Natural hybridization between *Gladiolus palustris* and *G. imbricatus* inferred from morphological, molecular and reproductive evidence

Přirozená hybridizace mezi *Gladiolus palustris* and *G. imbricatus*, zjištěná na základě morfologických, molekulárních a reprodukčních znaků

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While studying the extremely rare species, *Gladiolus palustris*, in Poland, putative hybrid plants were discovered. Natural hybridization between *G. palustris* and *G. imbricatus* was confirmed by chloroplast (*psb*A-*trn*H and *rpl32-trn*L) DNA and nuclear ribosomal DNA (ITS1) sequences, AFLP markers and macro-, micromorphological and reproductive characters. Based on molecular data, the hybridization events are likely to have occurred relatively recently with *G. palustris* as the maternal species and *G. imbricatus* as the pollen donor in interspecific crosses. The existence of a shared common cpDNA haplotype in all hybrids and *G. palustris* indicates unidirectional hybridization. A new nothospecies, *G.* ×*sulistrovicus*, is described. Analyses of AFLP data and polymorphisms of ITS1 sequences showed additive inheritance of parental genomic fragments in *G.* ×*sulistrovicus*. The hybrids exhibited either morphological similarity to *G. imbricatus* or intermediateness in phenotypic characters. The corm structure of flowering plants and seed capsules clearly distinguish the hybrid. The new taxon is characterized by a lower generative reproduction than the parental species, however hybrids produce ~50% viable pollen and seeds, which allows them to produce subsequent hybrid generations. The weak generative reproduction was enhanced by highly efficient vegetative propagation. The western part of the Balkan Peninsula and adjacent areas (Croatia, Bosnia and Hercegovina, Serbia, northern Italy) and central Europe (Poland, the Czech Republic, Slovakia, eastern Austria, Hungary) are the most likely areas where *G.* ×*sulistrovicus* will occur. Hybridity in the context of *G. palustris* conservation is discussed.

K e y w o r d s: AFLP, generative reproduction, *Gladiolus* ×*sulistrovicus*, interspecific hybridization, morphometrics, new nothospecies, nrDNA, plastid DNA, pollen viability, vegetative propagation

Introduction

Reticulate evolution by means of hybridization and introgression cycles have played an important role in the diversification and evolution of *Gladiolus* L. (Barnard 1972, Ohri & Khoshoo 1983a, b, van Raamsdonk & de Vries 1989, Goldblatt et al. 2001) and other genera of *Iridaceae* (e.g. Arnold et al. 1990, Lovo et al. 2012). Species of *Gladiolus* occur in Africa and Madagascar, in Europe and western Asia (Meusel et al. 1965, Hamilton 1980). The majority of the ~260 *Gladiolus* species originate from South Africa (Goldblatt 1996, Goldblatt & Manning 1999) and the Cape of Good Hope is considered to be the centre of diversity of this genus (Goldblatt & Manning 1999, Valente et al. 2011). The second centre of this species richness is in the Mediterranean region with only about seven species originating from this region (Valente et al. 2011).

The evolution of the European *Gladiolus* species has been largely affected by hybridization and polyploidy. The majority of the African species of *Gladiolus* are diploids (2n = 2x = 30) whereas the European species are polyploids (2n = 60–130), indicating the southern origin of the genus (Cantor & Tolety 2011, Valente et al. 2011). Identification of the European *Gladiolus* spp. is difficult because several morphological character ranges of the species overlap considerably (Hamilton 1980, van Raamsdonk & de Vries 1989) and natural interspecific hybridization occurs. Hybridization between closely related species, such as *G. imbricatus*, *G. italicus* and *G. illyricus* have occurred as a result of migrations due to climatic changes in glacial periods (van Raamsdonk & de Vries 1989). Moreover, *G. communis* subsp. *byzantinus* appears to hybridize with *G. illyricus* within its core range in southern Spain producing an evenly-graded range of morphological intermediates (Cantor & Tolety 2011). The lack of confidence in the identification of some *Gladiolus* plants in the field indicates that spontaneous hybridization between *G. communis* and *G. italicus* is highly likely to have occurred on Malta (Mifsud & Hamilton 2013).

It should be briefly mentioned that artificial hybridization has also been very important in producing *Gladiolus* cultivars. The present-day very decorative garden varieties are the result of ~180 years of breeding and selection in Europe and North America, far away from South Africa, the native range of the original species (Ohri & Khoshoo 1983b). Hybridization between wild species and cultivars of *Gladiolus* is an effective means of producing individuals (genotypes) with desirable features (e.g. flower form and shape, colour diversity or scent; Ohri & Khoshoo 1983b, Cantor & Tolety 2011). However, Eurasian species have not been used in developing modern cultivars of gladiolus (Ohri & Khoshoo 1983b) even though they can be valuable because of their relative hardiness and low sensitivity to fungal diseases (Rakosy-Tican et al. 2012).

Gladiolus palustris Gaudin and *G. imbricatus* L. are partially sympatric species (Fig. 1), indigenous to Europe and have the most northern and north-eastern distributions, respectively, recorded for *Gladiolus* species (Hamilton 1980). Natural, interspecific hybridization between these species is possible mainly due to their close genetic relatedness, their occurrence in the same habitats and overlapping flowering periods. Recently, the occurrence of putative hybrids, *G. palustris* × *G. imbricatus*, has been reported in Poland (central Europe) (Kamiński 2012, Cieślak et al. 2014).

Gladiolus palustris, the marsh gladiolus, and *G. imbricatus*, the sword lily, are rare and declining, perennial, polyploid (2n = 4x = 60) species (Skalińska et al. 1964, 1974, Schnittler & Günther 1999, Bilz et al. 2011, Cantor & Tolety 2011, Bilz 2013) with the

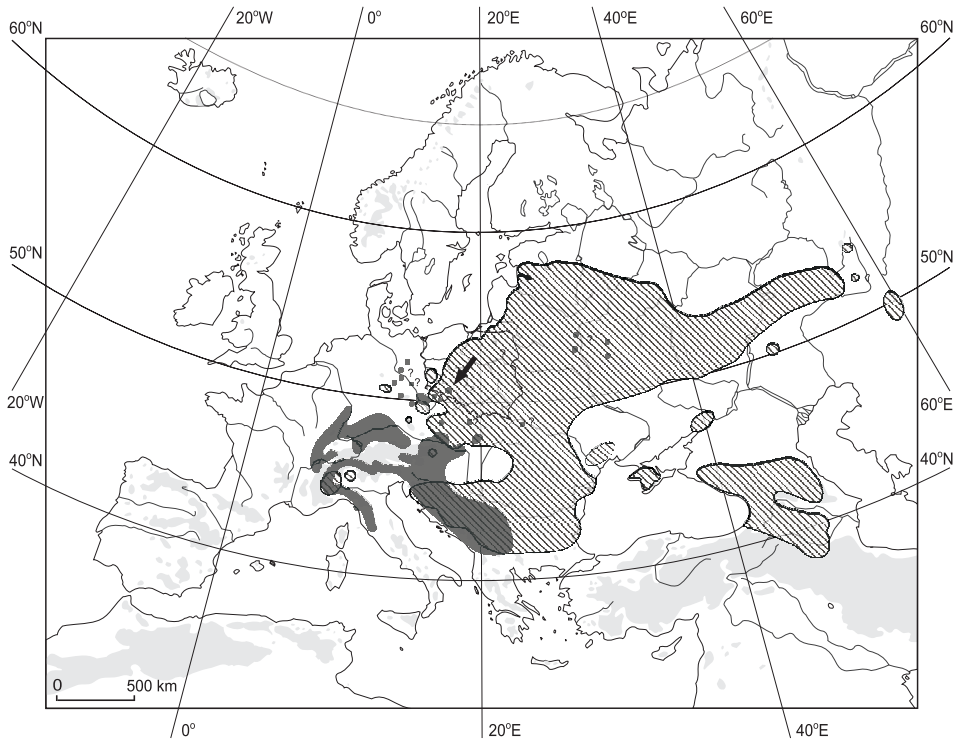

Fig. 1. – Map showing the distributions of ■ *Gladiolus palustris* and ▨ *G. imbricatus*, and the locus classicus of their hybrid *G.* ×*sulistrovicus* (arrow). The map is a compilation based on Meusel et al. (1965), which was revised and modified.

sub-Mediterranean–Pannonian–central European and sub-Mediterranean–central European–Pontic-Pannonian type of distribution, respectively (Meusel et al. 1965; Fig. 1), occurring mainly in wet Molinia meadows (Bilz 2013). Protogynous flowers are cross-pollinated and flowering period extends from June till August. Both species are cold-tolerant and their seeds and corms require a cooling period to germinate (Chrtek et al. 2007, Jõgar & Moora 2008).

Gladiolus palustris is considered to be one of the rarest elements in the Polish flora and at the greatest threat of extinction (Kamiński 2012, Towpasz et al. 2014). At present it occurs at one natural locality in the Łąka Sulistrowicka reserve on the Ślęża massif in Lower Silesia (south-western Poland), where it is continuously being monitored (Kamiński 2012). As part of the conservation effort an ex situ cultivar collection of this species is kept at the Botanical Garden of Wrocław University, Poland. Recent AFLP studies of the genetic diversity of *G. palustris* revealed the presence of putative hybrids in this ex situ culture (Cieślak et al. 2014). Hybrids between *G. palustris* and *G. imbricatus* have not been reported from outside of Poland to the best of our knowledge and these putative hybrid plants have never been formally described as a new hybrid taxon.

The aims of this study were: (i) to estimate the range in the variability of macro- and micromorphological characters and morphological distinctiveness of hybrids, (ii) to

confirm the occurrence of natural hybridization between *G. palustris* and *G. imbricatus* by the use additive profiles from both putative parents using AFLP markers and nrDNA (ITS) sequences, (iii) to infer the direction of the hybridization event based on cpDNA sequence data, (iv) to assess the level of hybrid stabilization and determine the ability of hybrids to survive as an independent, recognizable and self-reproducing unit, that is, as separate hybridogenous species.

Material and methods

Plant material

Plant samples used in the analyses of macro-, micromorphological and reproductive characters originated from different sources: (i) *Gladiolus palustris*, *G. imbricatus* and putative interspecific hybrids came from the ex situ culture at the Botanical Garden of Wrocław University. The ex situ culture of *G. palustris* was established from seeds harvested in 2004 and 2005 at the natural locality in the Łąka Sulistrowicka reserve. The hybrid individuals in ex situ culture were grown from seed of two plants morphologically identified as *G. palustris*. In addition, individuals of *G. palustris* that were introduced into ex situ culture from the Botanical Garden in Berlin Dahlem (Germany), *G. imbricatus* from the Botanical Garden in Utrecht (the Netherlands), in Munich (Germany) and from Moravia (Czech Republic) were also studied. Furthermore, (ii) individuals of *G. imbricatus* from three natural populations in southern Poland: Kraków – Łąki Królówki (N 50°04'26.2", E 19°50'17.4", 213 m a.s.l.), Pieniny National Park – Szczawnica, vicinity of Szafranówka, by the Slovakian border (N 49°24'57.8", E 20°27'52.3", 627 m a.s.l.) and Lower Silesia, Sudeten Foothills, Mt. Radunia, Łąka Sulistrowicka reserve (N 50°50'28.3", E 16°43'53.7", 310 m a.s.l.) were measured and estimated directly in the field without collecting individuals (macromorphological characters: PH, LN, LW, FN; Table 1).

The origin of the plant material used in the molecular study is listed in Electronic Appendix 1. We analysed the putative hybrid (24 individuals for AFLPs, 6 and 7 individuals for nrDNA and cpDNA sequence analyses, respectively), previously detected in the ex situ culture (Cieślak et al. 2014). In addition, putative parental species *G. palustris* (24 individuals for AFLPs, 10 and 18 individuals for the nrDNA and cpDNA analyses) and *G. imbricatus* (30 individuals for AFLPs, 7 and 17 individuals for the nrDNA and cpDNA analyses) from ex situ culture and from different natural localities were also included in the comparative analyses. Herbarium (KRA, KRAM) specimens of *G. palustris* and *G. imbricatus* were selected from the whole distribution ranges of these species. One herbarium specimen of the more distant relative, *G. illyricus*, was included in the cpDNA analyses for reference only. Vouchers were deposited in the herbaria of the W. Szafer Institute of Botany, Polish Academy of Sciences, Kraków (KRAM), Jagiellonian University, Kraków (KRA) and Museum of Natural History, University of Wrocław (WRSL). All sequences are stored in GenBank with accession numbers KM887188–KM887354 and KP027306–KP027328 (for details see Electronic Appendix 1).

Table 1. – Comparison of the characters of *Gladiolus palustris*, *G. imbricatus* and their hybrid *G. ×sulistrovicus*. Given are: N – number of plants (or pollen grains or seeds) used for measuring the morphological characters, mean ± standard deviation, minimum, maximum and one-way ANOVA results (F statistics and P values).

Code	Character	*G. palustris*	*G. ×sulistrovicus*	*G. imbricatus*	ANOVA	
	PH, LN, LW, FN	N = 103	N = 117	N = 225		
	PS	N = 100	N = 100	N = 100		
	PV	N = 2000	N = 2000	N = 2000		
	SL	N = 75	N = 114	N = 86		
	CN, SCN, ECN, SN	N = 26	N = 65	N = 28		
	COD, COT	N = 170	N = 301	N = 26	F	P
Quantitative macro- and micromorphological characters:						
PH	height of flowering plant [cm]	42.2±12.2 15.0–69.0	57.9±12.4 33.0–92.0	83.2±19.3 36.0–140.0	251.63	< 0.001
LN	leaf number	3.3±0.5 2.0–4.0	4.1±0.8 2.0–6.0	3.7±0.6 2.0–5.0	43.62	< 0.001
LW	leaf width [mm]	9.2±2.1 4.0–17.0	11.4±3.9 5.0–24.0	12.5±4.2 4,0–25,0	28.14	< 0.001
FN	flower number	5.4±1.7 2.0–9.0	8.8±2.6 3.0–16.0	9.4±2.7 3.0–17.0	93.10	< 0.001
PS	pollen size (diameter) [µm]	58.1±3.8 49.1–68.5	58.2±10.1 31.8–83.6	62.7±3.5 52.8–68.9	16.23	< 0.001
PV	pollen viability [%]	95.8±3.1 93.1–98.7	19.5±16.7 7.2–48.9	90.9±14.3 69.4–98.1	47.89	< 0.001
SL	seed length [mm]	4.8±0.7 3.5–6.4	4.3±0.7 2.1–5.4	4.3±0.5 3.0–6.0	17.27	< 0.001
CN	capsule number per individual (with and without seeds)	4.6±1.3 (2.0–7.0)	7.4±3.5 1.0–16.0	7.4±2.4 2.0–13.0	9.73	< 0.001
SCN	capsule number with seeds per individual	4.6±1.3 2.0–7.0	5.8±3.2 0.0–13.0	7.4±2.4 2.0–13.0	7.42	< 0.001
ECN	capsule number without seeds per individual	0.0	1.7±1.8 0.0–6.0	0.0	23.39	< 0.001
SN	seed number per individual	179.5±58.7 82.0–300.0	32.6±31.0 0.0–147.0	204.6±88.7 71.0–408.0	136.81	< 0.001
SN/SCN	seed number per capsule	39.6±9.5 22.5–55.0	5.3±3.7 0.0–21.0	27.4±5.6 11.8–38.5	366.15	< 0.001
SCN/FN	number of capsules with seeds over number of flowers ×100 [%]	77.9±18.0 33.3–100.0	63.8±26.1 0.0–100.0	70.8±15.2 20.0–91.7	3.83	< 0.050
SN/FN	number of seeds divided by number of flowers	29.8±6.7 13.7–42.2	3.6±2.9 0.0–16.3	19.4±6.2 7.1–30.5	337.70	< 0.001
COD	corm diameter of flowering plant [mm]	15.2±3.1 10.0–24.0	17.2±5.3 12.0–40.0	11.3±2.6 8.0–18.0	26.63	< 0.001

Code	Character	*G. palustris*	*G. ×sulistrovicus*	*G. imbricatus*
VP	vegetative propagation by cormlets	production of one or rarely two daughter corms and one or two short-lived cormlets per maternal corm per vegetative season; five- or six-year old population died out without generative reproduction	production of one or two daughter corms and one or two very viable cormlets per maternal corm per vegetative season; total number of cormlets formed by 36 two- and three-years old maternal corms in 2007–2014 was 155 (a greater than four-fold increase)	production of one or rarely two daughter corms and one or two short-lived cormlets per maternal corm per vegetative season
Qualitative macro- and micromorphological characters:				
FC	flower colour	rosy violet or magenta	rosy violet or magenta to red and reddish-purple	deep purple to carmine
EM	exine microstructure of pollen grains	verrucate ornamentation	more dense verrucate ornamentation than in *G. palustris* and *G. imbricatus*	verrucate ornamentation
SM	stigma shape and microstructure	three-partite, papillae on the margins – branches and papillae longer than in *G. imbricatus*	three-partite, papillae on the margins – branches and papillae intermediate, more similar to *G. palustris*	three-partite, papillae on the margins – branches and papillae shorter than in *G. palustris*
SCM	seed coat microstructure	papillose with collapsed cells in the wing area	papillose with collapsed cells in the wing area	papillose with collapsed cells in the wing area
COT	corm tunic of flowering plant	formed from remnants of old, dry leaf sheaths with a thick, distinct venation forming a reticulum with an irregular mesh mainly in the upper part and on the sides of corm	formed from remnants of old, dry leaf sheaths with slightly marked, fine fibres reticulated in the upper part and more or less parallel on the sides of corm	formed from remnants of old, dry leaf sheaths with a delicate, parallel venation on the whole surface of corm

Morphological analysis with emphasis on reproductive success

A set of 21 quantitative and qualitative characters of flowering and fruiting plants were analysed (Table 1), including reproductive characters and microstructural characters of pollen grains, stigma of the pistil and seed coat.

The relationships between the parental species and the hybrids were examined using univariate statistics of morphological characters: minimum, maximum, arithmetic mean and standard deviation. One-way analysis of variance (one-way ANOVA) was used to assess the significance of differences among these three taxa. Values of F statistic were used to identify characters that contribute most to the resulting patterns. The significance of differences between the means of the characters were tested using Tukey's HSD post hoc test for unequal sample sizes ($P < 0.001$; Sokal & Rolf 1981). In order to show morphological relationships among individuals of the parental species and the hybrids, a scatter diagrams of the most discriminating characters were plotted and box plots of diagnostic characters presented. Numeric analyses of morphological characters were carried out using STATISTICA ver. 5.1 G software (StatSoft Inc.).

Pollen size and viability test

Pollen was isolated from flowers previously fixed in a mixture of 96% ethanol and glacial acetic acid (v/v 3:1) and stained with Alexander dyes (Singh 2003). Viable pollen grains stained purple, nonviable pollen grains were green. Viability was determined for ~2000 pollen grains (one flower was taken from each of the five plants of each taxon, hybrid and parents). The diameter of 100 viable pollen grains of the hybrid and each of the parental species were measured. Measurements were performed along the equatorial axis including the exine, using an Eclipse E400 optical microscope (Nikon) equipped with NIS Elements ver. 4.0 program.

Pollen, stigma and seed microstructure

Flowers of five plants of each parental species and 10 hybrid individuals were fixed in a mixture of 96% ethanol and glacial acetic acid (v/v 3:1), then washed in cacodylate buffer and placed in 50% ethyl alcohol. The samples were dehydrated in solutions of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 95%, 100%). Pollen grains isolated from anthers and stigmas cut off from the pistil were dried in Anderson's apparatus at the critical point of carbon dioxide. Pollen shed from fresh flowers, dry seeds and dehydrated samples were glued onto holders and then gold-coated using a Jeol JFC-1100E ion sputter. The samples were analysed in a scanning electron microscope Jeol JSM-5410.

Vegetative reproduction of hybrids

In order to assess the effectiveness of vegetative reproduction, seeds of the putative hybrids were planted in autumn 2004 and 2005 in the ex situ culture in the Botanical Garden, Wrocław University. In 2007, 18 two-year old maternal corms and 18 three-year old maternal corms that developed from the seeds were replanted into two separate baskets. In the following years, inflorescences of the hybrids were cut before seed dispersal to

avoid propagation by seeds. The number of cormlets developed by maternal corms and the number of initial maternal corms that had died off were counted in 2014.

AFLP data generation and analyses

Total genomic DNA was isolated from ~15 mg of silica gel-dried leaf tissue using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol (Qiagen, Valencia, CA, USA). DNA quality and concentration were estimated against λ-DNA on 1% agarose gel stained with ethidium bromide. The extracted genomic DNA of each *Gladiolus* individual was amplified using primers and protocols specific for the respective markers.

The binary AFLP data matrix (coded as 0/1) obtained in our previous, initial examinations (Cieślak et al. 2014) were again analysed in more detail in the present study to verify additive genetic profiles from both putative parents. Individual AFLP stages are described by Cieślak et al. (2014). An analysis of 78 individuals of parental species and their putative hybrids with three selective primer pair combinations: *Eco*ACA-*Mse*CAC, *Eco*AGG-*Mse*CTG and *Eco*ACT-*Mse*CAG were used in the main part of the analysis.

To estimate the molecular distinctiveness of *G. palustris*, *G. imbricatus* and their hybrid, the number of species-diagnostic AFLP fragments, including private (i.e. the number of fragments present in all the individuals analysed of a respective species and absent elsewhere) and characteristic (i.e. the number of fragments present in some individuals analysed of a respective species and absent elsewhere) fragments were sought. Species-diagnostic AFLP fragments of each parental species shared with the hybrids were identified to confirm additive profiles of the hybrid and to determine the level of pairwise genetic affinity between the species. To represent the overall genetic relationships among parental species and hybrids, a Neighbor Net was constructed based on a matrix of Nei-Li coefficients (Nei & Li 1979) using SplitsTree version 4.6 (Huson & Bryant 2006).

cpDNA and nrDNA data generation and analyses

Five regions of the chloroplast genome (cpDNA) and the internal transcribed spacer region (ITS) of the nuclear ribosomal DNA (nrDNA) were analysed to test the reliability of the hypothesis that *G. palustris* and *G. imbricatus* had hybridized (Electronic Appendix 1). The following cpDNA regions were amplified and sequenced: *trn*L$^{(UAA)}$-*trn*F$^{(GAA)}$ (Taberlet et al. 1991), *psb*A-*trn*H (Hamilton 1999), *trn*S$^{(GCU)}$-*trn*G$^{(UCC)}$, *rpl32*-*trn*L$^{(UAG)}$ and *trn*Q$^{(UUG)}$-*rps16* (Shaw et al. 2007). The results of the analyses of the *psb*A-*trn*H and *trn*L-*trn*F regions are presented in detail because they were the most informative and polymorphic. The nrDNA ITS region (including ITS1, 18S and 5.8S genes) was amplified using universal primers ITS1 and ITS2 (White et al. 1990).

In the first stage of the analyses, PCRs were performed for all samples. The PCR mixture, in a total volume of 24.5 μL, contained: 1.25 U AmpliTaq Gold 360 polymerase (Applied Biosystems), 1× PCR Gold Buffer supplied with the enzyme (Applied Biosystems), 2.5 mM $MgCl_2$, 0.1 μM of each primer, 2.5 mM of each dNTP (Applied Biosystems), 10 × 0.2 μL BSA (1 mg/mL, New England Biolabs) and 1 μL of DNA template. For cpDNA, PCR was performed with the following cycling conditions: initial denaturation of 10 min at 95 °C; 30 cycles of 1 min at 95 °C and 1 min at 50 °C; ramp of

0.2 °C/s to 65 °C; primer extension of 4 min at 65 °C; final extension step of 5 min at 65 °C; cooling to 4 °C (Shaw et al. 2005). For ITS, the following PCR cycling profile was used: 10 min at 95 °C; 10 cycles of 30 s at 94 °C, 30 s at 60 °C (with a decrease of 1 °C per cycle) and 1 min at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C; final extension step of 7 min at 72 °C; cooling to 4 °C.

In the second stage of the analyses, if first PCR reactions did not give a positive result for either primer, the following procedure was used: the PCR mixture, in a total volume of 19 μL, contained: 1 μL AccuTaq™ LA DNA Polymerase (Sigma – Aldrich), 1× PCR (2 μL) AccuTaq™ Buffer supplied with the enzyme (Sigma – Aldrich), 0.8 μL of each primer, 1 μL dNTP (Sigma – Aldrich) and 1 μL of DNA template. PCR was performed with the following cycling conditions: initial denaturation 30 s at 96 °C; 35 cycles of 1 min at 94 °C, 45 s at 48 °C and 10 min at 68 °C, final extension step of 30 min at 68 °C; cooling to 4 °C. The PCR product was diluted 10× and was used in the sequencing reactions that were performed using the BigDye Terminator ver. 3.1 Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. Cycle sequencing products were purified using EDTA/ethanol precipitation, re-suspended in 12 μL formamide and separated on an ABI 3130 Genetic Analyser equipped with 36 cm capillaries and a POP-7 polymer (Applied Biosystems). In all samples, both strands were sequenced using the same primers as for the PCR.

The cpDNA and nrDNA sequences obtained were reviewed and verified based on sequences from forward and reverse sequencing directions using FinchTV ver. 1.4.0 (Geospiza Inc., Seattle, WA) and the consensus sequences were aligned manually using BioEdit 7.0.9.0 software (Hall 1999). Additive nucleotide polymorphisms of ITS sequences were analysed and coded using IUPAC nucleotide ambiguity codes.

Results

Phenotypic distinctiveness of parental species and intermediate nature of hybrids

Gladiolus palustris and *G. imbricatus* strongly differed in flowering plant height, the number of flowers and in the number and width of leaves (Table 1, Fig. 2A–C). High variability in the height of *G. imbricatus* was related to the fact that some specimens used in morphometric analysis originated from natural localities where interspecific competition leads to greater variation in plant growth. *Gladiolus palustris* was clearly distinct from *G. imbricatus* in that the corms of flowering plants are ovate and covered by a tunic of old leaf sheaths with a thick, distinct venation forming a reticulum with an irregular mesh. *Gladiolus imbricatus* corms are covered by a tunic of leaf sheaths with a delicate, parallel venation (Fig. 3A1–A2, C1–C2). In both parental species, corms cultivated from seeds were the largest in size at the time of first flowering and some of them also produced daughter corms and cormlets. The majority of the maternal and daughter corms died after the second flowering.

Interspecific hybrids were either morphological similar to *G. imbricatus* or intermediate in terms of the characters analysed. Hybrid individuals were indistinguishable from *G. imbricatus* in terms of flower number and leaf width (Table 1, Fig. 2C). Only flowering plant height of the hybrids was intermediate between those of *G. palustris* and *G. imbricatus* (Table 1, Fig. 2A). The hybrids had more leaves (Fig. 2B) and larger corms than the parental species (Table 1, Fig. 2H). Corms of hybrids were surrounded by a tunic

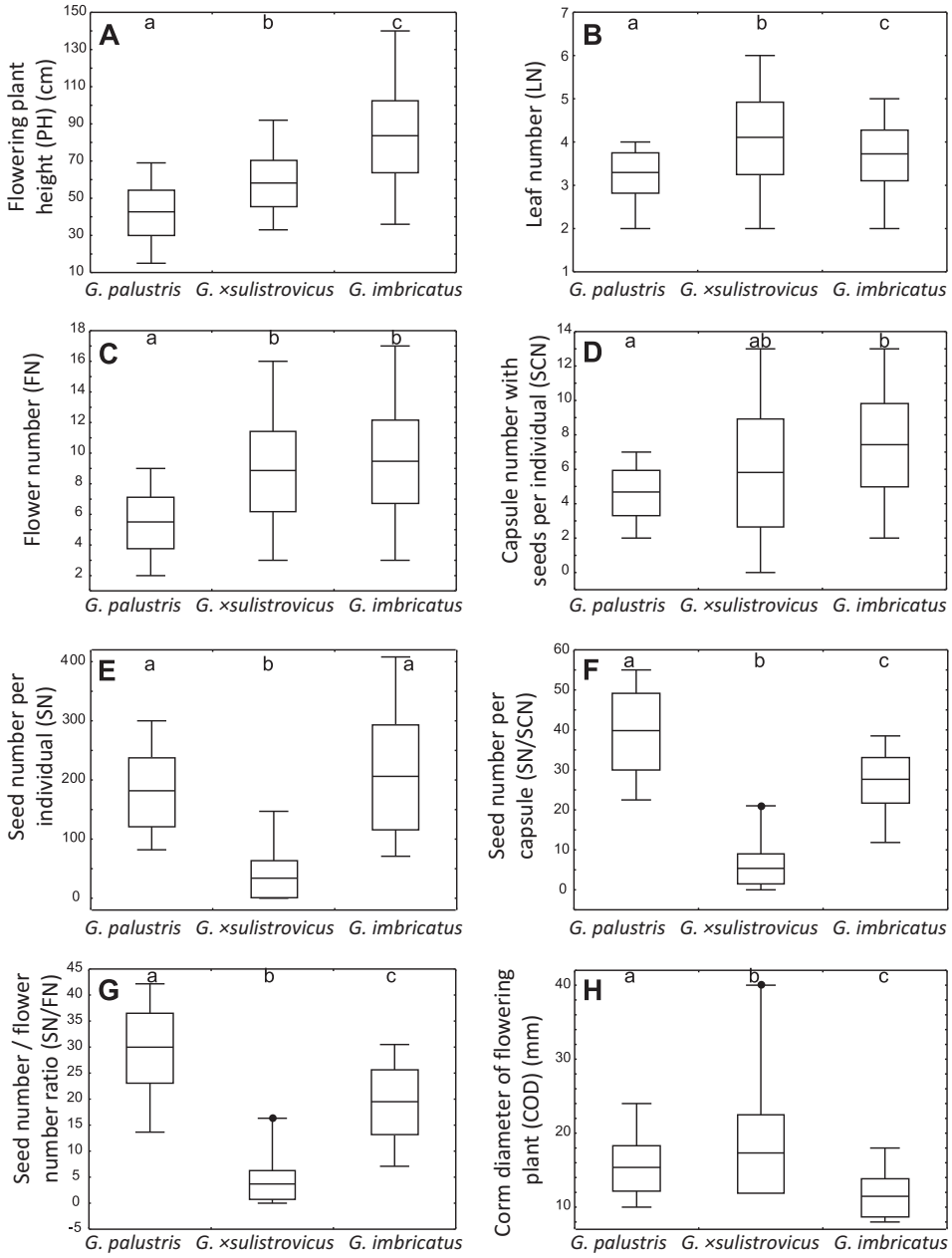

Fig. 2. – Variation in certain morphological characters of *Gladiolus palustris*, *G. imbricatus* and their hybrid *G.* ×*sulistrovicus*. Given are: mean (line), standard deviation (box), maximum and minimum (whisker) and outliers (filled circles); different letters indicate significant differences at $P < 0.05$.

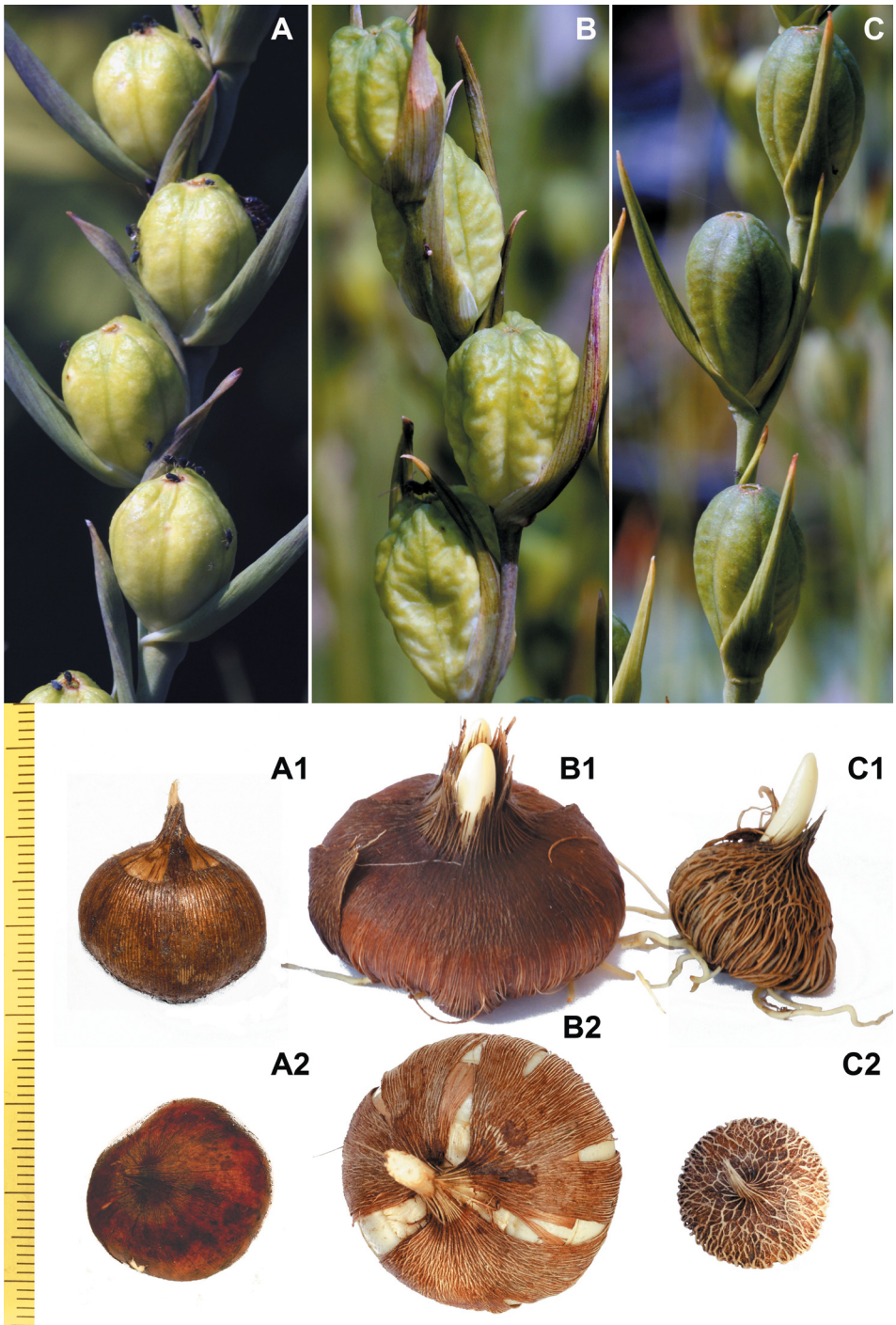

Fig. 3. – Maturing capsules and corms of flowering plants of *Gladiolus imbricatus* (A–A2), *G. palustris* (C–C2) and their hybrid *G.* ×*sulistrovicus* (B–B2), respectively (Photographer R. Kamiński).

with a poorly outlined reticulum of nerves in their upper part and clearly differed in this respect from those of the parental species (Table 1, Fig. 3B1–B2).

Comparison of reproduction in the hybrids with that in the parental species: pollen viability, size and exine microstructure

Pollen of both putative parental species was highly stainable, i.e. with a percentage viability of over 90%. In contrast, pollen viability of interspecific hybrids was very low and on average 19.5% (Table 1).

Pollen grains of *G. palustris* and *G. imbricatus* were uniform in size with no sharp intraspecific differences. *Gladiolus imbricatus* pollen was larger (mean 62.71 μm) than that of *G. palustris* (mean 58.07 μm). Viable pollen grains of hybrids were similar in size (mean 58.22 μm) to those of *G. palustris* (Table 1). The hybrids produced pollen grains differing in size from small (31.79 μm) to large (83.58 μm); some were shrunken, very small and non-viable. The SEM revealed that the dry pollen of all the taxa analysed was boat-shaped with one sunken aperture, whereas hydrated pollen was broadly oval or circular with verrucate exine ornamentation. The ornamentation was less dense in both parental species than in the hybrid (Table 1, Electronic Appendix 2).

Stigma characters

The style at the apex is divided into three filiform spoon-shaped branches, forming a three-partite stigma, papillate on the margins. The size of branches and papillae differed between the parental species being longer in *G. palustris* and intermediate in the hybrid but more similar to *G. palustris* than *G. imbricatus* (Table 1, Electronic Appendix 3).

Seed size, coat microstructure and seed production

Seeds of the parental species were brown, ovoid and winged with a noticeable pellet-like structure. *Gladiolus palustris* had longer seeds (4.79 mm) than *G. imbricatus* (4.27 mm; Table 1). The hybrids produced seed of two sizes, well developed seeds similar in length (4.29 mm) to those of *G. imbricatus* and small poorly developed seed. The frequency of large seeds varied per plant and capsule. There were no evident differences in the pattern of the microstructure of the seed coat between the parental taxa and the hybrid. The upper seed surface was papillose, with collapsed cells in the wing area.

The number of seeds was recorded only for hybrid plants growing far from the parental species, which eliminated the possibility of measuring the seed of backcrosses. Thus, all the seeds were from inter F_1 hybrid crosses. Seed production by hybrids was very low (Table 1, Fig. 2E–F). Some hybrid plants did not form capsules or formed empty capsules, unlike the parental species the capsules of which were full of seed (Figs 2D, F). The capsules of the putative hybrids were more or less wrinkled and shrivelled, which clearly distinguished them from those of their parents (Fig. 3A–C). Reproduction success of the hybrids, defined as the percentage of flowers that produced capsules with seeds (SCN/FN; Table 1) varied greatly (0 to 100%) and differed significantly from that recorded for the parental species. Another reproduction character, defined as the number of seeds produced by a single flower (SN/FN), was significantly lower for the hybrid plants and very different from that recorded for the parents (Figs 2G, 4).

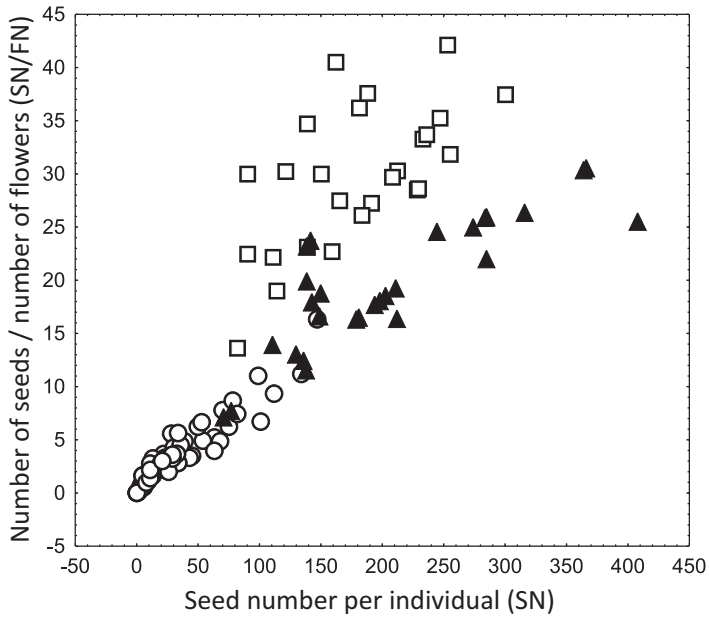

Fig. 4. – Scatter plot of the values of two characters connected with seed production (SN and SN/FN) by □ *Gladiolus palustris*, ▲ *G. imbricatus* and their hybrid ○ *G.* ×*sulistrovicus*. See Table 1 for character codes.

Vegetative reproduction

The efficiency of vegetative propagation of the putative hybrids was determined in an experiment carried out in the ex situ culture. After seven years 18 two-year old and 18 three-year old maternal corms of hybrids produced a total of 155 cormlets, which is an increase of 430%. One to twelve cormlets developed from each maternal corm. Three two-year old (17%) and two three-year old (11%) maternal corms died during the experiment. Three-year old corms produced more cormlets (90) after replanting than two-year old corms (65 cormlets). This could be related to the greater maturity of three-year old maternal corms as planting them well separated favourably influenced their growth and increased vegetative reproduction. Production of cormlets by hybrids increased with maturity (4–5 years after planting). In contrast, fewer, smaller and shorter-lived cormlets, were recorded for *G. palustris* than the hybrids (Table 1).

In the flowering period the maternal corm of hybrids may also divide horizontally. A young daughter corm formed at the top from the material in the old maternal corm, which flowered as early as the following year. In other cases, rarely seen in the parental species, new corms of hybrids, formed on the upper part and still connected to the maternal corm, can flower in the first or the second year. The maternal corm then divided vertically, initially into two and those into more daughter corms in the following years. Sometimes, a flowering daughter plant originated from the maternal corm and its own corm formed after flowering.

Genetic evidence for hybridization between Gladiolus palustris and G. imbricatus: AFLP variation

Altogether 239 AFLP fragments were obtained using three pairs of primers in our previous study (Cieślak et al. 2014). One-hundred-and-twenty-nine AFLP fragments were detected for *G. palustris* of which 61 (25.5%) were species-diagnostic (including 46 private and 15 characteristic fragments). Of 174 AFLP fragments generated for *G. imbricatus*, 77 (32.2%) were species-diagnostic (including 30 private and 77 characteristic fragments). We identified three characteristic AFLP fragments (1.3%) that occurred in the hybrid plants but not in either of the parental species. Of the total number of 198 AFLP fragments recorded for the putative F_1 hybrids, 67 (28.5%) fragments were common to both parental species. Fifty-four (27.3%) AFLP fragments were common to hybrids and *G. palustris* and 74 (37.4%) to hybrids and *G. imbricatus*. Based on the AFLP analyses, the contribution of both genomes of the putative parental species to the hybrid genome was determined. The greater total number of AFLP fragments in the hybrids in comparison with their progenitors indicates additive inheritance.

The mean coefficient of genetic similarity between the hybrids and *G. palustris* was slightly higher than their similarity to *G. imbricatus*, 0.81 and 0.77, respectively (Cieślak et al. 2014). In addition, the greater genetic similarity to *G. palustris* may be attributed to the fact that the hybrids inherited 88.5% (54 out of 61) of the species-diagnostic AFLP fragments of *G. palustris* and only 69.2% (74 out of 107) of those of *G. imbricatus*.

The Neighbor Net analysis of AFLP data revealed three clearly distinct groups corresponding to *G. palustris*, *G. imbricatus* and putative hybrids, separated by a well-supported split (bootstrap: 100%; Fig. 5). *Gladiolus imbricatus* showed greater AFLP variation than *G. palustris*. All hybrid individuals were grouped together and were more closely related to *G. palustris*.

nrDNA variation

The sequences of nrDNA were 325 bp long in *G. palustris* and 332 bp long in *G. imbricatus* (Electronic Appendix 1), and included the end of gene 18S, the entire ITS1 region and the beginning of gene 5.8S. On the other hand, the 147 bp long region that included the end of the 18S gene and the entire ITS1 was successfully amplified for putative hybrids. The analysis of the aligned sequences revealed eight polymorphic sites in the ITS1 region that distinguished *G. palustris* and *G. imbricatus*. The ITS1 sequences of four hybrid individuals showed clear additive polymorphisms for seven of the eight sites, whereas Gim-Gpa (WR0105) and Gim-Gpa (WR0601) individuals displayed an additivity at three and six sites, respectively (Table 2). Examination of the pattern in the polymorphism at these sites indicated *G. palustris* and *G. imbricatus* were the parental species of *G.* ×*sulistrovicus* (Table 2).

cpDNA variation

The *psb*A-*trn*H region was the most informative and polymorphic of the cpDNA regions analysed (Table 3). In addition, there were nucleotide polymorphisms in the *trn*L-*trn*F region. The remaining cpDNA regions analysed (*rpl32*-*trn*L, *trn*Q-*rps16* and *trn*S-*trn*G)

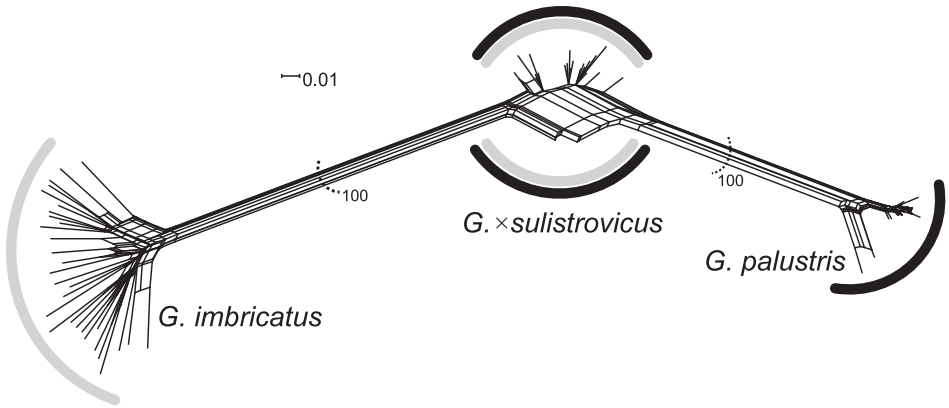

Fig. 5. – Neighbor Net for AFLP data using Nei-Li distances for 78 individuals of *Gladiolus palustris*, *G. imbricatus* and their hybrid *G.* ×*sulistrovicus*. Numbers along the branches are bootstrap values derived from an analysis of 2,000 replicates.

Table 2. – Variable sites of the ITS1 region of *Gladiolus palustris* (10 individuals), *G. imbricatus* (7 individuals) and their hybrid *G.* ×*sulistrovicus* (6 individuals). IUPAC ambiguity symbols are used to show polymorphisms. GenBank accession numbers are given in Electronic Appendix 1.

Taxon/nucleotide position in the consensus sequence	55	75	88	93	109	118	126–129	135
G. palustris	A	T	C	C	C	T	– – – –	C
G. imbricatus	C	C	Y	T	T	C	CTCT	A
G. ×*sulistrovicus*								
Gim-Gpa (WR0101)	M	Y	Y	Y	Y	Y	CTCT	M
Gim-Gpa (WR0105)	C	C	C	Y	Y	Y	CTCT	A
Gim-Gpa (WR0107)	M	Y	Y	Y	Y	Y	CTCT	M
Gim-Gpa (WR0115)	M	Y	Y	Y	Y	Y	CTCT	M
Gim-Gpa (WR0505)	M	Y	Y	Y	Y	Y	CTCT	M
Gim-Gpa (WR0601)	M	Y	C	Y	Y	Y	CTCT	M

were either incomplete or did not add more informative and polymorphic sites (Electronic Appendix 1).

A total of 18 polymorphisms were detected in the sequences of the *psb*A-*trn*H and *trn*L-*trn*F regions of the *Gladiolus* individuals, distinguishing five different haplotypes (Table 3). Two main cpDNA haplotypes H1 and H2 occurred in almost all the individuals of *G. palustris* and *G. imbricatus*, respectively. Haplotype H3 was found only in one individual of *G. palustris* from Poland and H4 in one individual of *G. palustris* from Austria. Haplotype H5 was found in one individual of *G. imbricatus* from Romania. All hybrid individuals shared haplotype H1 in common with *G. palustris*. The greater genetic affinities in terms of cpDNA markers of hybrids with *G. palustris* indicates that *G. palustris* was the maternal species in interspecific crosses.

Table 3. – Polymorphism of the plastid *psb*A-*trn*H and *trn*L-*trn*F regions determining five haplotypes (H1–H5) in *Gladiolus palustris* (18 individuals), *G. imbricatus* (17 individuals) and their hybrid *G.* ×*sulistrovicus* (7 individuals). All the samples (except those listed in the table below) of individual taxa had identical sequences of cpDNA regions. Individuals examined within each taxon and their GenBank accession numbers are listed in Electronic Appendix 1; ? – sequences of specific cpDNA regions were not obtained for the herbarium specimens below (see Results for details).

Chloroplast region	*psb*A-*trn*H											*trn*L-*trn*F							
Taxon / nucleotide position in the consensus sequence	62	66–69	72–73	78	79	84–85	95	418	423–430	431	433	51	87	88	93	565	682	698	Haplotype
G. palustris	T	– – – –	GT	C	T	CA	G	T	TTTTTTTT	A	A	A	A	A	C	A	–	G	H1
Gpa (KRAM355575)	C	– – – –	GT	C	T	CA	G	T	TTTTTT – –	C	T	?							H3
Gpa (KRA272251)	C	ATAT	TG	A	G	AC	A	T	TTTTTTTT	A	A	?							H4
G. imbricatus	C	ATAT	TG	A	G	AC	A	A	TTTTTTTT	A	A	G	–	G	T	G	A	C	H2
Gim (KRAM217157)	C	ATAT	TG	A	G	AC	A	A	TTTTTTTT	A	A	G	–	G	C	G	A	C	H5
G. ×*sulistrovicus*	T	– – – –	GT	C	T	CA	G	T	TTTTTTTT	A	A	A	A	A	C	A	–	G	H1

The plastid *psb*A-*trn*H primers amplified partial sequences of the photosystem II protein D1 (psbA) gene and the *psb*A-*trn*H intergenic spacer of 539 nucleotides in length in all *G. palustris* and the putative hybrid, and a region of 543 nucleotides in all *G. imbricatus* samples (Electronic Appendix 1). Sequence variation between the taxa was detected at 11 variable sites of the consensus assemblage of the *psb*A-*trn*H intergenic spacer region. *Gladiolus imbricatus* samples have *psb*A-*trn*H sequences with an indel, which were not found in the *G. palustris* samples and these species also differed in seven substitutions and two inversions (Table 3). Intraspecific variation in the *psb*A-*trn*H region was found in *G. palustris*: the individual from Poland (KRAM 355575) had the poly-T chain shorter by two nucleotides than in other *G. palustris* individuals and had two additional substitutions (431 and 433 sites), while the individual from Austria (KRA 272251) exhibited the same sequence as in *G. imbricatus* except for consensus site 418, which contained T and not A as in other *G. imbricatus*. Sequences of the *psb*A-*trn*H region for all the hybrids were identical to those in *G. palustris*.

The partial sequences of the *tRNA-Leu* (*trn*L) gene and complete sequences of the *trn*L-*trn*F intergenic spacer were amplified for *Gladiolus* individuals (Electronic Appendix 1). The length of the *trn*L-*trn*F regions (765 bp) were the same for *G. imbricatus*, *G. palustris* and the hybrids. Among these taxa, seven variable sites were detected: three nucleotide substitutions (51, 88 and 93 sites) and one deletion (87 site) in *G. imbricatus* sequences within the coding region of the *trn*L gene and two additional substitutions (565, 698 sites) and one deletion (682 site) in non-coding regions of the *trn*L-*trn*F intergenic spacer (Table 3). There was no intraspecific variation in the *trn*L-*trn*F region except for one substitution (93 site) in one individual from Romania (KRAM 217157), morphologically identified as *G. imbricatus*. The sequences of the hybrid individuals were identical with those of *G. palustris*.

Discussion

Genetic and morphological distinctiveness of Gladiolus ×sulistrovicus

This paper reports a comprehensively documented account of the spontaneous hybridization between *G. palustris* and *G. imbricatus*, which was first mentioned by Cieślak et al. (2014). Consequently, a new nothospecies, *Gladiolus ×sulistrovicus* is described. Our research indicates that *G. ×sulistrovicus* individuals in terms of several characters are more morphologically similar to *G. imbricatus* (Fig. 2) and based on these characters were erroneously recognized as this species (Cieślak et al. 2014). Analysis of cpDNA indicate that hybridization is likely to have occurred relatively recently with *G. palustris* as the maternal species and *G. imbricatus* as the pollen donor in interspecific crosses. The existence of a shared common cpDNA haplotype in all hybrids and *G. palustris* indicate unidirectional hybridization. The fact that the extremely rare species *G. palustris* is the maternal parent of the hybrid may be a consequence of higher pollen availability of the more frequent *G. imbricatus*. The reciprocal hybridization with *G. imbricatus* as the maternal species and *G. palustris* as the paternal species was not explicitly confirmed by our analyses. A few hybrid plants were found in 2014 at the Łąka Sulistrowicka reserve, which indicates the ability of the hybrid to survive at this natural locality.

The contribution of both genomes of the putative parental species to the newly described taxon was clearly indicated by AFLP markers and ITS1 sequences. Hybrids were grouped together and their closer relatedness to *G. palustris* confirmed by cpDNA markers. The finding of only three characteristic AFLP fragments in the hybrids and the nonconcerted evolution of nrDNA polymorphisms towards either of the parental rybotypes indicates that the hybridization events are likely to have occurred relatively recently.

Intraspecific variation in the nucleotides in the cpDNA regions in *G. palustris* and *G. imbricatus* was very limited. Two main haplotypes H1 and H2 were characteristic of these species and detected in the majority of individuals of both *G. palustris* and *G. imbricatus*. Another sample of *G. palustris* (KRA 272251), however, was almost identical with *G. imbricatus* and distinguished by one unique substitution. The inconclusive behaviour of some chloroplast haplotypes among *Gladiolus* species and their lack of species-specificity are reported, especially in allopolyploids and may be an indication of incomplete lineage sorting (Comes & Abbott 2001).

Corm characters appear to be very useful for identifying European species of *Gladiolus* (van Raamsdonk & de Vries 1989). The corms of flowering plants of *G.* ×*sulistrovicus* are larger, more viable and longer lived, up to 10 years, than those of the parental species. The corm structure of flowering plants of the hybrids is distinctive. They are covered by a tunic of remnants of leaf sheaths, with a poorly defined venation in their upper part, which is intermediate in character to that of the corms of the parents. It is noteworthy, however, that determining a species based on the appearance of young corms can be inaccurate. We noted during observations of the ex situ culture that young, one-year old corms selected as those of *G. palustris* based on morphological characters turned out to be hybrids with intermediate corms when 5–6 years old, which was also confirmed in our present and previous genetic analyses (Cieślak et al. 2014). The wrinkled, shrunken and sometimes not fully developed seed capsule also clearly distinguished the hybrids.

Stabilization of the hybrid species Gladiolus ×*sulistrovicus*

In angiosperm evolution hybridization has an important role in the formation of new genotypes and even of novel species by homoploid hybrid speciation or by allopolyploidization. In homoploid hybrid speciation the resulting hybrids are interfertile and partially sterile with both parents (Arnold 1997, Rieseberg 1997, Buerkle et al. 2000, Mallet 2007, Wissemann 2007, Soltis & Soltis 2009). The arising hybrids can be less fertile or less viable than parental species, but their fertility strongly depends on the genetic relatedness of the parental species. The more closely the species are related, the more fertile the hybrids (Yakimowski & Rieseberg 2014 and references cited herein). Fertility is one of the factors stabilizing hybrid-derived individuals and in effect gives rise to new lineages of species that become an independent, morphologically and genetically recognizable and self-reproducing unit (Rieseberg 1997, Mallet 2007, Soltis & Soltis 2009). Gene flow by pollen and seed dispersal allows new populations to be established. Pollen viability of *G.* ×*sulistrovicus* was very low but some plants produced partly viable pollen (~50%). Therefore, some backcrossing to one or both parental species can be expected. Effective introgression of a low-fertility F_1 hybrid with any of fully-fertile parents is more probable than formation of other hybrids (Rieseberg et al. 2000).

On the other hand, the data and observations on the ex situ culture indicate that hybrids produced the next hybrid generation. Seed production by *G.* ×*sulistrovicus* was distinctly less than that of the parental species. Its winged seeds can be dispersed by wind and can colonize new habitats. In the ex situ culture, 69.5% (829 seeds planted in 2009) of *G.* ×*sulistrovicus* seed, planted in soil that originated from the natural locality in the Łąka Sulistrowicka reserve, germinated, which is similar to the 68.5% of the seed of *G. palustris* that germinated (2271 seeds planted in 2009). Comparable values of seed germination for the hybrid and parental species indicate that the hybrid can also disperse effectively and produce seedlings in nature.

Many species of *Gladiolus* can self-reproduce and increase their numbers by vegetative propagation by the production of daughter corms and cormlets in various ways (Goldblatt & Manning 1999). The decreased ability of *G.* ×*sulistrovicus* to reproduce generatively and produce an abundance of viable seed is compensated for by their highly efficient vegetative reproduction by cormlets. Based on our findings, it is likely that the annual regeneration and vegetative reproduction as well as relative corm longevity (up to ten years) of *G.* ×*sulistrovicus* are sufficient to ensure their continued survival. Maternal corms of the hybrid begin to form young cormlets earlier than the parental species and when the maternal corm flowers it may also divide horizontally. Our observations indicate that the number of cormlets of *G.* ×*sulistrovicus* in the ex situ population increased four-fold over seven years. A single maternal corm of *G.* ×*sulistrovicus* produced from one to as many as 12 very viable cormlets, which would enable a hybrid population to survive over a long period of time at a natural locality. Results of 10-year long ex situ observations indicate that vegetative reproduction in *G. palustris* was more restricted and insufficient for the survival of a population for longer than five to six years. In the ex situ culture, only 196 corms remained out of 571 annual *G. palustris* corms after three years (decrease of 66%) and only a few survived after six years after which the population became extinct (R. Kamiński, unpublished data). *Gladiolus imbricatus* was also characterized by limited vegetative spread, and the production of more than one daughter corm within one season was rare (Klimeš et al. 1997).

The occurrence of hybrids in the context of Gladiolus palustris conservation

Natural hybridization may have a significant effect on the genetic structure of rare taxa when they come into contact with a more numerous relative (Arnold et al. 1999, Wolf et al. 2001, Gómez et al. 2015). Recurrent hybridization events occurred in a highly impoverished natural population of very rare *G. palustris* in the Łąka Sulistrowicka reserve, where there are only a few flowering specimens. This was confirmed by finding that plants that were cultured in ex situ conditions from seed collected in both 2004 and 2005 proved to be hybrids (determined as WR0501–WR0507, WR0601 from 2004 seed and WR0101–WR0116 from 2005 seed in this study). In 2004 the 125 seeds were collected from four plants that were morphologically identified as *G. palustris*. From 21 seeds of one of these *G. palustris* plants hybrids developed. Hence, the percentage of hybrid seeds in the seed set of *G. palustris* was 16.8%. The very small population size of *G. palustris* at the natural locality resulted in a decrease in the ability of individuals of the same species to cross and increase in the probability of interspecific crossing with the significantly more abundant *G. imbricatus*. There are no accurate records of the long-term survival of

G. ×*sulistrovicus* at a natural locality; however, ex situ observations confirmed that the percentage germination of the seed of the hybrids is similar to that of *G. palustris*. In 2014, four individuals of *G.* ×*sulistrovicus* were recorded in the *G. palustris* population in the Łąka Sulistrowicka reserve indicating that hybrids can develop flowers and produce viable seeds at a natural locality. *Gladiolus* ×*sulistrovicus* may be better adapted to changeable environmental conditions of wet *Molinia* meadows than *G. palustris*, whose ability to survive in nature is considerably limited. Therefore, the hybrid may compete with a rare parental species, especially as its potential for vegetative reproduction is considerably greater than that of *G. palustris*. Natural hybridization with extensive introgression could lead to the replacement of parental forms by hybrid individuals (Arnold et al. 1999, Wolf et al. 2001). In an extreme case, a pure species may be hybridized out of existence (Stace 1975, Wolf et al. 2001, Gómez et al. 2015). Hybridity should be considered as one of many factors (e.g. habitat degradation, genetic impoverishment of a population and the absence of insect pollinators) that may potentially bring about the extinction of *G. palustris*, an undoubtedly sensitive species (Kamiński 2012). It was very important in our studies to determine if there were genotypically pure individuals of *G. palustris* and interspecific hybrids in the ex situ culture as this is of crucial importance for the current conservation programmes of *G. palustris* in Poland (Kamiński 2012, Cieślak et al. 2014).

Gladiolus* ×*sulistrovicus Kamiński, Szczepaniak et Cieślak, **nothosp. nova** (Fig. 6)

[*Gladiolus palustris* Gaudin × *Gladiolus imbricatus* L.]

D e s c r i p t i o: *Gladiolus* ×*sulistrovicus* – inter speciem *Gladiolus palustris* Gaudin et *G. imbricatus* L. hybrida. Geophytum bulbotuberibus diam. 12–40 mm, tunica squamosa involutis, ex siccis volvis foliaceis anno proximo formata; tunica gracilibus, tenuibus nervis in parte superiore leniter delineatum reticulum bulbotuberum facientibus. Tempore vegetationis bulbotuber matriculare in parte superiore 1–2 bulbotubera descendentia et in basi 1–2 bulbotubera advenientia facit. Germen florescens cylindricum, recte ascendens, 33–92 cm altum. Folia ensiformia, plana, acute coartantia, facientia volvam, quae continet germen, cum 5–6 distincte emergentibus nervis nec non relictis nervis minutioribus et modice crassioribus, margines crassiores. Folia in parte media germinis 30–45 cm longa nec non 0.5–2.4 cm lata, basi brevissima. Inflorescentia spica apte conferta, cum floribus in duobus ordinibus alternatim dispositis; folia membranacea duo, viridia, ab 8 mm (in flore altissimo inflorescentiae) ad 130 mm (in flore humillimo inflorescentiae) longa, internum adaxiale paulo minus abaxiali. Flores hermaphroditici, dorsales, rosaceo-violacei et colore magentae et rubro-violacei, florum numerus: 3–16, circa 30 mm longorum (flos latissimus) ad 42 mm (flos humillimus inflorescentiae); tubus perianthii bene formatus, circa 10 mm longus, in basi faucis incurvatus, petala perianthii brevior; petale perianthii 6, distincte divisae, inaequales (25–40 mm × 6–16 mm), superiores ovales nec non laterales rhombiformes, in duabus verticibus accumulati; tres superiores (dorsales) latiores et arcuate stamina contegentes; duae inferiors, mediales petalae angustiores; tres inferiores petalae in parte media clariores, cum obscuriore rhombi forma; rarissime aliae petalae cum tenui forma; stamina tres, staminum fila bis longiora thecis; pistillum unum, cum filiformi collo et tripartito naevo in marginibus verrucoso; ovarium inferius, ovoideo-oblongatum. Fructus capsula, saepissime rugosa et in lateribus procisa, nonnumquam evacuata, ovalis vel globosa, 7–15 mm longa, intra cum 6 tenuibus oblongis striis. Semina quaedam apta ad vivendum, circa 4.3 mm longa, lateraliter applanata, ovalia, fusca, laxe alata. Florescit inde a mense Iulio usque ad mensem Augustum.

H o l o t y p e: SW Poland, Lower Silesia, Sudety Foothils, Mt. Radunia, Łąka Sulistrowicka reserve, near Sulistrowice village, wet, mid-forest meadow of the *Molinion caerulae* alliance, N 50°50'20.2", E 16°44'49.8", ATPOL: BE 77, alt. 310 m a.s.l., 23 VII 2014 (flowering plant), coll. R. Kamiński (KRAM 617586) (Fig. 6). P a r a t y p e: from the same site, 15 X 2014 (part of inflorescence with seeds).

Fig. 6. – Holotype and paratype of *Gladiolus ×sulistrovicus* from the Łąka Sulistrowicka reserve, SW Poland, deposited at the herbarium of W. Szafer Institute of Botany, Polish Academy of Sciences (KRAM 617586). Scale bar = 1 cm.

D e s c r i p t i o n: Hybrid between *Gladiolus palustris* (maternal species) and *G. imbricatus* (parental species). Perennial geophyte with corms of 12–40 mm in diameter, covered by tunic of dry leaf sheaths with slightly marked, fine fibres, more or less parallel on the sides and cross-linked in the upper part. One or two new daughter corms are produced each growing season above the mother corm and the same number of cormlets from buds located at the base. Flowering stems terete, simple, erect, 33–92 cm tall. Leaves flat, lanceolate, sheathed, stem-clasping, with 5–6 clearly raised midribs and other veins only slightly thickened, margins also thickened, acuminate tapering, middle stem leaves 30–45 cm long × 0.5–2.4 cm wide, basal leaves are the shortest on the stem. Inflorescence: spike, relatively dense, the flowers distichous; floral bracts: two, green, relatively large, from 8 mm (the highest flower in spike) to 130 mm (the lowest flower in spike) long, the inner (adaxial) usually slightly smaller than the outer (abaxial); flowers: hermaphroditic, bilaterally zygomorphic, rosy violet or magenta to red and reddish-purple, flowers 3–16, from ~30 mm (the highest flowers) to 42 mm (the lowest flowers in inflorescence) long; perianth tube: well developed, ~10 mm long, curved near throat, shorter than the floral bracts; petals: 6, noticeably split-apart, unequal (25–40 × 6–16 mm), upper petals ovate, lateral and lower rhomboidal, arranged in two whorls: upper three (dorsal) petals wider and arched to hooded over the stamens, the two inside, lower petals narrower, three lower petals (sometimes one upper petal) are brighter in the middle and have characteristic darker, rhomboidal pattern; very rare that other petals are also indistinctly marked; stamens: three, filaments are twice as long as anthers; pistil: one, with filiform style and three-partite stigma, papillate on the borders; ovary: inferior, ovoid to oblong. Fruit: capsule, usually wrinkled and shrunken, sometimes empty, ovoid or globose, 7–15 mm long, rounded at the top, with 6 shallow, longitudinal furrows. Seeds: some large, viable and fully developed, ~4.3 mm long and some much smaller, without embryo, laterally flattened, ovoid, brown, with a broad membranous and pellet-like wings; seed coat microstructure: upper surface papillose, with collapsed cells in the wing area. Flowering time: July to August.

E t h y m o l o g y: The name of the new nothospecies refers to the geographical name of the Sulistrowice village (Lower Silesia, south-western Poland) and the neighbouring Łąka Sulistrowicka reserve, where the hybrids were discovered.

D i s t r i b u t i o n: *Gladiolus* ×*sulistrovicus* is known only from one natural locality in the Łąka Sulistrowicka reserve on Mt. Radunia on the Mt. Ślęża massif in the Polish Sudeten foothills (Lower Silesia) where it occurs with the parental species *G. palustris*, *G. imbricatus* and other rare species such as *Dianthus superbus*, *Dactylorhiza majalis* and *Iris sibirica*. It should be noted that only a few flowering individuals of *G. palustris* occur at this locality while there are two fairly numerous neighbouring populations of *G. imbricatus* (Kamiński 2012). *Gladiolus* ×*sulistrovicus* does not seem to be a very frequent taxon as the parental species, and especially *G. palustris*, are very rare in their entire range and the probability for spontaneous hybridization is limited. The western part of the Balkan Peninsula and adjacent areas (Croatia, Bosnia and Hercegovina, Serbia, northern Italy) and central Europe (Poland, the Czech Republic, Slovakia, eastern Austria, Hungary) are the most probable areas of the occurrence of *G.* ×*sulistrovicus* (Fig. 1). It may be present in populations where *G. palustris* and *G. imbricatus* occur alongside each other. A detailed analysis of corm and capsule characters could be used to increase the probability of finding *G.* ×*sulistrovicus* in such populations.

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Souhrn

Při výzkumu velmi vzácného druhu *Gladiolus palustris* v Polsku byly nalezeny rostliny pravděpodobně hybridního původu. Kombinace sekvenování chloroplastové a jaderné ribosomální DNA, markeru AFLP, morfologických porovnání a reprodukčního chování potvrdila, že se jedná o přirozené křížence druhů *G. palustris* a *G. imbricatus*. Ti byli platně popsáni pod jménem *G.* ×*sulistrovicus*. Všechny hybridní rostliny měly identický chloroplastový haplotyp, který sdílely s druhem *G. palustris*; křížení tudíž probíhalo jen jedním směrem a *G. palustris* byl vždy mateřskou rostlinou. Fenotypově je hybrid intermediární mezi rodiči nebo podobnější druhu *G. imbricatus*. Od obou rodičovských druhů se kříženec nejvíce odlišuje utvářením hlíz a tobolek. Sice má nižší fertilitu, ale přibližně poloviční produkce životaschopných pylových zrn a semen je dostatečná, aby kříženci umožnila vytváření dalších generací. Snížené generativní rozmnožování je vyváženo vysoce efektivním vegetativním množením. Kromě Polska může být kříženec nalezen i v jiných územních se sympatrickým rozšířením rodičovských druhů, což jsou Balkánský poloostrov a přilehlá území (Chorvatsko, Bosna a Hercegovina, Srbsko, severní Itálie) a střední Evropa (Česká republika, Slovensko, východní Rakousko, Maďarsko).

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