Biodiv. Res. Conserv. 19: 23-32, 2010

10.2478/v10119-010-0006-2

BRC
www.brc.amu.edu.pl

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

Zbigniew Celka[1], Monika Szczecińska[2] & Jakub Sawicki[2]

[1]Department of Plant Taxonomy, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznań, Poland, e-mail: zcelka@amu.edu.pl
[2]Department of Botany and Nature Protection, University of Warmia and Mazury in Olsztyn, Plac Łódzki 1, 10-727 Olsztyn, Poland, e-mail: monika.szczecinska@uwm.edu.pl, jakub.sawicki@uwm.edu.pl

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 (Zając 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; 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 Naggar 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* whithin 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°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 µl of a reaction mixture containing 40 ng genomic DNA, 1 µM primer, 1.5 mM $MgCl_2$, 200 µM dNTP (dATP, dGTP, dCTP, dTTP), 1x PCR buffer (Sigma, supplied with polymerase), 1µl 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°C, (2) denaturation – 1 minute at 94°C, (3) annealing – 1 minute at 49°C, (4) elongation – 1'30'' at 72°C, final elongation – 7 minutes at 72°C. Stages 2-4 were repeated 34 times. The following reaction conditions were applied to ISJ primers: (1) initial denaturation – 3 minutes at 94°C, (2) denaturation – 1 minute at 94°C, (3) annealing – 1 minute at 50°C, (4) elongation – 2'50'' at 72°C, final elongation – 5 minutes at 72°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	Seguence (5'- 3')	Number of the amplified bands	Number of polymorphic bands
IS810	$(GA)_8T$	8	7
IS813	$(CT)_8T$	19	16
IS822	$(TC)_8A$	21	18
IS825	$(AT)_8G$	22	22
IS828	$(TG)_8A$	14	14
IS831	$(ACC)_6$	29	29
IS843	CATGGTGTTGGTCATTGTTCCA	21	18
IS846	$GGGT(GGGGT)_2G$	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 polymorfic loci, number of species specific bands and private alleles of studied *Malva* taxa

	M. alcea	*M. alcea* var. *fastigiata*	*M. excisa*	*M. moschata*	*M. neglecta*	*M. pusilla*	*M. sylvestris*	*M. verticillata*
% of polymorfic 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	*M. alcea*	*M. alcea* var. *fastigiata*	*M. excisa*	*M. moschata*	*M. neglecta*	*M. pusilla*	*M. sylvestris*	*M. verticillata*
M. alcea		0.818	0.936	0.623	0.518	0.479	0.432	0.504
M. alcea var. *fastigiata*	5		0.830	0.713	0.582	0.570	0.535	0.600
M. excisa	0	6		0.641	0.518	0.475	0.440	0.514
M. moschata	31	25	28		0.691	0.634	0.692	0.677
M. neglecta	63	50	61	33		0.670	0.759	0.771
M. pusilla	62	52	60	30	29		0.735	0.672
M. sylvestris	64	53	63	28	19	26		0.821
M. verticillata	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

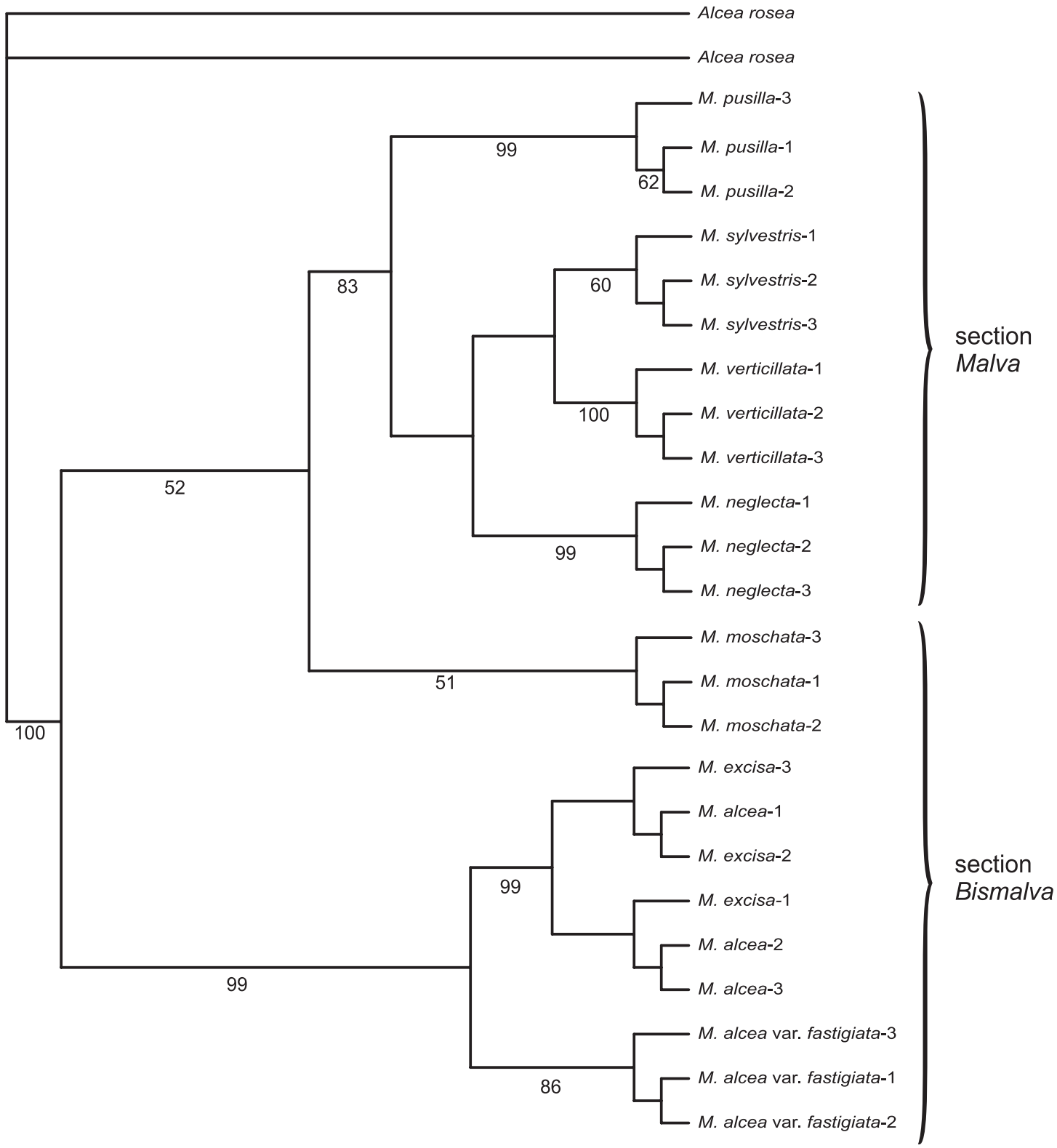

Fig. 1. Strict consensus tree of 180 most parsimonious trees (CI=0.62; RI=0.87) obtained by a parsimony analysis of 24 specimens of *Malva*. Bootstrap values (bs) higher than 50% are indicated below branches

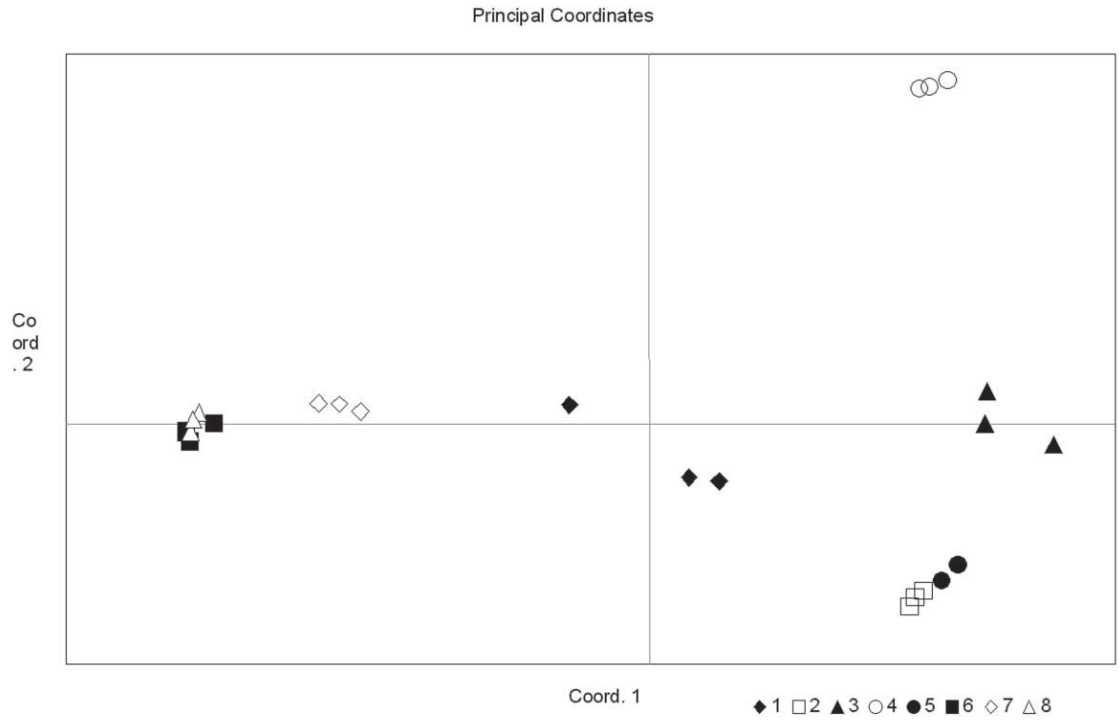

Fig. 2. Principal coordinates analysis of 24 *Malva* specimens
Explanations: 1 – *Malva moschata*, 2 – *M. pusilla*, 3 – *M. sylvestris*, 4 – *M. verticillata*, 5 – *M. neglecta*, 6 – *M. excisa*, 7 – *M. alcea* var. *fastigiata*, 8 – *M. alcea*

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 separate 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.

Acknowledgements. Authors sincerely thank the herbarium curators: Dr I. V. Tatanov from the Komarov Botanical Institute of the Russian Academy of Sciences in Saint Petersburg, Dr P. Szkudlarz from the Department of Plant Taxonomy of the A. Mickiewicz in Poznań, and Prof. T. Korniak

and Dr. M. Środa from the Department of Botany and Nature Protection of the University of Warmia and Mazury in Olsztyn for making available materials used in the DNA studies. Authors are also very grateful to Prof. Dr. N. N. Tzvelev and Dr G. Yu. Konechnaya from the Komarov Botanical Institute of the Russian Academy of Sciences in Saint Petersburg for their kind help and donating their own herbarium specimens. Scientific work financed from resources earmarked for science in years 2005-2008 as Research Project no. 2 P04G 050 29.

References

Abromeit J., Jentzsch A. & Vogel G. 1898. Flora von Ost- und Westpreussen. I, 690 pp. In Komission bei R. Friedländer & Sohn, Berlin.

Ascherson P. & Graebner P. 1898-1899. Flora des Nordostdeutschen Flachlandes (ausser Ostpreussen). xii+875 pp. Verlag von Gebrüder Borntraeger, Berlin.

Bączkiewicz A., Sawicki J., Buczkowska K. Polok K. & Zieliński R. 2008. Application of different DNA markers in studies on cryptic species of *Aneura pinguis* (Hepaticae, Metzgeriales). Cryptogamie Bryologie 29(1): 3-21.

Boisselier-Dubayle M. C., Jubier M. F., Lejeune B. & Bischler H. 1995. Genetic variability in the three subspecies of *Marchantia polymorpha* (Hepaticae): Isozymes, RFLP and RAPD markers. Taxon 44: 363-376.

Bonin A., Bellemain E., Bronken Eidesen P., Pompanon F., Brochmann C. & Taberlet P. 2004. How to track and assess genotyping errors in population genetic studies. Mol. Ecol. 13: 3261-3273.

Celka Z., Drapikowska M., Buczkowska K., Bączkiewicz A. & Marciniak J. 2006a. Morphological variability of *Malva alcea* L. populations from Poland. In: P. Kočárek, V. Plášek & K. Malachová (eds.). Environmental changes and biological assessment. III. Scripta Facultatis Rerum Naturalium Universitatis Ostraviensis 163:159-165.

Celka Z., Szkudlarz P. & Biereżnoj U. 2006b. Morphological variation of hairs in *Malva alcea* L. (Malvaceae). Biodiv. Res. Conserv. 3-4: 258-261.

Celka Z., Drapikowska M., Lembicz M., Kubiak L. & Szkudlarz P. 2008. The population of *Malva alcea* at the medieval settlement: from distribution to the reproduction of individuals. In: P. Kočárek, V. Plášek, K. Malachová & Š. Cimalová (eds.). Environmental changes and biological assessment. IV. Scripta Facultatis Rerum Naturalium Universitatis Ostraviensis 186: 224-230.

Celka Z., Drapikowska M., Ogrodowicz K., Shevera M. V. & Szkudlarz P. 2007. Differentiation of petals in the *Malva alcea* populations from the region of Central and Eastern Europe. Biodiv. Res. Conserv. 5-8: 17-24.

Cronberg N. 1996. Isozyme evidence of relationships within *Sphagnum* sect. *Acutifolia* (Sphagnaceae, Bryophyta). Pl. Syst. Evol. 203: 41-64.

Dalby D. H. 1968. *Malva* L. In: T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters & D. A. Weeb (eds.). Flora Europea. Rosaceae to Umbelliferae, 2, pp. 249-251. Cambridge University Press.

Daniels R. E. & Eddy A. 1990. Handbook of European Sphagna. 263 pp. Institute of Terrestrial Ecology, Natural Environment Research Council, HMSO. London.

Dogan B., Duran A. & Hakki E. E. 2007. Phylogenetic analysis of *Jurinea* (Asteraceae) species from Turkey based on ISSR amplification. Ann. Bot. Fenn. 44: 353-358.

Dostál J. 1989. Nová kvetena ČSSR. 1548+viii pp. Academia Praha.

El Naggar S. M. 2004. Pollen Morphology of Egyptian Malvaceae: An Assessment of Taxonomic Value. Turk. J. Bot. 28: 227-240.

Escobar García P., Schönswetter P., Fuertes Aguilar J., Nieto Feliner G., & Schneeweiss G. M. 2009. Five molecular markers reveal extensive morphological homoplasy and reticulate evolution in the *Malva* alliance (Malvaceae). Molecular Phylogenetics and Evolution 50: 226-239.

Ferriol M., Pico B. & Nuez F. 2003. Genetic diversity of a germplasm collection of *Cucurbita pepo* using SRPA and AFLP markers. Theor. Appl. Genet. 107: 271-282.

Furmanowa M., Michalska Z., Parczewski A. & Zarębska I. 1959. Lecznictwo renesansowe w Polsce na podstawie „Herbarza" Marcina z Urzędowa. Studia i Materiały z Dziejów Nauki Polskiej. Seria B – Historia nauk biologicznych i medycznych 2: 233-313.

Garcke A. 1972. Illustrierte Flora Deutschland und angrenzende Gebiete. 1607 pp. Verlag Paul Parey, Berlin und Hamburg.

Gunnarsson U., Hassel K. & Soderstrom L. 2005. Genetic structure of the endangered peat moss *Sphagnum angermanicum* in Sweden: a result of historic or contemporary processes? Bryologist 108: 194-203.

Hamrick J. L. & Godt M. J. 1989. Allozyme diversity in plant species. In: A. H. D. Brown, M. T. Clegg, A. L. Kahler & B. S. Weir (eds.). Plant Population Genetics, Breeding and Germplasm Resources, pp. 43-63. Sinauer, Sunderland, Mass.

Hegi G. 1925. Malvaceae. In: Illustrierte Flora von Mitteleuropa 5(1): 453-498. J. F. Lehmanns Verlag München.

Hey J. & Wakeley J. 1997. A coalescent estimator of the population recombination rate. Genetics 145: 833-846.

Hlavaček A. 1982. Malvaceae. In: J. Futák & L. Bertová (eds.). Flora Slóvenska, 3, pp. 372-405. VEDA, vydavateľstvo Slovenskej akadémie vied Bratislava.

Inamdar J. A. & Chohan A. J. 1969. Epidermal Structure and Stomatal Development in Some Malvaceae and Bombacaceae. Oxford Journals – Annals of Botany 33: 865-878.

Inamdar J. A., Balakrishna Bhat R. & Ramana Rao T. V. 1983. Structure, ontogeny, classification, and taxonomic significance of trichomes in Malvales. Korean J. Bot. 26(3): 151-160.

Jundziłł B. S. 1791. Opisanie roślin w prowincyi W. X. L. naturalnie rosnących według układu Linneusza. 570 pp. + Regester. W Drukarni J. K. Mci y Rzeplitey u XX. Piarow.

Kluk K. 1808. Dykcyonarz roślinny, 2, 256 pp. Drukarnia Xięży Piarów, Warszawa.

Kumar P. & Singh Dalbir 1991. Development and structure of seed coat in *Malva* L. Phytomorphology 41(1-2): 147-153.

Liu X.-Q., Gituru R.W. & Chen L.-Q. 2007. Genetic variation in the endangered fern *Adiantum reniforme* var. *sinense* (Adiantaceae) in China. Ann. Bot. Fenn. 44: 25-32.

Mabberley D. J. 1987. The plant-book. 858 pp. Cambridge University Press, Cambridge.

Marcin z Urzędowa 1595. Herbarz Polski. Drukarnia Lazarzowa, Kraków.

Mirek Z., Piękoś-Mirkowa H., Zając A. & Zając M. 2002. Flowering plants and pteridophytes of Poland. A checklist. In: Z. Mirek (ed.). Biodiversity of Poland 1, 442 pp. W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków.

Mosyakin S. L. 1999. Malvaceae. In: S. L. Mosyakin & M M. Fedoronchuk (eds.). Vascular plants of Ukraine a nomenclatural checklist, pp. 249-250. M. G. Kholodny Institute of Botany, Kiev.

Nei M. & Li W. H. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76: 5269-5273.

Olyanitskaya L. G. & Tzvelev N. N. 1996. Malvaceae. In: N. N. Tzvelev (ed.). Flora Europae Orientalis, pp. 231-255. Mir i Semia Petropoli.

Peakall R. & Smouse P. E. 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Not. 6: 288-295.

Percifield R. J., Hawkins J. S., McCoy J.-A., Widrlechner M. P. & Wendel J. F. 2007. Genetic diversity in *Hypericum* and AFLP markers for species-specific identification of *H. perforatum* L. Planta Med 73: 1614-1621.

Plášek V. & Sawicki J. 2010. Is the hairy vaginula an diagnostic feature in the taxonomy of the genus *Orthotrichum*? Acta Soc. Bot. Pol. 79: 73-80.

Polok K. 2005. Evolutionary status of closely related *Lolium* taxa. In: W. Prus-Głowacki & E. Pawlaczyk (eds.). Variability and Evolution – New Perspectives, Biologia 72: 195-208. Adam Mickiewicz University, Poznań.

Polok K. 2007. Molecular evolution of the genus *Lolium* L. 317 pp. Studio Poligrafii Komputerowej „SQL", Olsztyn.

Ray M. F. 1995. Systematics of *Lavatera* and *Malva* (*Malvaceae, Malveae*) – a new perspective. Pl. Syst. Evol. 198: 29-53.

Ray M. F. 1998. New Combinations in *Malva* (Malvaceae: Malveae). Novon 8: 288-295.

Reichenbach L. 1841. Icones florae germanicae et helveticae. Apud Fridericum Hofmeister, Lipsiae.

Rothmaler W., Jäger E. J. & Werner K. 2005. Exkursionsflora von Deutschland. Band 4, Gefäßpflanzen: Kritischer Band. 980 pp. Spektrum Akademischer Verlag, Elsevier Gmbh, München.

Rutkowski L. 2004. Klucz do oznaczania roślin naczyniowych Polski niżowej. 814 pp. Wyd. Nauk. PWN, Warszawa.

Sawicki J. & Szczecińska M. 2007. Semi-specific intron-exon splice junction markers in bryophyte studies. Biodiv. Res. Conserv. 5-8: 25-30.

Sawicki J., Szczecińska M., Korniak T. & Hołdyński Cz. 2006. Genetic structure of *Phyteuma spicatum* subsp. *coeruleum* R. Schulz and *Ph. spicatum* subsp. *spicatum* populations. Biodiv. Res. Conserv. 1-2: 46-49.

Sawicki J. & Zieliński R. 2008. Phylogenetic relationships between five *Sphagnum* species of the section *Acutifolia* based on DNA markers. Cas. Slez. Muz. Opava 57: 63-80.

Sawicki J., Plášek V. & Szczecińska M. 2010. Molecular studies resolve *Nyholmiella* (Orthotrichaceae) as a separate genus. J. Syst. Evol. 48: 183-194.

Schlechtendal D. F. L., Langethal L. E. & Schenk E. 1885. Flora von Deutschland, 21, 304 pp. Verlag von Fr. Eugen Köhler Gera-Untermhaus.

Slavík B. 1992. Malvaceae Juss. – slézovité. In: B. S. Hejný & B. Slavík (eds.). Květena České republiky, 3, pp. 282-316. Academia, Praha.

Slavík B. 2002. Malvaceae. In: K. Kubát (ed.). Klíč ke květeně České republiky, 286-290 pp. Praha.

Swofford D. L. 2003. PAUP*:* Phylogenetic Analysis Using Parsimony (and Other Methods) (Sinauer, Sunderland, MA), 4.0 Beta

Syreniusz 1613. Zielnik Simona Syrenniusa z 1613 r. www.zielnik-syrenniusa.art.pl.

Szczecińska M., Sawicki J., Polok K., Hołdyński Cz. & Zieliński R. 2006. Comparison of the *Polygonatum* species from Poland on the basis of different DNA markers. Ann. Bot. Fenn. 43(5): 379-388.

Szczecińska M., Sawicki J., Wąsowicz K. & Hołdyński Cz. 2009. Genetic variation of the relict and endangered population of *Chamaedaphne calyculata* (Ericaceae) in Poland. Dendrobiology 62:23-33.

Vanderpoorten A., Hedenas L. & Jacquemart A.-L. 2003. Differentiation in DNA fingerprinting and morphology among species of the pleurocarpous moss genus, *Rhytidiadelphus* (Hylocomiaceae). Taxon 52: 229-236.

Walas J. 1959. Malvaceae, Ślazowate. In: W. Szafer & B. Pawłowski (eds.). Flora polska. Rośliny naczyniowe

Polski i ziem ościennych, 8, pp. 278-301. PWN, Warszawa.

WEINING S. & LANGRIDGE P. 1991. Identification and mapping of polymorphisms in cereals based on polymerase chain reaction. Theor Appl Genet 82: 209-216.

WERNER O., JIME´NEZ J. A., ROS R. M., CANO M. J. & GUERRA J. 2005. Preliminary investigation of the systematics of *Didymodon* (Pottiaceae, Musci) based on nr ITS sequence data. Syst. Bot. 30: 461-470.

WOLFE A. D. & LISTON A. 1998. Contributions of PCR-based methods to plant systematics and evolutionary biology. In: D. E. SOLTIS, P. S. SOLTIS & J. J. DOYLE (eds.). Molecular Systematics in plants. II. DNA sequencing, pp. 43-86. Kluwer Academic Press.

WÜNSCHE O. 1932. Die Pflanze Deutschalnds. Eine Anleitung zu ihrer Kenntnis. II. Die höheren pflanzen. 603 pp. Verlag und Druck von. B. G. Teubner, Leipzig und Berlin.

ZAJĄC A. 1979. Pochodzenie archeofitów występujących w Polsce. Rozpr. habil. Uniw. Jagiell. 29: 1-213. Druk UJ w Krakowie.

ZIETKIEWICZ E., RAFALSKI A. & LABUDA D. 1994. Genome fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification. Genomics 20: 176-183.

Appendix. Localities of analyzed samples

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