
Research Paper

Interspecific hybridization among cultivars of hardy *Hibiscus* species section *Muenchhusia*

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Rose mallows belong to the *Muenchhusia* section of the *Hibiscus* genus. They represent a small group of cold tolerant North American plants and are popular ornamentals mainly because of their abundant, large and colorful flowers. Due to their geographical origin they are well suited for garden use in temperate regions worldwide. The aim of the study was to investigate hybridization barriers in crosses among cultivars of *Hibiscus* species from the *Muenchhusia* section: *H. coccineus*, *H. laevis* and *H. moscheutos*. Crossing barriers were identified as both pre- and post-zygotic. The analysis of pollen tube growth revealed inhibition of pollen tubes and their abnormal growth. In specific crosses the fertilization success was low. The pre-fertilization barriers did not cause a complete reproductive isolation between the hybridization partners. In relation to post-fertilization barriers, the occurrence of hybrid incompatibilities such as unviability, chlorosis, necrosis, stunted growth and albinism were the main drawback in production of hybrids. The appearance of symptoms of hybrid incompatibilities was dependent upon specific parental plants. The obtained progeny had intermediate leaf morphology and flower morphology compared to parental plants. Hybridity state was verified by morphological analysis and RAPD markers. Based on the overall plant morphology, 472 hybrid progenies were obtained.

Key Words: albinism, hybrid incompatibility, hybrid inviability, pollen tube, pre-fertilization barrier, post-fertilization barrier.

Introduction

Hybridization among different species and genera is one of the most important breeding approaches used for improvement of ornamental plants. The main aim of this strategy is to merge distant gene pools, hence broadening the genetic variability. However, hybridization barriers often occur in interspecific crosses hampering production of hybrids. Traditionally two types of hybridization barriers have been defined (Morgan *et al.* 2011, Van Tuyl and De Jeu 1997). The first group, pre-fertilization barriers, includes lack of stigma receptivity or pollen viability at the time of pollination, low adhesion and germination of pollen grains, and abnormal growth of pollen tubes in the style and ovary (Rieseberg and Carney 1998). Moreover, different abnormalities in the growth of pollen tubes can influence their ability to successfully deliver sperm cells to ovules (Winkelmann *et al.* 2010). The second group comprises post-fertilization barriers

that affect development of hybrid embryos and further development of hybrid plants. In some cases, failure in hybridization process is due to the breakdown of endosperm development. Moreover, post-fertilization barriers often involve deleterious hybrid characteristics, collectively called hybrid incompatibilities (HIs), such as unviability, lethality, sterility, albinism and abnormality in phenotypic traits of hybrids (Johnson 2010, Kinoshita 2007). These symptoms have been associated with hyperactivation of pathogen response genes due to discrepancies among components of immune systems in *Arabidopsis*, tomato and lettuce (Bombliès 2010, Johnson 2010).

Hibiscus L. is a genus within the Malvaceae family. It comprises around 300 species mainly distributed in tropical and subtropical regions with a few species extending into temperate zones of the world (Akpan 2006). The species are characterized by pentamerous and regular flowers that exhibit a variety of colors. The leaf morphology is very diverse in the genus. They vary from alternate and petiolated to simple or entire, lobbed and parted (Lawton 2004). *Hibiscus* species produce a wide range of growth habits ranging from small trees and shrubs to annual and perennial

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herbs (Akpan 2006).

Hibiscus species are generally classified as either tropical or hardy types. The most appreciated tropical cultivars belong to the *H. rosa-sinensis* group, however, they have limited use as outdoor plants in temperate regions due to their chilling sensitivity (Lawton 2004, Vazquez-Thello *et al.* 1996). The hardy *Hibiscus* plants include cultivars derived from a few species that are mainly native to North America such as *H. mutabilis* and *H. moscheutos* and a few Asiatic species, which comprise *H. syriacus* (Lawton 2004, Van Laere *et al.* 2007).

The *Hibiscus* Muenchhusia section is a North American taxon that includes the five species *H. coccineus*, *H. dasycalyx*, *H. grandiflorus*, *H. laevis* and *H. moscheutos*, commonly known as Rose mallows. These species share common growth habit being hardy herbaceous perennials and are primarily wetland species. Rose mallows are popular ornamental plants mainly due to their abundant, large and colorful flowers. The species of the Muenchhusia section are cold resistant, and thus suitable for garden use in temperate regions. All species of the Muenchhusia section have a basic chromosome number of $n = 19$ and they are known to form hybrids relatively easy (Small 2004, Winters 1970). However, there is a continuous need for improvement of hardy *Hibiscus* cultivars in relation to their growth habit and flower characteristics.

The objective of this study was to evaluate the occurrence of hybridization barriers in crosses between hardy cultivars of *Hibiscus* species. We investigated the occurrence of pre- and post-fertilization barriers as well as hybrid characters of the obtained seedlings.

Materials and Methods

Plant material and sexual hybridization

Five garden cultivars belonging to three hardy *Hibiscus* species were used to perform reciprocal crosses. They included *H. moscheutos*: ‘Disco Belle’, ‘Pale Pink’ and *H. moscheutos* ssp. *palustris* ‘Pink Mallow’ as well as cultivars of *H. coccineus* ‘Red Star’ and *H. laevis*—an unnamed breeding line. Plants were propagated from seeds at Graff Breeding A/S, Sabro, Denmark and then transported to University of Copenhagen, Taastrup, Denmark, where they were maintained in the greenhouse under 16/8 h photoperiod and 22/18°C ± 4°C, day/night with additional light of 180 μmol s⁻¹ m⁻² (Philips Master SON-T PIA Green Power 400 W, Eindhoven, The Netherlands). The plants were irrigated weekly with fertilizer (Pioneer NPK Makro 14-3-23, Tilst, Denmark) with an electrical conductivity of 1.3 mS cm⁻¹.

Interspecific crosses were performed from June to August in 2012 and 2013 using six to ten plants per genotype. Flowers were emasculated in the bud stage and hand pollinated. Pollination was performed by applying fresh pollen on the stigma of a fully open flower. An overview of the performed crosses is given in **Table 1**. The flowers left for natural self-pollination served as controls to determine self-incompatibility of the plants.

Pollen viability

Pollen was collected at the point of anther dehiscence i.e. in the day of flower opening before noon. Pollen from five flowers was immersed in a drop of 1% (w/v) acetocarmine solution and examined under a light microscope (Leitz DMRD, Leica, Germany). Pollen grains were classified as viable when stained red and unviable when unstained

Table 1. Characterization of interspecific crosses in relation to pollen tube growth and fruit, seed and plant production

Cross combination	Pollen tubes ¹		No. of pollinated flowers	Fruit set ² (%)	Av. no. of seeds/ fruit (S.E.)	Germination percentage (%) (S.E.)	Seedling survival (%) (S.E.)	Seedlings/plants with HIs ³ (%)	Total adult plants	Total hybrids ⁴
	Stigma	Ovary								
<i>H. coccineus</i> × <i>H. laevis</i> reciprocal crosses										
<i>H. c.</i> ‘Red Star’ ⁵ × <i>H. laevis</i>	5/5***	5/5*	109	8.2	16.9 (3.6)	96.0 (2.3)	93.0 (1.5)	0	67	67
<i>H. laevis</i> × <i>H. cocc.</i> ‘Red Star’	5/5***	5/5***	69	13	39.6 (4.8)	97.3 (1.3)	93.1 (3.7)	0	68	68
<i>H. coccineus</i> × <i>H. moscheutos</i> reciprocal crosses										
<i>H. c.</i> ‘Red Star’ × <i>H. m.</i> ⁶ ssp. <i>palustris</i> ‘Pink Mallow’	5/5***	5/5***	90	36.7	23.1 (1.6)	96.0 (2.3)	0.0 (0.0)	100	0	–
<i>H. c.</i> ‘Red Star’ × <i>H. m.</i> ‘Disco Belle’	5/5**	5/5*	68	19.1	15.8 (2.8)	88.0 (10.1)	93.3 (6.7)	100	61	60
<i>H. c.</i> ‘Red Star’ × <i>H. m.</i> ‘Pale Pink’	2/5*	1/5*	110	11.8	13.8 (2.0)	54.7 (5.8)	92.5 (0.8)	0	38	38
<i>H. m.</i> ssp. <i>palustris</i> ‘Pink Mallow’ × <i>H. c.</i> ‘Red Star’	5/5***	5/5***	62	64.5	73.6 (2.6)	80.0 (12.0)	0.0 (0.0)	100	0	–
<i>H. m.</i> ‘Disco Belle’ × <i>H. c.</i> ‘Red Star’	5/5**	3/5**	98	2	58.0 (9.0)	56.0 (6.1)	88.2 (11.8)	100	36	27
<i>H. m.</i> ‘Pale Pink’ × <i>H. c.</i> ‘Red Star’	5/5***	5/5***	73	41.1	29.3 (1.5)	54.7 (3.5)	93.3 (6.7)	0	38	38
<i>H. laevis</i> × <i>H. moscheutos</i> reciprocal crosses										
<i>H. laevis</i> × <i>H. m.</i> ssp. <i>palustris</i> ‘Pink Mallow’	5/5***	5/5***	60	28.3	23.5 (2.2)	92.0 (6.1)	4.7 (2.9)	96	3	0
<i>H. laevis</i> × <i>H. m.</i> ‘Disco Belle’	5/5***	5/5***	67	35.8	33.0 (1.8)	94.7 (3.5)	98.5 (1.5)	0	70	69
<i>H. laevis</i> × <i>H. m.</i> ‘Pale Pink’	5/5***	5/5***	60	31.7	36.1 (1.9)	44.0 (15.1)	96.1 (3.9)	0	31	30
<i>H. m.</i> ssp. <i>palustris</i> ‘Pink Mallow’ × <i>H. laevis</i>	5/5***	5/5***	66	62.1	93.5 (2.9)	26.7 (10.9)	0.0 (0.0)	100	0	–
<i>H. m.</i> ‘Disco Belle’ × <i>H. laevis</i>	5/5***	5/5***	118	26.3	52.9 (3.1)	54.7 (17.3)	83.3 (8.5)	31	32	29
<i>H. m.</i> ‘Pale Pink’ × <i>H. laevis</i>	5/5***	5/5***	64	75	34.5 (1.4)	64.0 (4.0)	95.6 (4.4)	0	46	46

¹ Pollen tubes were examined two places in the pistil: stigma and in the entrance to the ovary, the numbers indicate no. of pistils in which pollen tubes were observed, asterisks indicate number of observed pollen tubes: * up to 20 pollen tubes ** 20 to dozens pollen tubes *** hundreds pollen tubes; ² No. of fruits/ no. of pollinated flowers; ³ HIs: hybrid incompatibilities; ⁴ Based on morphological observations; ⁵ *H. c.*: *H. coccineus*; ⁶ *H. m.*: *H. moscheutos*.

(Singh 2002). At least 100 pollen grains were analyzed per genotype. Each experiment was performed once in three technical replicates. The significance of differences was determined using one-way analysis of variance followed by Tukey's honestly significant difference test (HSD) in the SPSS 22.0 for Windows statistical software package (SPSS Inc., Chicago, IL, USA).

Pollen tube growth

Pollen germination and growth of pollen tubes were examined according to Kuligowska *et al.* (2015b). Pistils were softened for 20 minutes. Pollen tubes were examined with fluorescence microscope (Leica DM2000 LED, Leica, Germany; excitation filter BP 340–360 nm) equipped with a digital camera (Leica DFC420, Leica, Germany). The relative numbers of pollen tubes was assessed in two parts of the pistil—stigma and at the entrance to the ovary. Numbers of tubes were quantified according to the criteria: *—up to 20 pollen tubes, **—20 to dozens pollen tubes, ***—dozens to hundreds pollen tubes.

Fruit and seed set, seed germination and plant production

Fruit and seed set were evaluated around 60 days after pollination. Number of fruits and average number of seeds were registered. To determine germination percentage, 20 seeds in three replicates were sown in peat in the greenhouse with conditions as described for parental species. Number of seedlings and seedling survival was determined one month after seed sowing. Number of obtained plants and hybrids as well as plants with HIs was recorded.

Evaluation of hybrid incompatibilities

After germination plants were transplanted and were grown in the greenhouse as described for parental species. The numbers of plants exhibiting symptoms of HIs i.e. chlorosis, necrosis, stunted growth and albinism were determined following visual assessments of seedlings and adults plants.

Morphology of hybrids

The progenies obtained from crosses were morphologically analyzed. Leaf morphology, flower morphology and flower color of F1 plants that had intermediate phenotype compared to the parental plants were determined.

Random Amplified Polymorphic DNA analysis

For each cross-combination, the DNA from four individuals of the progeny exhibiting intermediate phenotype and parental plants were used to compare the amplification products using the RAPD technique (Williams *et al.* 1990).

DNA was extracted from 150 mg fresh leaf tissue. The leaf tissue was frozen in liquid nitrogen and ground to a powder. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Due to the large quantity of polysaccharides present in the extract the volume of AP1 buffer and P3 buffer were increased to 500 µl and 150 µl, respectively, and 0.02% (w/v) Polyvinylpyrrolidone (40,000) (Sigma-Aldrich, Steinheim, Germany) and 3.75% (v/v) β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) were added to AP1 buffer to prevent polyphenol oxidation. The DNA concentration was determined using NanoDrop1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Amplification reactions were carried out in a volume of 20 µl containing 25 µM TAPS (pH 9.3), 50 µM KCl, 2 mM MgCl₂, 0.1 mM DTT, 100 µM [H³]-dTTP, 0.25 mg/ml activated salmon sperm DNA, 2% (v/v) DMSO, 200 µM of each dNTP, 1 µM primer, 1 U Taq DNA polymerase (TaKaRa Ex Taq, TAKARA BIO INC., Shiga, Japan) and 50 ng plant genomic DNA in thermal cycler (MyCycler, Biorad, Hercules, CA, USA). The selection of reaction conditions was made following a preliminary study in which different concentrations of polymerase, primer and template DNA were tested (data not shown).

Ten random decamer primers were tested and four primers (A01, A07, A08, A09; primer kit A, Carl Roth, Karlsruhe, Germany) were selected depending on specific cross-combinations (Table 2). Thermal cycling was conducted with 5 min initial denaturation at 94°C, followed by 45

Table 2. RAPD markers used for hybrid confirmation with the respective number of bands obtained for female and male parents, both parents, and the total number of bands

Cross combination	Primer	♀	♂	Both	Total number of bands	No. of female-specific bands in the progeny ¹	No. of male-specific bands in the progeny ¹
<i>H. c.</i> ² 'Red Star' × <i>H. laevis</i>	A07	6	8	4	10	3	3
<i>H. laevis</i> × <i>H. c.</i> 'Red Star'	A07	8	6	4	10	2 or 3	1
<i>H. c.</i> 'Red Star' × <i>H. m.</i> ³ 'Disco Belle'	A09	6	6	4	8	1 or 2	1
<i>H. m.</i> 'Disco Belle' × <i>H. c.</i> 'Red Star'	A07	4	6	2	8	2	1 or 2
<i>H. c.</i> 'Red Star' × <i>H. m.</i> 'Pale Pink'	A07	6	7	4	9	2	1 or 2
<i>H. m.</i> 'Pale Pink' × <i>H. c.</i> 'Red Star'	A07	7	6	4	9	1	2
<i>H. laevis</i> × <i>H. m.</i> 'Disco Belle'	A08	7	5	3	9	2	1
<i>H. m.</i> 'Disco Belle' × <i>H. laevis</i>	A07	4	8	3	9	2	2
<i>H. laevis</i> × <i>H. m.</i> 'Pale Pink'	A07	8	7	4	11	2 or 3	1
<i>H. m.</i> 'Pale Pink' × <i>H. laevis</i>	A01	5	6	3	8	1	2

¹ based on 5 F1 plants; ² *H. c.*: *H. coccineus*; ³ *H. m.*: *H. moscheutos*.

cycles of 1 min at 94°C, 1 min at 35°C and 2 min at 72°C, and a final extension step for 10 min at 72°C. Amplification products were separated by electrophoresis in 1.5% agarose gels in 1× TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0, 20 mM glacial acetic acid; pH 8.4), detected by staining with Gelred (Biotium, Hayward, CA, USA) and visualized under UV light. The banding patterns were evaluated by visual inspection. All amplification reactions were repeated twice. Only bands present in both amplification reactions were considered in the analysis. Plants were classified as hybrids when at least one male-specific band was present in the banding profile of the progeny.

Results

Pollen viability

The pollen viability was high for all tested cultivars. Levels of pollen viability were found to be 87.5% ± 2.5% for *H. moscheutos* ‘Pale Pink’, 88.0% ± 1.7% for *H. moscheutos* ssp. *palustris* ‘Pink Mallow’, 88.2% ± 2.2% for *H. moscheutos* ‘Disco Belle’, 92.5% ± 2.9% for *H. coccineus* ‘Red Star’ and 95.5% ± 1.2% for *H. laevis*. Pollen viability was not significantly different between tested cultivars.

Pollen tube growth

The analysis of pollen tube growth revealed lower numbers of pollen tubes in the stigmas from three cross-combinations compared to other crosses. They included reciprocal crosses between *H. coccineus* ‘Red Star’ and *H. moscheutos* ‘Disco Belle’ as well as crosses between *H. coccineus* ‘Red Star’ and *H. moscheutos* ‘Pale Pink’ (Table 1). The numbers of pollen tubes reaching the ovaries were lower in four crosses: *H. coccineus* ‘Red Star’ × *H. moscheutos* ‘Disco Belle’, *H. moscheutos* ‘Disco Belle’ × *H. coccineus* ‘Red Star’, *H. coccineus* ‘Red Star’ × *H. moscheutos* ‘Pale Pink’ and *H. coccineus* × *H. laevis*. Additionally, in crosses between *H. coccineus* ‘Red Star’ and *H. moscheutos* ‘Pale Pink’ as well as *H. moscheutos* ‘Disco Belle’ and *H. coccineus* ‘Red Star’ no growth of pollen tubes was observed in some of the pistils (Table 1).

Qualitative analysis of pollen tube germination and growth revealed occurrence of different abnormalities in all tested interspecific crosses. They included germination of multiple pollen tubes from single pollen grains (Fig. 1A), branching of pollen tubes (Fig. 1B) and occurrence of spiky pollen tubes (Fig. 1C). Normal pollen tubes were also observed in high numbers. Thus, the normal pollen tubes were growing through the style and entering ovaries without any disruption.

Self-pollinations and intraspecific pollinations of parental cultivars revealed germination of multiple pollen tubes from single pollen grains but in less extend compared to interspecific crosses. No other abnormalities were observed in control pollinations (data not shown).

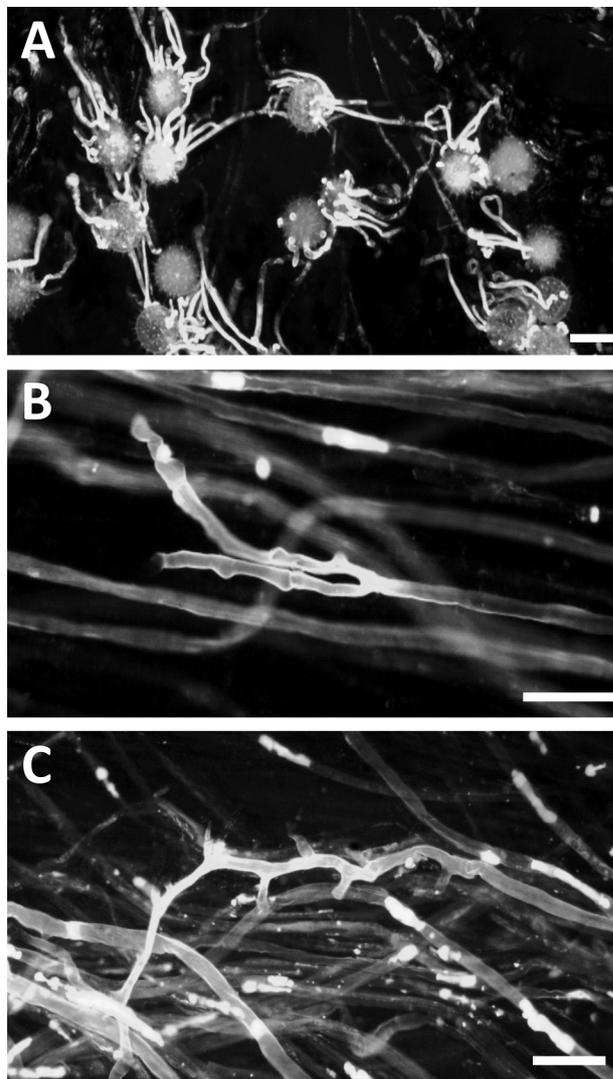


Fig. 1. Abnormal pollen germination and pollen tube growth. (A) Pollen grains of *H. laevis* on stigma of *H. moscheutos* ‘Pale Pink’, abnormal germination with visible multiple pollen tubes emerging from single pollen grains. (B) Branching of pollen tube, *H. coccineus* ‘Red Star’ × *H. moscheutos* ‘Pale Pink’. (C) Spiky pollen tube, *H. coccineus* ‘Red Star’ × *H. moscheutos* ssp. *palustris* ‘Pink Mallow’; Scale bars: 100 μm.

Fruit and seed set, seed germination and plant production

The lowest fruit set was observed in the cross between *H. moscheutos* ‘Disco Belle’ and *H. coccineus* ‘Red Star’ (2.0%). The values lower than 20% were obtained following reciprocal crosses between *H. coccineus* ‘Red Star’ and *H. laevis*, crosses between *H. coccineus* ‘Red Star’ and *H. moscheutos* ‘Disco Belle’ as well as *H. coccineus* ‘Red Star’ and ‘Pale Pink’ (Table 1).

The lowest observed average number of seeds (less than 20) was found in three cross-combinations also characterized by low fruit set. They were: *H. coccineus* ‘Red Star’ × *H. laevis*, *H. coccineus* ‘Red Star’ × *H. moscheutos* ‘Disco Belle’ and *H. coccineus* ‘Red Star’ × ‘Pale Pink’ (Table 1).

Germination rate of seeds was generally high (around 50% or higher) for all examined cross-combinations. The only exception was observed for crosses between *H. moscheutos* ssp. *palustris* 'Pink Mallow' × *H. laevis*, where the germination rate was 26.7 ± 10.9 (Table 1).

No abnormal seeds were obtained following interspecific crosses.

Survival of seedlings was generally high for all cross-combinations (from 83.3 ± 8.5 for *H. moscheutos* 'Disco Belle' × *H. laevis* to 98.5 ± 1.5 for *H. laevis* × *H. moscheutos* 'Disco Belle') except the crosses where *H. moscheutos* ssp. *palustris* 'Pink Mallow' was used as one of the parental plants. In these crosses no seedling of intermediate phenotype developed into an adult plant (Table 1).

In our study selected cultivars could be easily self-pollinated and yielded high fruit and seed set in intraspecific crosses (data not shown).

Evaluation of hybrid incompatibilities

Following several cross-combinations, the obtained hybrids demonstrated signs of HIs. In the crosses where *H. moscheutos* ssp. *palustris* 'Pink Mallow' was used either as a paternal or maternal plant, all obtained seedlings showed signs of chlorosis and necrosis (Fig. 2A). They eventually died after developing one pair of true leaves (Table 1—seedling survival). Crosses between *H. moscheutos* 'Disco Belle' × *H. laevis* resulted in 9 out of 29 (31%) plants exhibiting partial albinism (Fig. 2B). The progeny obtained after reciprocal crosses between *H. coccineus* 'Red Star' and *H. moscheutos* 'Disco Belle' exhibited stunted growth, chlorosis and necrosis (Fig. 2C). These symptoms became more severe during the growth of the plants. The remaining crosses yielded vigorously growing plants.

Morphological assessment

The adult plants obtained after interspecific crosses mainly exhibited intermediate characteristics. The percentage of plants with intermediate phenotype is presented in Table 1. The flower characteristics were intermediate between both hybridization partners in respect to petal shapes. Progeny resulting from crosses where *H. coccineus* 'Red Star' contributed as one of the parents resulted in red flowers. The plants obtained from reciprocal crosses between *H. laevis* and *H. moscheutos* 'Disco Belle' had intermediate pink color. An example of hybrid flower compared to parental plants is shown in Fig. 2D.

The leaf morphologies had clearly intermediate shapes between parental genotypes (Table 3). Reciprocal crosses between *H. coccineus* 'Red Star' and *H. moscheutos* 'Pale Pink' as well as *H. laevis* resulted in palmate leaves with indentations of lesser deepness than *H. coccineus* 'Red Star'. When *H. coccineus* 'Red Star' was crossed with *H. moscheutos* 'Disco Belle' that has deltoid to cordate leaves, the progeny exhibited lobed leaves with depth of indentations depending on the maternal genotype. All the reciprocal crosses between *H. laevis* and *H. moscheutos*

resulted in lobed leaves, while the depth of indentation depended on the specific cross-combination. An example of hybrid leaf compared to parental plants is shown in Fig. 2E.

Based on the overall plant morphology, 472 hybrid progenies were obtained in the study.

RAPD analysis

Four selected RAPD primers generated informative bands using RAPD analysis to confirm hybridity of the progeny at the molecular level. The numbers of bands obtained for parental species and polymorphic bands in specific cross combinations are included in Table 2. Total number of bands is the sum of the bands present in female and male parents subtracted from the number of bands shared by both parents.

The band pattern was evaluated and all analyzed plants were verified as hybrids. A representative example of confirmation of hybrid status is presented in Fig. 3. Male-informative bands are present in the sample of the progeny, confirming hybrid origin of the plants.

Discussion

The objective of the study was to investigate the occurrence of hybridization barriers in interspecific crosses among cultivars belonging to *Hibiscus* species section *Muenchhusia*. In our study control pollinations yielded high fruit and seed set (data not shown). The pollen viability was high for all examined parental genotypes, thus pollen viability is not a limiting factor during hybridization. The only problematic cultivar was *H. moscheutos* 'Disco Belle' that was characterized by low fertility despite high pollen viability. Nevertheless, we can conclude that self-incompatibility did not occur in the cultivars used in the current investigation.

In four crosses of *H. coccineus* 'Red Star' we observed lower numbers of pollen tubes or even total lack of pollen tubes in the pistils (Table 1). The pollen adhesion is a first step of reproduction that relies on successful interaction between pollen and stigma. This stage is especially important in species with dry stigma type as the one occurring in *Hibiscus*. Furthermore, pollen germination on stigma is dependent upon pollen rehydration and controlled by both physiological and genetic factors. Thus, pollen-stigma interactions determine the first selective barrier for successful pollen tube growth in the pistil tissue (Gao *et al.* 2010). Low numbers of pollen tubes in stigmas can indicate disturbed adhesion and germination of pollen in our crosses. Further impaired growth of pollen tubes to the ovaries suggests specific differences in pollen-pistil interactions. Different factors such as components of extracellular matrix, water gradient potential, concentration gradients of Ca^{2+} , concentration of γ -aminobutyric acid, lipid molecules and proteins in the style are likely to influence the growth of pollen tubes (Gao *et al.* 2010, Swanson *et al.* 2004). Additional evidence for the disruption of pollen tube growth in our crosses comes from microscopic analysis that revealed the occurrence of a number of abnormalities (Fig. 1). A similar

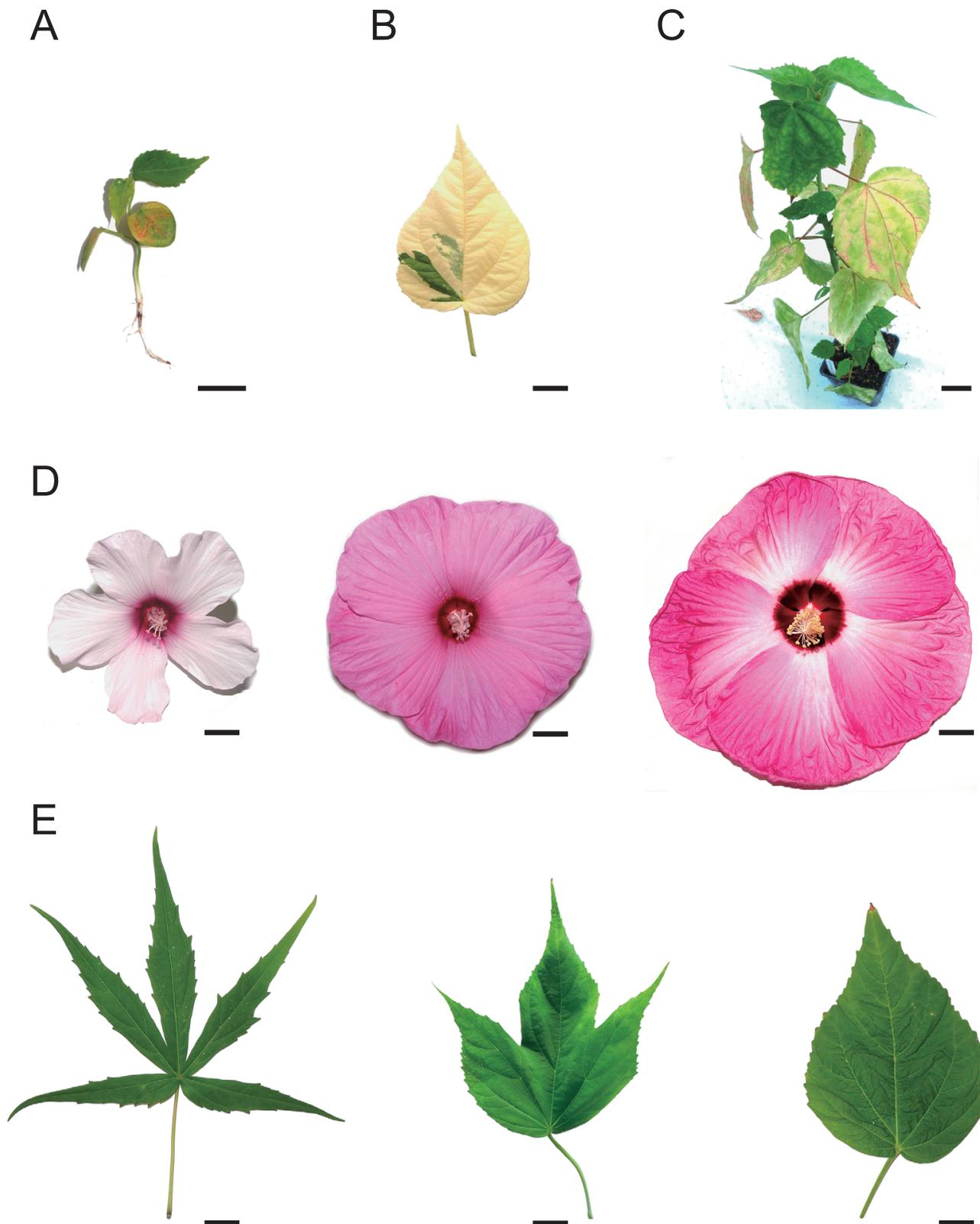


Fig. 2. Hybrid incompatibilities and morphology of *Hibiscus* hybrids. (A) Two months old seedlings from cross *H. coccineus* ‘Red Star’ × *H. moscheutos* ssp. *palustris* ‘Pink Mallow’ showing signs of chlorosis and necrosis. (B) Partial albinism of interspecific hybrid from cross between *H. moscheutos* ‘Disco Belle’ and *H. laevis*. (C) Adult interspecific hybrid with signs of chlorosis and stunted growth. (D) Flower morphology of parental cultivars: *H. laevis* (♀) (left) and *H. moscheutos* ‘Disco Belle’ (♂) (right) and their representative interspecific hybrid (middle). (E) Leaf morphology of parental cultivars: *H. coccineus* ‘Red Star’ (♀) (left) and *H. moscheutos* ‘Disco Belle’ (♂) (right) and their representative interspecific hybrid (middle). Scale bars A, B, D, E: 2 cm, C: 10 cm.

Table 3. General characterization of leaf shapes of parental cultivars and hybrids

Parental cultivars and hybrids	Leaf shape
<i>H. c.</i> ‘Red Star’	Digitate
<i>H. laevis</i>	Hastate
<i>H. m.</i> ‘Disco Belle’	Deltoid to cordate
<i>H. m.</i> ‘Pale pink’	Deltoid with 3 lobes, shallow indentation
<i>H. c.</i> ‘Red Star’ × <i>H. laevis</i>	Palmetly-lobed, 5 lobes
<i>H. laevis</i> × <i>H. c.</i> ‘Red Star’	Palmetly-lobed, 3–5 lobes
<i>H. c.</i> ‘Red Star’ × <i>H. m.</i> ‘Disco Belle’	Deltoid to cordate, one pair of lobes, deep indentation
<i>H. m.</i> ‘Disco Belle’ × <i>H. c.</i> ‘Red Star’	Deltoid to cordate, one pair of shallow lobes, shallow indentation
<i>H. c.</i> ‘Red Star’ × <i>H. m.</i> ‘Pale pink’	Palmetly-lobed, 3–5 lobes, deep indenta- tion
<i>H. m.</i> ‘Pale pink’ × <i>H. c.</i> ‘Red Star’	Palmetly-lobed, 3–5 lobes, deep indenta- tion
<i>H. laevis</i> × <i>H. m.</i> ‘Disco Belle’	Deltoid to cordate, sometimes with a pair of small lobes
<i>H. m.</i> ‘Disco Belle’ × <i>H. laevis</i>	Deltoid to cordate, sometimes with a pair of small lobes
<i>H. laevis</i> × <i>H. m.</i> ‘Pale pink’	Deltoid, usually with one pair of small lobes
<i>H. m.</i> ‘Pale pink’ × <i>H. laevis</i>	Deltoid, usually with one pair of small lobes

¹ *H. c.*: *H. coccineus* ² *H. m.*: *H. moscheutos*.

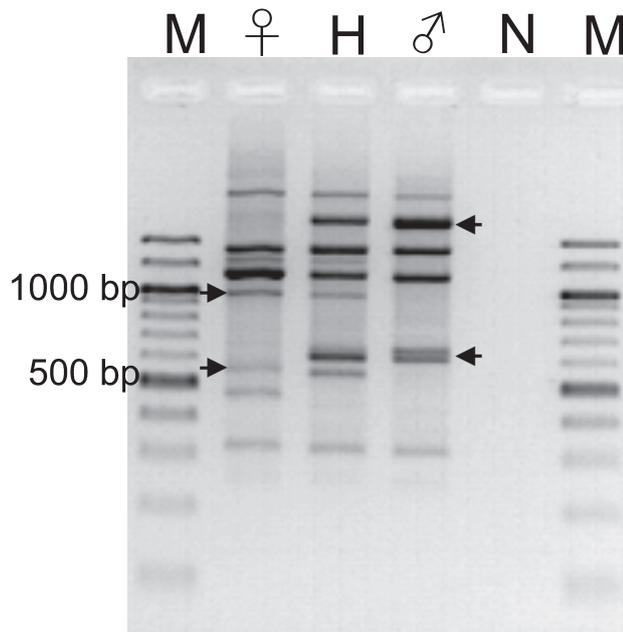


Fig. 3. RAPD binding pattern of hybrid plant (H) obtained from the cross between *H. moscheutos* ‘Pale Pink’ (♀) and *H. coccineus* ‘Red Star’ (♂) with the primer A07, M—100 bp ladder, N—negative control; arrows indicate female and male parent-specific markers.

situation was observed in interspecific crosses among *H. rosa-sinensis*, *H. syriacus* and *H. moscheutos* (Kuligowska *et al.* 2012), *Tulipa* species (Van Creij *et al.* 1997) and within the *Vigna* genus (Barone *et al.* 1992).

Low numbers of pollen tubes and lack of pollen tubes in the pistil corresponded to lower fruit set and number of

seeds in crosses between *H. coccineus* ‘Red Star’ and *H. laevis*, *H. coccineus* ‘Red Star’ × *H. moscheutos* ‘Pale Pink’ and *H. coccineus* ‘Red Star’ × *H. moscheutos* ‘Disco Belle’. In the crosses between *H. moscheutos* ‘Disco Belle’ and *H. coccineus* ‘Red Star’ the low fruit set is likely a result of impaired pollen tube growth (Table 1).

When *H. moscheutos* ssp. *palustris* ‘Pink Mallow’ was used as hybridization partners in reciprocal crosses with both *H. laevis* and *H. coccineus* ‘Red Star’ the obtained seedlings exhibited symptoms similar to HIs and as a result died shortly after germination. Stunted growth and chlorosis were also observed in plants resulting from reciprocal crosses of *H. coccineus* ‘Red Star’ and *H. moscheutos* ‘Disco Belle’ (Table 1). HIs occurring after interspecific hybridization are due to genomic conflicts i.e. the situation when different parts of the genome of the same organism have conflicting genetics. This is considered to be a byproduct of genetic divergence between hybridization partners (Johnson 2010). Similar HIs were reported in hybrids of *Capsicum* (Inai *et al.* 1993), *Oryza* (Chu and Oka 1972) and *Solanum* (Sawant 1956).

In crosses between *H. moscheutos* ‘Disco Belle’ and *H. laevis*, 31% of the progeny exhibited partial albinism (Fig. 3B). Albinism is a frequently occurring HI in hybrids and has been reported among others for *Hibiscus* (Van Laere *et al.* 2007), *Lonicera* (Miyashita and Hoshino 2010) and *Campanula* (Röper *et al.* 2015). In case of the crosses between *H. moscheutos* ‘Disco Belle’ and *H. laevis*, the albinism is likely a result of improper interactions between nuclear genes from the male parent and cytoplasmic elements from the maternal plants (Bombliès and Weigel 2007). Thus, the appearance of HIs was only present in this cross when *H. moscheutos* ‘Disco Belle’ was used as maternal plant.

Based on hybridization and phylogenetic studies, the species within section *Muenchusia* can be divided into two groups. It was stated that plants within the groups hybridize easily, while intergroup hybridization is usually hampered by hybridization barriers. According to the division *H. moscheutos* belongs to group I, while *H. coccineus* and *H. laevis* belong to group II (Small 2004, Wise and Menzel 1971). Our results can only partly confirm previous reports about reproductive isolation. A possible explanation of the differences in hybrid productivity in crosses among *H. moscheutos* cultivars and *H. coccineus* as well as *H. laevis* cultivars might be their specific genetic background for hybrid production.

The morphological observations of the obtained progeny revealed that the majority of plants had intermediate phenotypes between both hybridization partners. Only a small fraction of these plants had maternal-like phenotypes (Table 1) that can be due to unintentional self-pollination. The occurrence of intermediate phenotypes of the progeny is a typical feature of interspecific hybridization and was described for several plant genera such as *Dianthus* (Nimura *et al.* 2003), *Chrysanthemum* (Cheng *et al.* 2011), *Streptocarpus* (Afkhami-Sarvestani *et al.* 2012) and *Kalanchoë* (Kuligowska *et al.*

2015a). It can be explained by inheritance pattern based on polygenic control with additive effects (Schwarzbach *et al.* 2001). All plants obtained from crosses where *H. coccineus* was used as one of the parents had red flowers suggesting dominance of this trait. Several of the obtained interspecific hybrids exhibited favorable characteristics such as vigorous growth and branching that can be useful for development of new cultivars.

Future analysis of the obtained interspecific hybrids will require evaluation of their cold tolerance and environmental requirements for flowering such as thermal and photoperiodic conditions. The *Hibiscus* plants used in our experiments are North American species and their range of latitude is between 27 and 42°N that corresponds to hardiness zones from 10 to 5 (down to -26.1°C or -15°F) (Magarey *et al.* 2008, Warner and Erwin 2001). At the same time these species have high requirements in relation to light intensities (Warner and Erwin 2003). Therefore, the usefulness of species and hybrids in different locations such as Northern Europe can be limited. Screening of our hybrids for their environmental requirements is of great importance.

The interspecific hybrids produced during the study have provided insights into breeding of hardy *Hibiscus* species and presence of hybridization barriers. The main drawback in production of hybrid progeny was the presence of post-fertilization barriers associated with the occurrence of HIS that limit the usefulness of the obtained plants for further breeding purposes. A clear influence of the genetic background of the hybridization partners was recognized for the production of hybrids. Moreover, the cross direction was another factor that influenced the creation of progeny. Thus, for future breeding activities it is necessary to screen available cultivars of hardy *Hibiscus* species and perform reciprocal crosses.

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