: (1) initial denaturation – 5 minutes at a temperature of  $94^{\circ}\text{C}$ , (2) denaturation – 1 minute at  $94^{\circ}\text{C}$ , (3) annealing – 1 minute at  $49^{\circ}\text{C}$ , (4) elongation – 1'30'' at  $72^{\circ}\text{C}$ , final elongation – 7 minutes at  $72^{\circ}\text{C}$ . Stages 2-4 were repeated 34 times. The following reaction conditions were applied to ISJ primers: (1) initial denaturation – 3 minutes at  $94^{\circ}\text{C}$ , (2) denaturation – 1 minute at  $94^{\circ}\text{C}$ , (3) annealing – 1 minute at  $50^{\circ}\text{C}$ , (4) elongation – 2'50'' at  $72^{\circ}\text{C}$ , final elongation – 5 minutes at  $72^{\circ}\text{C}$ . The products of the PCR reaction were separated on 2% (ISSR) or 1.5% (ISJ) agarose gel, followed by DNA staining with ethidium bromide. After rinsing in deionized water, agarose gels were analyzed in a transilluminator

**Table 1.** Sequence of 12 primers successfully used in the ISSR and ISJ analysis and number of amplified bands per primer

Primer	Sequence (5' - 3')	Number of the amplified bands	Number of polymorphic bands
IS810	(GA) <sub>8</sub> T	8	7
IS813	(CT) <sub>8</sub> T	19	16
IS822	(TC) <sub>8</sub> A	21	18
IS825	(AT) <sub>8</sub> G	22	22
IS828	(TG) <sub>8</sub> A	14	14
IS831	(ACC) <sub>6</sub>	29	29
IS843	CATGGTGTTGGTCATTGTTCCA	21	18
IS846	GGGT(GGGGT) <sub>2</sub> G	15	15
ISJ 2	ACTTACCTGAGGCGCCAC	6	2
ISJ 4	GTCGGCGGACAGGTAAGT	18	18
ISJ 5	CAGGGTCCCACCTGCA	12	11
ISJ 6	ACTTACCTGAGCCAGCGA	9	8
	Total	194	178

under UV light at a wavelength of 302 nm, with the application of the Felix 1010 gel documentation

### 2.2. Data analysis

Bands were scored for presence (1) or absence (0), and transformed into a binary 0/1 character matrix. Fragments that could not be scored unambiguously were excluded from the analysis. The reproducibility of the ISJ and ISSR markers was checked by randomly selecting 5 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 four differences per 885 comparisons, giving an error rate of 0.45%.

Phylogenetic analysis of a binary data matrix was accomplished by using maximum parsimony analysis in PAUP\* 4.0b10 (Swofford 2003). Multiple most-parsimonious trees were summarized with a strict consensus tree and bootstrapped with 1000 replicates. Bootstrap support was considered to be good >70%, moderate <70% and >50%, and poor <50% (Werner *et al.* 2005). Genetic diversity measured as the percentage of polymorphic bands (P) and the number of private alleles was calculated using GenAlEx 6.1 (Peakall & Smouse 2006). The number of fixed allelic differences among specimens was estimated for all pairwise combinations of taxa using Sites program (Hey & Wakeley 1997). Since this program supports sequence data only, binary matrix was transformed into A/T dataset (1=A, 0=T). A Principal Coordinate Analysis (PCO) was performed on the binary data matrix using GenAlEx 6.1 (Peakall & Smouse 2006). The degree of genetic similarity was determined with the Nei's formula (Nei & Li 1979).

### 3. Results

An analysis of 24 specimens of the eight *Malva* taxa, performed using twelve primers representing two DNA marker categories, enabled 194 bands to be distinguished, of which 92% were polymorphic (Table 1). Four ISJ primers amplified a total of 45 bands (11.3 bands per primer), while eight ISSR primers revealed 149 bands (18.6 bands per primer). All primers amplified fragments across the 26 samples studied, including an outgroup, with the number of amplified fragments ranging from six (ISJ-2) to 29 (ISSR-831). The highest number of bands was identified in *M. moschata* (109), followed by *M. excisa* (98) and *M. alcea* (97). The lowest number of bands was amplified in *M. neglecta* (45) and *M. pusilla* (44).

The highest degree of polymorphism was observed in *M. moschata* with 34.4% of polymorphic loci. The degree of polymorphism in *M. sylvestris* (P=16.3) was less than half of that noted in *M. moschata*. Polymorphism in 9% of analyzed loci was reported in *M. alcea* var. *fastigiata* and *M. excisa*, and 5% in *M. alcea*. The lowest degree of polymorphism was observed in *M. neglecta*, *M. pusilla* and *M. verticillata*: 1.5%, 2.3% and 3% of polymorphic loci, respectively (Table 2).

The applied primers revealed a total of 16 species-specific bands (Table 2). Bands occurring only within a given species and showing no polymorphism at the intra-specific level were considered to be species-specific markers. The highest number of marker bands (9) was determined for *M. moschata*. Three bands were characteristic of *M. verticillata*, and one marker band for each was determined for *M. neglecta*, *M. pusilla* and *M. sylvestris*.

A much higher number of diagnostic bands was determined for different species pairs (Table 3). The highest

**Table 2.** Percentage of polymorphic loci, number of species specific bands and private alleles of studied *Malva* taxa

	<i>M. alcea</i>	<i>M. alcea</i> var. <i>fastigiata</i>	<i>M. excisa</i>	<i>M. moschata</i>	<i>M. neglecta</i>	<i>M. pusilla</i>	<i>M. sylvestris</i>	<i>M. verticillata</i>
% of polymorphic loci	5%	9%	9%	34.6%	1.5%	2.3%	16.5%	3%
Species specific bands	0	0	0	7	1	1	1	3
Private alleles	1	1	1	9	1	1	1	3

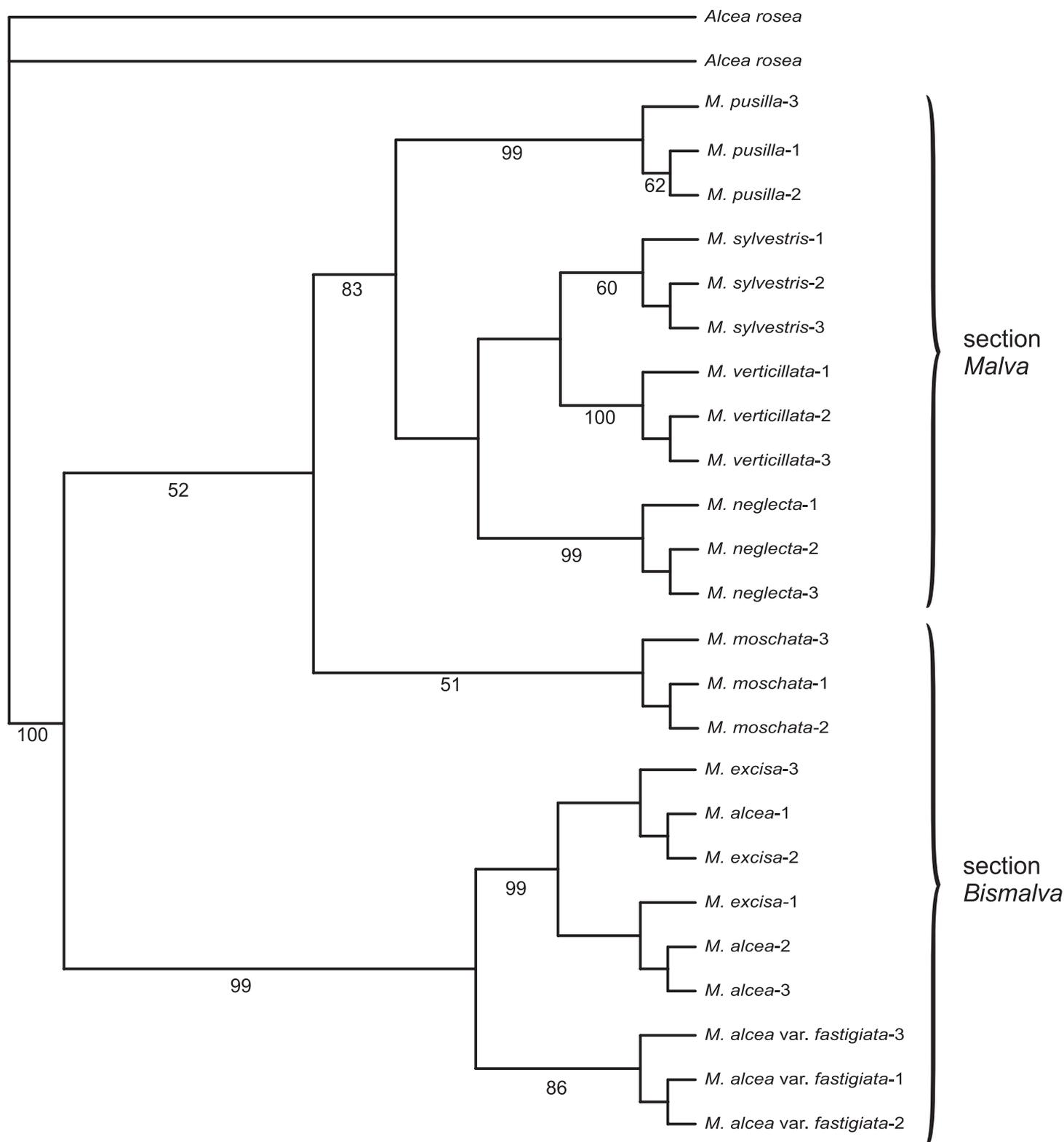
**Table 3.** Nei genetic identity (above) and fixed allelic differences (below) among analyzed *Malva* taxa

Species	<i>M. alcea</i>	<i>M. alcea</i> var. <i>fastigiata</i>	<i>M. excisa</i>	<i>M. moschata</i>	<i>M. neglecta</i>	<i>M. pusilla</i>	<i>M. sylvestris</i>	<i>M. verticillata</i>
<i>M. alcea</i>		0.818	0.936	0.623	0.518	0.479	0.432	0.504
<i>M. alcea</i> var. <i>fastigiata</i>	5		0.830	0.713	0.582	0.570	0.535	0.600
<i>M. excisa</i>	0	6		0.641	0.518	0.475	0.440	0.514
<i>M. moschata</i>	31	25	28		0.691	0.634	0.692	0.677
<i>M. neglecta</i>	63	50	61	33		0.670	0.759	0.771
<i>M. pusilla</i>	62	52	60	30	29		0.735	0.672
<i>M. sylvestris</i>	64	53	63	28	19	26		0.821
<i>M. verticillata</i>	76	54	65	36	41	42	27	

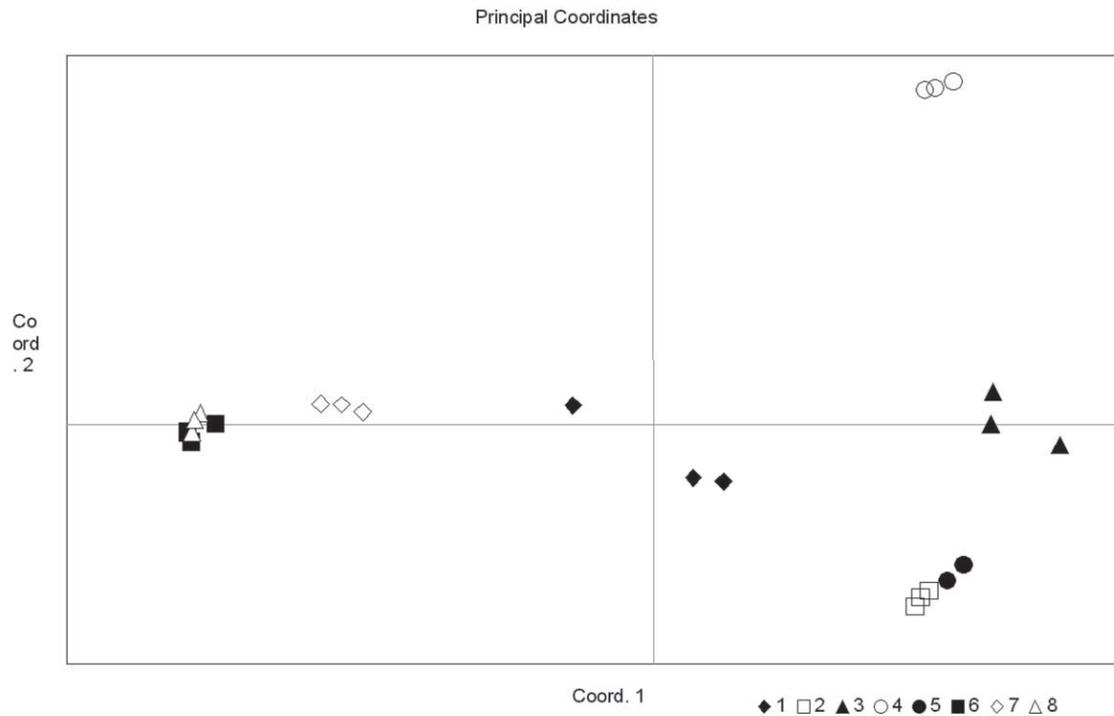
number of fixed band differences was observed between *M. alcea* and *M. verticillata* (76 bands). The applied primers did not amplify bands supporting the molecular identification of *M. alcea* and *M. excisa*. Molecular identification was possible for *M. alcea* var. *fastigiata*, which differed from *M. alcea* and *M. excisa* by 5 and 6

bands, respectively. Nineteen (*M. sylvestris* and *M. neglecta*) to 69 (*M. excisa* and *M. verticillata*) bands were noted for the remaining species pairs.

The values of Nei's genetic identity of eight *Malva* taxa, analyzed using DNA markers, ranged from 0.432 to 0.936 (Table 3). The highest value of the coefficient



**Fig. 1.** Strict consensus tree of 180 most parsimonious trees (CI=0.62; RI=0.87) obtained by a parsimony analysis of 24 specimens of *Malva*. Bootstrap values (bs) higher than 50% are indicated below branches



**Fig. 2.** Principal coordinates analysis of 24 *Malva* specimens  
 Explanations: 1 – *Malva moschata*, 2 – *M. pusilla*, 3 – *M. sylvestris*, 4 – *M. verticillata*, 5 – *M. neglecta*, 6 – *M. excisa*, 7 – *M. alcea* var. *fastigiata*, 8 – *M. alcea*

$I_N$  was recorded for *M. alcea* and *M. excisa* (0.936). High genetic similarity was also observed between these taxa and *M. alcea* var. *fastigiata*: 0.818 and 0.830, respectively. The lowest value of the genetic identity coefficient ( $I_N = 0.432$ ) was found between *M. alcea* and *M. sylvestris*. Insignificantly higher values of genetic identity were noted for *M. excisa* and *M. sylvestris* (0.440) as well as *M. excisa* and *M. verticillata* (0.475). The noted genetic identity values were consistent with the results of Maximum Parsimony analysis (Fig. 1) and the Principal Coordinates Analysis (Fig. 2). A maximum parsimony analysis resulted in 180 most parsimonious trees (length 98, consistency index (CI) 0.9673, retention index (RI) 0.9532). The strict consensus tree with bootstrap values for supported nodes is presented in Figure 1. The resulting tree had two main clusters. *M. alcea* and *M. excisa* specimens formed a shared well supported (99% bs) clade. Within this group *M. alcea* var. *fastigiata* formed an isolated, well supported (86% bs) clade, and *M. excisa* did not form a distinct clade from *M. alcea*. The specimens of the remaining taxa were grouped in a second, moderately supported clade (52% bs). Among species of this clade, the most distant was *M. moschata* that formed separate, but moderately supported clade (51% bs). Remaining taxa formed a well supported clade (83% bs) and grouped in accordance with their taxonomic classification from moderately (60% bs in case of *M. sylvestris*) to good supported (99% and 100% bs for *M. neglecta* and *M. verticillata*) clades.

A similar structure was revealed by the Principal Coordinates Analysis. The analyzed specimens formed two main groups. The first group comprised *M. alcea*, *M. alcea* var. *fastigiata* and *M. excisa* samples, while the other one accounted for *M. neglecta*, *M. pusilla*, *M. sylvestris*, *M. moschata* and *M. verticillata* (Fig. 2).

#### 4. Discussion

Genetic relationships between the investigated species are consistent with the results of previous molecular analyses of the genus *Malva* relying on ITS sequences (Ray 1995, 1998; Escobar García *et al.* 2009). ISJ and ISSR markers revealed a significant genetic divergence of *M. alcea* and *M. excisa* from the remaining taxa. This results validates the division of species of the genus *Malva* into the Malvoid group consisting of *M. neglecta*, *M. pusilla*, *M. sylvestris* and *M. verticillata*, and the Lavateroid group which, according to analysis of seed structure (Ray 1995), includes *M. alcea* and *M. excisa*. The position of *M. moschata*, placed outside the above groups based on an ITS sequence analysis, is also consistent with Ray's findings (1995, 1998). The obtained results also support the internal division of the genus *Malva* proposed by Dalby (1968). The presented dendrogram clearly divides the studied taxa into two groups corresponding to the sections *Bismalva* (*M. alcea*, *M. excisa* and *M. moschata*) and *Malva* (*M. neglecta*, *M. pusilla*, *M. sylvestris* and *M. verticillata*) (Fig. 1). The division of the genus *Malva* into four sections

(Olyanitskaya & Tzvelev 1996) is less supported by the obtained results. *M. neglecta* and *M. pusilla*, grouped in the section *Planocentrae*, did not form a shared clade. Genetic identity values noted for representatives of the remaining sections, *Malva* (*M. sylvestris*) and *Conocentrae* (*M. verticillata*), were also relatively high.

Species described in view of their morphological and anatomical properties are not always good biological species, which should have isolated gene pools and typically show the presence of many specific markers. A sound example of the above are *L. perenne* and *L. multiflorum*, grass species of the genus *Lolium*, which have a common gene pool despite good morphological features (Polok 2005). The above is also noted in lower plants. Despite a high number of morphological characters supporting their identification, the species of the genus *Sphagnum* – *S. fimbriatum* and *S. girgensohnii* – exhibit a high degree of genetic similarity (Cronberg 1996; Sawicki & Zieliński 2008). However, the applied DNA markers, enabled the molecular identification of both species (Polok 2007; Sawicki & Zieliński 2008).

Not all members of the genus *Malva* analyzed in this study meet the criterion of distinct biological species. The observed genetic identity values and the absence of species-specific bands suggest that *M. alcea* and *M. excisa* have a common gene pool. The genetic identity value noted for those taxa ( $I_N=0.965$ ) is characteristic of populations within a single species (Hamrick & Godt 1989). As regards closely related species of the genus *Polygonatum*, the genetic identity index, identified based on DNA markers, was  $I_N=0.57$  (Szczecińska *et al.* 2006). Much lower values of the genetic identity index were also reported for *Sphagnum capillifolium* and *S. rubellum* whose identity as distinct botanical species is frequently questioned (Daniels & Eddy 1990). Genetic similarity between those species was determined within the range of 0.670 for isoenzymatic markers (Cronberg 1996) to 0.754 for DNA markers (Sawicki & Zieliński 2008). A high degree of genetic identity and the absence of species-specific markers for *M. alcea* and *M. excisa* noted in this study correspond to the results of previous morphological research into those species. A morphological analysis of diagnostic parameters, such as corolla petals or the structure of stem hairs, did not reveal significant differences between those species (Celka *et al.* 2006b; Celka *et al.* 2007).

In contrast to the absence of genetic differences between *M. alcea* and *M. excisa*, the applied DNA markers supported the molecular identification of *M. alcea* var. *fastigiata*. The reported genetic identity values between the *fastigiata* form and typical *M. alcea* proved to be much lower than for *M. alcea* and *M. excisa*, and reached  $I_N=0.818$ . *Malva alcea* L. var. *fastigiata* Cav. (*M. fastigiata* Cav.) is rarely distinguished as a sepa-

rate taxon. In Central Europe, the only references to that variety can be found in older sources covering the area of Germany (Reichenbach 1841; Schlechtendal *et al.* 1885; Wünsche 1932; Garcke 1972), in particular northern Germany (Abromeit *et al.* 1898; Ascherson & Graebner 1898-1899; Hegi 1925). The division of *Malva alcea* into lower taxonomic units is based on the depth of incision of main stem leaves, stem hairs and fruits (Hegi 1925). This taxon has not been recognized in the most recent floras of Germany (Rothmaler *et al.* 2005), the Czech Republic, Slovakia (Hlavaček 1982; Slavík 1992, 2002) and Poland (Walas 1959; Mirek *et al.* 2002; Rutkowski 2004). In view of the biological species concept, the present taxonomic status of *M. alcea* var. *fastigiata* seems to be justified as genetic similarity values are generally lower for subspecies (Hamrick & Godt 1989). The genetic identity of *Phyteuma spicatum* subspecies analyzed with the use of isoenzymatic and DNA markers was  $I_N=0.65$  (Sawicki *et al.* 2006). The observed genetic similarity was even lower among *Marchantia polymorpha* subspecies where  $I_N$  values ranged from 0.515 for *montivagans* and *ruderalis* subspecies to 0.618 for *montivagans* and *polymorpha* subspecies (Boisselier-Dubayle *et al.* 1995). However, as regards some taxa, genetic identity values between subspecies are much higher. An AFLP marker analysis of *Hypericum perforatum* subspecies revealed genetic identity at a level of  $I_N=0.925$  for West European subspecies and  $I_N=0.828$  for Asian and East European subspecies (Percifield *et al.* 2007). Genetic identity values reported for *Cucurbita pepo* subspecies in SRAP and AFLP marker analyses were slightly lower, in the range of 0.71 to 0.80 (Ferriol *et al.* 2003).

Not all of the analyzed mallow taxa are good biological species. A high degree of genetic identity was reported for *M. neglecta* and *M. sylvestris* ( $I=0.821$ ) as well as for *M. neglecta* and *M. pusilla* ( $I_N=0.771$ ). However, despite relatively high levels of genetic similarity, both taxa were characterized by species-specific markers as well as a high number of fixed allelic differences. The close affinity between the studied species was also validated by an analysis of ITS sequences (Ray 1995).

The results reported in this study are largely consistent with the findings of Ray (1995, 1998) as well as with the division into sections proposed by Dalby (1968). ISJ and ISSR marker analyses revealed the absence of genetic differences between *M. alcea* and *M. excisa*, implying the need for follow-up research into the taxonomic status of the latter.

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**Appendix.** Localities of analyzed samples

Species	Locality	Herbarium
<i>Alcea rosea</i>	Olsztyn (Warmińsko-Mazurskie region, Poland)	OLS
<i>Alcea rosea</i>	Mragowo (Warmińsko-Mazurskie region, Poland)	OLS
<i>Malva alcea</i>	Teterow (Mecklenburg-Vorpommern, Germany)	POZ
<i>Malva alcea</i>	Tum near Łęczycza (Łódzkie region, Poland)	POZ
<i>Malva alcea</i>	Olevsk (Zhytomyr region, Ukraine)	POZ
<i>Malva alcea</i> var. <i>fastigiata</i>	Caprino (Venice region, Italy)	LE
<i>Malva alcea</i> var. <i>fastigiata</i>	Mamensee (Thüringen, Germany)	POZ
<i>Malva alcea</i> var. <i>fastigiata</i>	Koblenz (Rhineland-Palatinate, Germany)	POZ
<i>Malva excisa</i>	Zabiel'e (Pskov region, Russia)	POZ
<i>Malva excisa</i>	Anniskoe (Pskov region, Russia)	POZ
<i>Malva excisa</i>	Velikolutzk (Pskov region, Russia)	LE
<i>Malva moschata</i>	Kwieciszewo (Zachodniopomorskie region, Poland)	POZ
<i>Malva moschata</i>	Saint Petersburg (Leningrad region, Russia)	POZ
<i>Malva moschata</i>	Olsztyn (Warmińsko-Mazurskie region, Poland)	OLS
<i>Malva neglecta</i>	Rydzyna (Wielkopolskie region, Poland)	POZ
<i>Malva neglecta</i>	Góra (Mazowieckie region, Poland)	OLS
<i>Malva neglecta</i>	Posorty (Warmińsko-Mazurskie region, Poland)	OLS
<i>Malva pusilla</i>	Damaśławek (Wielkopolskie region, Poland)	POZ
<i>Malva pusilla</i>	Ciechanów (Mazowieckie region, Poland)	OLS
<i>Malva pusilla</i>	Olsztyn (Warmińsko-Mazurskie region, Poland)	OLS
<i>Malva sylvestris</i>	Słupca (Wielkopolskie region, Poland)	POZ
<i>Malva sylvestris</i>	Miłakowo (Warmińsko-Mazurskie region, Poland)	OLS
<i>Malva sylvestris</i>	Szczytno (Warmińsko-Mazurskie region, Poland)	OLS
<i>Malva verticillata</i>	Brody (Wielkopolskie region, Poland)	POZ
<i>Malva verticillata</i>	Brody (Wielkopolskie region, Poland)	POZ
<i>Malva verticillata</i>	Olsztyn (Warmińsko-Mazurskie region, Poland)	OLS

Explanations: LE – Herbarium of the Komarov Botanical Institute, Russian Academy of Science, Saint Petersburg; POZ – Herbarium of the Department of Plant Taxonomy of the A. Mickiewicz University of Poznań; OLS – Herbarium of the Department of Botany and Nature Protection of the University of Warmia and Mazury in Olsztyn