Isolation and characterization of 15 SSR loci for the endangered European tetraploid species *Gladiolus palustris* (Iridaceae)

Tamás Malkócs¹, Shyryn Almerekova², Judit Bereczki¹, Judit Cservenka³, Emese Meglécz³, and Gábor Sramkó¹,6,7

Manuscript received 25 October 2018; revision accepted 11 December 2018.

1 Department of Botany, University of Debrecen, Debrecen, Hungary
2 Department of Biodiversity and Bioresources, Al-Farabi Kazakh National University, Almaty, Kazakhstan
3 Department of Evolutionary Zoology and Human Biology, University of Debrecen, Debrecen, Hungary
4 Balaton Uplands National Park Directorate, Cospák, Hungary
5 Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France
6 MTA-DE “Lendület” Evolutionary Phylogenomics Research Group, Debrecen, University of Debrecen, Hungary
7 Author for correspondence: sramko.gabor@science.unideb.hu

Citation: Malkócs, T., S. Almerekova, J. Bereczki, J. Cservenka, E. Meglécz, and G. Sramkó. 2019. Isolation and characterization of 15 SSR loci for the endangered European tetraploid species *Gladiolus palustris* (Iridaceae). *Applications in Plant Sciences* 7(5): e1245. doi:10.1002/apps.3.1245

PREMISE: *Gladiolus palustris* (Iridaceae) is an endangered European perennial tetraploid herb with special conservation interest in the European Union. Microsatellite markers can serve as effective tools for the conservation genetics of this species.

METHODS AND RESULTS: We utilized a 454 pyrosequencing approach to identify simple sequence repeat (SSR) regions in a microsatellite-enriched library. Of all SSR regions, 46 were screened for specific PCR amplification, and 15 were found to be applicable in the target species. We found 1.62–3.08 alleles per population (effective alleles: 1.58–2.08) that indicated moderate to high genetic diversity values (0.28–0.44) in three pilot populations. Cross-species amplification was less effective in *G. imbricatus* and *G. tenuis*.

CONCLUSIONS: The primers reported here can be used for the population genetic characterization of *G. palustris*. They will help us to better understand the conservation genetics of this highly endangered species.

KEY WORDS: conservation; *Gladiolus palustris*; Habitats Directive; microsatellite; polyploid; population genetics.

The genus *Gladiolus* L. comprises approximately 260 species confined to the Old World, which are mainly found on the African continent. Only seven species are native to Europe, with the highest diversity in the Mediterranean (Valente et al., 2011), but some extend farther to the north and occur in temperate regions. One of these species is *G. palustris* Gaudich., an endemic species of Central Europe that occurs from eastern France to Hungary, and from southern Germany to northern Italy and the northern Balkans (Szczepaniak et al., 2016). It has a reported chromosome number of 2n = 60, which corresponds to the tetraploid level in this genus (Hamilton, 1980). Because this species lives in a highly endangered habitat, the ecotone between wet meadows and dry grasslands, it is declining throughout its distribution (Schnittler and Günther, 1999) and is included in Annex II of the Habitats Directive of the European Union (Council Directive 92/43/EEC, Council of the European Union, 1992). It is included on several national red lists (National Red List Project [https://www.nationalredlist.org/]) and is incorporated in the International Union for Conservation of Nature (IUCN) Red List as data deficient (Bilz, 2011). For the effective conservation of *G. palustris*, it is necessary to develop genetic markers providing resolution at the population level.

Here, we report the development and characterization of 15 microsatellite markers to investigate the conservation genetics of *G. palustris*. We also tested the cross-species utility of 11 polymorphic loci in two related European species, *G. imbricatus* L. and *G. tenuis* M. Bieb. The former species forms hybrids with *G. palustris*, which indicates the close genetic relationship between the two species (Szczepaniak et al., 2016). *Gladiolus tenuis* is also included as an Eastern European relative of *G. imbricatus* (Gabrielian, 2001). Previous genetic studies of *G. palustris*, which are scarce, have utilized commonly used plastid markers and amplified fragment length polymorphism ( AFLP) analysis (Szczepaniak et al., 2016). The markers evaluated here are expected to provide a fundamental tool for population genetic studies for the purpose of conservation and management of this species with high conservation importance.
METHODS AND RESULTS

Leaves were dried in silica gel, and genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1987) described in detail elsewhere (Sramkó et al., 2014). Microsatellite isolation followed a protocol based on 454 sequencing (Sinama et al., 2011), in which DNA of one plant from five geographically distant populations (Appendix 1) was mixed equimolarly and enriched for microsatellites using the following probes: (TG)$_{10}$, (TC)$_{10}$, (AAC)$_{9}$, (AAG)$_{9}$, (AGG)$_{9}$, (ACG)$_{10}$, (ACAT)$_{10}$, and (ACTC)$_{10}$. The purified and enriched library was used in the 454 GS-FLX Titanium library preparation (Roche Applied Science, Penzberg, Germany) following the manufacturer’s protocols performed by Genoscreen Ltd. (Lille, France). The software QDD version 3.1.2 (Méglécz et al., 2014) was used for the selection of sequences for primer design using default parameters. For contamination detection, sequences were compared with the nucleotide database of the National Center for Biotechnology Information (NCBI; downloaded in July 2015) using BLAST. Sequences were screened for transposable elements using the RepeatMasker Libraries (NCBI; downloaded in July 2015) using BLAST. Sequences were tested in a sample size of five plants from the Raposka population (Appendix 1), searching for potentially polymorphic loci using the same electrophoretic conditions as above. Twenty-five loci presented specific products. These were tested in a sample size of five plants from the Nyirád population (Appendix 1), searching for potentially polymorphic loci using the same electrophoretic conditions as above. The PCR cycling conditions were: denaturation at 95°C for 2 min; 40 cycles of 15 s at 95°C, 30 s at 62°C, and 1 min at 72°C; with a final extension step at 72°C for 10 min. All PCRs were run using the above conditions. Twenty-five loci presented specific products. These were tested in a sample size of five plants from the Nyirád population (Appendix 1), searching for potentially polymorphic loci using the same electrophoretic conditions as above. Fifteen loci were selected for further analyses based on apparent length differences between the five samples as assessed visually. Forward primers of the selected loci were labeled with a fluorophore dye at their 5′ end (Table 1), and the SSR loci were PCR amplified in three Hungarian populations of our target species represented by 25, 20, and 12 individuals.

### TABLE 1. Characteristics of 15 microsatellite loci developed in *Gladiolus palustris*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | Fluorescent dye | GenBank accession no. |
|-------|--------------------------|--------------|------------------------|-----------------|----------------------|
| GIPa01 | F: TTTCTGCAAAAGCTCAAAGGCG | (CT)$_{9}$ | 201 | PET | MG266909 |
|       | R: CCACTGGCCATTATCGGTCA | | | | |
| GIPa03 | F: TTTTAGACTGCTGCGGACCTCC | (CT)$_{9}$ | 211 | NED | MG266910 |
|       | R: CAGGTTCGATTTGCTAGGAAA | | | | |
| GIPa04 | F: TTTTGAGGCTATAGGGCTAG | (GA)$_{4}$ | 137 | 6-FAM | MG266911 |
|       | R: TTTGCTTTCCTGGAGGTCCA | | | | |
| GIPa08 | F: ATGCCCTTGGCTCTGCACTT | (CT)$_{9}$ | 137–141 | VIC | MG266912 |
|       | R: TTTGTCTCCAATGGAACAGGTC | | | | |
| GIPa11 | F: CGGAAATAGCAACTCCCCGG | (AG)$_{4}$ | 136–138 | PET | MG266913 |
|       | R: GTTGCTCCCTGCGTGTATC | | | | |
| GIPa13 | F: ATCTGCAAAGGAGTTACGCG | (GAA)$_{4}$ | 95–107 | 6-FAM | MG266914 |
|       | R: GCCTACCCACTGCTTCTCCT | | | | |
| GIPa14 | F: CCAAGTAGTTATAGGGC | (GAG)$_{4}$ | 189–207 | 6-FAM | MG266915 |
|       | R: GGCTTCTAGAAGGCTTGGG | | | | |
| GIPa21 | F: GTTGGTCTGCTAAGTGCTATG | (GA)$_{6}$ | 122–142 | NED | MG266916 |
|       | R: TCATGGGCGCGCAAGAG | | | | |
| GIPa22 | F: TGAAACCATACAGTACCCCT | (AG)$_{4}$ | 293–305 | 6-FAM | MG266917 |
|       | R: ATGTAGGGTGATGGTGGCTG | | | | |
| GIPa24 | F: ACATGCTTATATAGGCATTCT | (TC)$_{4}$ | 291–297 | VIC | MG266918 |
|       | R: AGTCCGCTTCTGATCATG | | | | |
| GIPa39 | F: ACAGTGAGTACATTAAAGGGCC | (AAC)$_{6}$ | 261–270 | NED | MG266919 |
|       | R: CACAGACCTGTAGGACCC | | | | |
| GIPa41 | F: ATCTCTTTCTCATAATTCCTTTCCC | (AG)$_{4}$ | 238–240 | 6-FAM | MG266920 |
|       | R: TGATGAGAGGATCTGCTGCAGAC | | | | |
| GIPa42 | F: TAAAGTGCCTAGCTACACACTG | (GA)$_{4}$ | 287 | PET | MG266921 |
|       | R: TCTGGAGAAGGTACACAG | | | | |
| GIPa46 | F: GGTTCTCCTGGCTCAG | (GAA)$_{4}$ | 207–219 | VIC | MG266923 |
|       | R: TCCTATGGCCTTGTGGGCTG | | | | |

*Annealing temperature (T$_{a}$) for all primers is 64°C.

*Monomorphic in the test sample set.
TABLE 2. Genetic diversity of 11 polymorphic microsatellite markers in three populations of *Gladiolus palustris* as calculated in GenoDive version 2.0b27.*

| Locus   | Raposka (n = 25) | Nyrőd (n = 20) | Bátónyterenye (n = 12) |
|---------|------------------|----------------|-------------------------|
|         | *A* | *A*<sub>e</sub> | *H*<sub>e</sub> | *A* | *A*<sub>e</sub> | *H*<sub>e</sub> | *A* | *A*<sub>e</sub> | *H*<sub>e</sub> |
| GlPal08 | 3   | 2.048          | 0.522             | 2   | 2.000          | 0.513           | 2   | 2.000          | 0.522           |
| GlPal11 | 2   | 1.992          | 0.508             | 2   | 2.000          | 0.513           | 2   | 2.000          | 0.522           |
| GlPal13 | 2   | 1.986          | 0.512             | 2   | 1.978          | 0.512           | 1   | 1.000          | 0.000           |
| GlPal14 | 5   | 2.183          | 0.557             | 4   | 2.442          | 0.609           | 2   | 1.578          | 0.387           |
| GlPal21 | 4   | 3.041          | 0.682             | 4   | 3.221          | 0.702           | 2   | 2.000          | 0.522           |
| GlPal22 | 5   | 2.405          | 0.601             | 6   | 3.058          | 0.694           | 1   | 1.000          | 0.000           |
| GlPal24 | 3   | 1.162          | 0.144             | 4   | 1.223          | 0.190           | 1   | 1.000          | 0.000           |
| GlPal37 | 4   | 3.550          | 0.727             | 4   | 3.849          | 0.751           | 3   | 3.000          | 0.686           |
| GlPal39 | 2   | 1.683          | 0.418             | 2   | 1.090          | 0.087           | 1   | 1.000          | 0.000           |
| GlPal42 | 3   | 2.100          | 0.534             | 4   | 2.164          | 0.551           | 2   | 2.000          | 0.522           |
| GlPal46 | 5   | 2.026          | 0.521             | 4   | 2.048          | 0.526           | 2   | 1.912          | 0.502           |
| Overall | 3.077 | 2.014        | 0.440             | 3.077 | 2.083        | 0.434           | 1.615 | 1.576        | 0.282           |

Note: *A* = number of alleles; *A*<sub>e</sub> = effective number of alleles; *H*<sub>e</sub> = expected heterozygosity; *n* = number of individuals.

*Locality and voucher information are provided in Appendix 1.*

The fluorescent-labeled PCR products were run on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). The products were multiplexed according to their predicted fragment length and fluorescent label type, containing varying amounts of PCR product per locus (1.5–4 μL) based on band intensity (Appendix S1). One microliter of the multiplexed PCR products was added to 0.25 μL of GeneScan 500 LIZ Size Standard (Applied Biosystems) and 14.75 μL of Hi-Di formamide (genetic analysis grade; Applied Biosystems) before loading on the Genetic Analyzer. Genotype calling was carried out manually using PeakScanner version 1.0 (Applied Biosystems). Because of the tetraploid nature of our target species, genotypes were evaluated and analyzed using GenoDive version 2.0b27 (Meirmans and Van Tienderen, 2004) as this software can take allele copy number ambiguity into account in partial heterozygotes.

Although we expected all 15 loci to be variable based on visual inspection, only 11 loci proved to be variable. We report population sizes, number of alleles, effective number of alleles, and levels of expected heterozygosity (Table 2). We do not report observed heterozygosities, as these calculations are not based on the corrected allele frequencies and are therefore biased (Meirmans and Van Tienderen, 2004). The number of alleles and effective number of alleles ranged from one to 6 and 1.000 to 3.849 per locus in the three studied populations, respectively. The mean level of expected heterozygosity was 0.385 (0.000–0.751) (Table 2). Cross-amplification was tested on the loci found to be polymorphic in the focal species and the species of the *G. imbricatus* group.

### CONCLUSIONS

We identified the first polymorphic microsatellite markers in *G. palustris*. These 11 polymorphic markers are crucial in population genetic studies in the species, providing information on genetic diversity, levels of inbreeding, and gene flow. Thus, they present a much-needed tool in the conservation and management of this critically endangered species.
**LITERATURE CITED**

Bilz, M. 2011. *Gladiolus palustris*. The IUCN Red List of Threatened Species 2011: e.T1621888A5555329. Website https://doi.org/10.2305/iucn.uk.2011-1. dts1621888a5555329.en [accessed 9 October 2018].

Council of the European Union. 1992. Council Directive 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora. *Official Journal of the European Communities* 35(L 206): 31992L0043. Website https://eur-lex.europa.eu/eli/dir/1992/43/oj [accessed 22 April 2019].

Doyle, J. J., and J. L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19: 11–15.

Gabrielian, E. 2001. The genus *Gladiolus* (Iridaceae) in southern Transcaucasia. *Bocconea* 13: 445–455.

Hamilton, A. P. 1980. *Gladiolus L.* In T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters, and D. A. Webb [eds.], *Flora Europaea*, vol. 5, Alismataceae to Orchidaceae (Monocotyledones), 101–102. Cambridge University Press, Cambridge, United Kingdom.

Meglécz, E., N. Pech, A. Gilles, V. Dubut, P. Hingamp, A. Trilles, R. Grenier, and J. F. Martin. 2014. QDD version 3.1: A user-friendly computer program for microsatellite selection and primer design revisited: Experimental validation of variables determining genotyping success rate. *Molecular Ecology Resources* 14: 1302–1313.

Meirmans, P. G., and P. H. Van Tienderen. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792–794.

Schnitzius, M., and K. F. Günther. 1999. Central European vascular plants requiring priority conservation measures—an analysis from national Red Lists and distribution maps. *Biodiversity and Conservation* 8(7): 891–925.

Sinama, M., V. Dubut, C. Costedoat, A. Gilles, M. Junker, T. Malaza, J. F. Martin, et al. 2011. Challenge for microsatellite development in Lepidoptera: *Euphydryas aurinia* (Nymphalidae) as a case study. *European Journal of Entomology* 108: 261–266.

Sramko, G., A. V. Molnár, J. A. Hawkins, and R. M. Bateman. 2014. Molecular phylogeny and evolutionary history of the Eurasian orchid genus *Himantoglossum* s.l. (Orchidaceae). *Annals of Botany* 114(8): 1609–1626.

Szczepaniak, M., R. Kamiński, E. Kuta, A. Slomka, W. Heise, and E. Cieslak. 2016. Natural hybridization between *Gladiolus palustris* and *G. imbricatus* inferred from morphological, molecular and reproductive evidence. *Preslia* 88(1): 137–161.

Valente, L. M., V. Savolainen, J. C. Manning, P. Goldblatt, and P. Vargas. 2011. Explaining disparities in species richness between Mediterranean floristic regions: A case study in *Gladiolus* (Iridaceae). *Global Ecology and Biogeography* 20: 881–892.

**APPENDIX 1. Geographic and voucher information of *Gladiolus* populations represented in this study.**

| Species                     | Location, ISO country code | n  | Geographic coordinates | Voucher no.* |
|-----------------------------|----------------------------|----|------------------------|--------------|
| *Gladiolus tenius* M. Bieb. | Horodyshche, UA            | 1  | 49°02′19.4″N, 39°39′32.7″E | DE-Soo-45670 |
| *Gladiolus tenius*          | Krasny Kurgan, RU           | 1  | 43°58′19.8″N, 42°35′54.8″E | DE-Soo-45671 |
| *Gladiolus tenius*          | Mikhailovka, RU             | 1  | 49°50′19.3″N, 43°07′48.9″E | DE-Soo-45672 |
| *Gladiolus imbricatus* L.   | Prejmer, RO                 | 1  | 45°43′36.8″N, 25°44′04.5″E | DE-Soo-45681 |
| *Gladiolus imbricatus*      | Lunca de Jos, RO            | 1  | 46°34′18.2″N, 25°59′22.9″E | DE-Soo-45679 |
| *Gladiolus imbricatus*      | Zakopane, PL                | 1  | 49°16′40.7″N, 19°54′11.0″E | DE-Soo-45682 |
| *Gladiolus imbricatus*      | Kornláska-Újhuta, Zsidó-rét, HU | 1  | 48°21′58.8″N, 21°28′23.0″E | DE-Soo-09423 |
| *Gladiolus imbricatus*      | Cluj-Napoca, Valea morilor, RO | 1  | 46°41′51.5″N, 23°35′56.4″E | DE-Soo-38677 |
| *Gladiolus palustris* Gaudich. | Radoska, HU                | 25 | 46°5′1′39.9″N, 17°25′01.3″E | DE-Soo-45673 |
| *Gladiolus palustris*       | Nyrád, HU                  | 20 | 47°00′02.1″N, 17°25′31.5″E | DE-Soo-45674 |
| *Gladiolus palustris*       | Bátontyerenye, Lengyendi-Galya, HU | 12 | 47°55′05.8″N, 19°54′12.8″E | DE-Soo-09419 |
| *Gladiolus palustris*       | Ásotthalom, Csodáret, HU    | 1  | 46°1′55.0″N, 19°49′48.1″E | DE-Soo-45683 |
| *Gladiolus palustris*       | Schwangau, DE              | 1  | 47°33′38.5″N, 10°43′53.7″E | DE-Soo-45684 |
| *Gladiolus palustris*       | Augsburg, DE               | 1  | 48°18′24.3″N, 10°55′43.2″E | DE-Soo-45685 |

Note: ISO = International Organization for Standardization; n = number of individuals included in this study.

*Voucher specimens are deposited at the herbarium of the University of Debrecen (DE), Debrecen, Hungary.

*Five individuals from this population were included in searching for potentially polymorphic loci during marker evaluation.

*DNA of a single individual from this population was used for the construction of the 454 sequencing library.

*This individual was used for the initial test of specific PCR amplification of 46 primers.