American Journal of Botany

American Journal of Botany: e277–e279. 2011.

*AJB* Primer Notes & Protocols in the Plant Sciences

# Isolation, characterization, and cross-amplification of microsatellite markers for the *Petunia integrifolia* (Solanaceae) complex[1]

Raquel A. Kriedt[2,6], Aline M. C. Ramos-Fregonezi[2,6], Luciano B. Beheregaray[3], Sandro L. Bonatto[4], and Loreta B. Freitas[2,5]

[2]Molecular Evolution Laboratory, Department of Genetics, Universidade Federal do Rio Grande do Sul, CP 15053, 91501-970 Porto Alegre, Rio Grande do Sul, Brazil; [3]Molecular Ecology Laboratory, School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide 5001, South Australia, Australia; and [4]Genomic and Molecular Biology Laboratory, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610-001 Porto Alegre, Rio Grande do Sul, Brazil

- *Premise of the study*: Microsatellite markers were developed for *Petunia integrifolia* subsp. *depauperata* with an intent to clarify taxonomic questions on the *P. integrifolia* complex, and to identify a purple-flowered parent of *P. hybrida*.
- *Methods and Results*: We characterized 11 microsatellite loci by screening primers developed using an SSR-enriched library. Genotyping of two populations resulted in eight polymorphic loci. Cross-species transferability was tested for other members of the *P. integrifolia* complex.
- *Conclusions*: The development of these markers may contribute to population genetics studies in *Petunia*, and cross-amplification among related species could be a useful tool for research on hybridization and introgression.

**Key words:** congeneric transferability; microsatellite; *Petunia integrifolia* complex.

The genus *Petunia* is commonly known worldwide as the garden petunia, an artificial hybrid obtained in the beginning of the 19th century from a crossing between *P. integrifolia* (Hook.) Schinz & Thell. and *P. axillaris* (Lam.) Britton, Sterns & Poggenb. (Stehmann et al., 2009). Garden petunias are one of the world's most important ornamental plants, with seed trading generating millions of dollars annually. There is great potential for enhancing this cultivated species utilizing native *Petunia* species as a source of genetic variability and agronomic features (Gerats and Vandenbussche, 2005). Conservation programs for native species of the genus are urgently needed to ensure appropriate management of this genetic resource. *Petunia* shows very low variability in nuclear sequence markers (Chen et al., 2007), both among and within species (Kulcheski et al., 2006). Mitochondrial regions, which are usually capable of discriminating among species of the same genus, do not show any variability in *Petunia* (Kulcheski et al., 2006). Therefore, there is an urgent need for new, more variable genetic markers for this genus.

*Petunia integrifolia* is composed of a complex of morphologically similar species that differ in habitat use, geographical distribution, and minor details in floral and vegetative structures. All species present $2n = 14$, and chromosome counts assign the basic number of $x = 7$. This similarity has led to many changes in taxonomy and species delimitation in the past. In this work, we adopt the most recent classification, based also on molecular data (Stehmann et al., 2009). Therefore, the *P. integrifolia* complex comprises five taxonomic entities: *P. bajeensis* T. Ando & Hashim., *P. inflata* R. E. Fr., *P. integrifolia* subsp. *depauperata* (R. E. Fr.) Stehmann, *P. integrifolia* subsp. *integrifolia*, and *P. interior* T. Ando & Hashim. These taxa have one of the largest distributions of *Petunia* species, found in southern Brazil, Uruguay, Paraguay, and Argentina (Stehmann et al., 2009). In this study, we describe the isolation and characterization of 11 loci for *P. integrifolia* subsp. *depauperata* and test their transferability to the other members of the complex.

## METHODS AND RESULTS

Genomic DNA was extracted from an individual of *P. integrifolia* subsp. *depauperata* according to Roy et al. (1992), and repeat motifs were isolated using an enrichment technique (Beheregaray et al., 2004). Briefly, genomic DNA was digested with *Rsa*I and *Hae*III, and fragments linked to two oligo adaptors. Biotinylated oligo probes $(dGT)_{10}$, $(dGA)_{10}$, $(dAGAT)_{10}$, $(dAACT)_{10}$, and $(dACAT)_{10}$ were hybridized to the digested DNA and selectively retained using streptavidin magnetic particles (Promega, Madison, Wisconsin, USA). PCRs were performed on the microsatellite-enriched eluate using one of the oligo adaptors as a primer. The enriched library was purified using an UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Carlsbad, California, USA), linked into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, California, USA) and transformed into One Shot TOP10 Chemically Competent Cells (Invitrogen). The plasmid DNA was PCR-amplified using M13(−20) forward and M13(−40) reverse primers, purified, and 279 positive clones were sequenced with MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA). A total of 28 clones presented perfect unique microsatellites, but only 13 were suitable for primer design using Primer3 (Rozen and Skaletsky, 2000; http://www-genome.wi.mit.edu/genome_software/other/primer3.html).

[1]Manuscript received 13 April 2011; revision accepted 16 May 2011.

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundaçao de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and the Australian Research Council (ARC) for financial support and grants.

[5]Author for correspondence: loreta.freitas@ufrgs.br

[6]These authors have contributed equally to this work, the order of authorship being arbitrary.

doi:10.3732/ajb.1100178

TABLE 1. Characterization of microsatellite loci indicating GenBank accession number, repeat motif, primer sequence, annealing temperature ($T_a$), allele size range (bp), and number of alleles per locus ($A$) for *Petunia integrifolia* subsp. *depauperata*, as well as expected heterozygosity ($H_e$) and observed heterozygosity ($H_o$) for the two analyzed populations.

| | | | | | | Taim[a] (n = 20) | | | Garopaba[b] (n = 23) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Primer | Repeat motif | Primer sequence (5′–3′) | GenBank Accession No. | $T_a$ (°C) | Size range (bp) | $A$ | $H_e$ | $H_o$ | $A$ | $H_e$ | $H_o$ |
| PID1D6 | $(TGG)_6$ | F: TGGCTATAGAGGAACATACCAATAG | JF720334 | 62 | 263–275 | 5 | 0.760 | 0.500 | 3 | 0.626 | 0.350[d] |
| | | R: CTGCTAAACATTTGGACATGG | | | | | | | | | |
| PID1F1[c] | $(CT)_7$ | F: ACCATTCTTAACATCCCAATCC | JF720335 | 62 | 171–175 | 3 | 0.664 | 0.470 | 3 | 0.552 | 0.173[d] |
| | | R: GAGTGGAGATCAGAGTGTATTTTCC | | | | | | | | | |
| PID1G6 | $(TG)_7$ | F: TTGGTAAGGCTGCTACTCACTC | JF720336 | 63 | 216 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 |
| | | R: AAAAGGAGATCTGCCAGGAG | | | | | | | | | |
| PID2F2 | $(TC)_{12}$ | F: TGCAAGTCAGTCGCAAAAAC | JF720337 | 58 | 235–241 | 5 | 0.841 | 0.375[d] | 2 | 0.369 | 0.000[d] |
| | | R: TGCGTTTGTGATTAGACCATC | | | | | | | | | |
| PID3C4 | $(CT)_{13}$ | F: CTGAAGGTTGTTGCCTGTTG | JF720338 | 63 | 247–259 | 5 | 0.784 | 0.500 | 5 | 0.725 | 0.904 |
| | | R: CATCCCCTGTGTATGGAAATG | | | | | | | | | |
| PID3G3 | $(TAGA)_7$ | F: TTTTGGACTCAAGATCATTTTATG | JF720339 | 58 | 180–256 | 9 | 0.846 | 0.055[d] | 4 | 0.500 | 0.100[d] |
| | | R: TCATATGGTTTACTAGTTTGGATGC | | | | | | | | | |
| PID3G5 | $(TTC)_8$ | F: GGTCATGCCAGGTGAACATC | JF720340 | 62 | 170 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 |
| | | R: CATATCCCGGCTCCTAACTG | | | | | | | | | |
| PID3G7[c] | $(CA)_{10}$ | F: TGTGCCACTGATAATGTGTCC | JF720341 | 59 | 170–176 | 3 | 0.342 | 0.266 | 3 | 0.416 | 0.523 |
| | | R: AAAACCTTTCCCTACTGTATTTCC | | | | | | | | | |
| PID3H7 | $(GAA)_6$ | F: GGGCAGTTTCCATGAAATTAAAC | JF720342 | 58 | 132–135 | 2 | 0.212 | 0.076 | 3 | 0.448 | 0.473 |
| | | R: AAGAGACACTCCATTTCTTTG | | | | | | | | | |
| PID4C6 | $(GAA)_{13}$ | F: GGCTTGGAAAATGTTGAAGAAC | JF720343 | 62 | 176 | 1 | 0.000 | 0.000 | 1 | 0.000 | 0.000 |
| | | R: GCTGATCCATCCCCAGAAG | | | | | | | | | |
| PID4G8 | $(CA)_8$ | F: TCAGTCAGGCTGAATAAGTTCG | JF720344 | 58 | 228–230 | 2 | 0.506 | 0.636 | 1 | 0.000 | 0.000 |
| | | R: CCTTAAACTCGTATCTTTGCACAT | | | | | | | | | |
| Mean | | | | | | | 0.450 | | | 0.330 | |

[a] Taim, Rio Grande do Sul, Brazil (30°32′32″S, 52°34′34″W).
[b] Garopaba, Santa Catarina, Brazil (28°01′26″S, 48°36′50″W).
[c] Linked loci in one population after correction for multiple tests ($P < 0.0009$).
[d] Deviation from Hardy–Weinberg equilibrium after correction for multiple tests ($P < 0.004$).

Primers were tested for amplification in two populations of *P. integrifolia* subsp. *depauperata* from the Brazilian Coastal Plain (Taim, n = 20; Garopaba, n = 23). Ten of the 13 primer pairs were successfully amplified, and eight of them showed polymorphism and were therefore further characterized. Amplifications were performed in a 15 µL reaction containing ~10–100 ng of template DNA and 200 µM of each dNTP (Invitrogen). Reactions included 2 pmol fluorescent-labeled M13(−21) primer and reverse primer, 0.4 pmol forward primer with 5′-M13(−21) tail, 2.0 mM $MgCl_2$ (Invitrogen), and 0.5 U *Taq* Platinum DNA polymerase and its reaction buffer (Invitrogen). Table 1 lists the sequences and the annealing temperature of each primer pair. Fragment analysis was performed on the MEGABACE 1000, with ET-ROX 550 size ladder (GE Healthcare). Fragment length and microsatellite genotyping were determined using GENETIC PROFILER 2.0 (GE Healthcare). Analysis of allele numbers, expected and observed heterozygosity, Hardy–Weinberg equilibrium (HWE), and genotypic disequilibrium were performed in GENEPOP 4.0 (Raymond and Rousset, 1995). Tests for null alleles were performed in MICRO-CHECKER 2.2.3 (Van Oosterhout et al., 2004).

All PCR products met the expected sizes based on sequence information, and the number of bands per individual was consistent with the diploidy condition of these species (one or two bands per locus per individual). Two loci (PID2F2 and PID3G3) significantly deviated from HWE in both populations, and another two loci (PID1D6 and PID1F1) showed significant deviations for the Garopaba population (Table 1). These results are likely a consequence of the nonrandom mating system. Although this species is an outcrosser, it presents

TABLE 2. Transspecies amplification of microsatellite markers developed for *Petunia integrifolia* subsp. *depauperata* in four congeneric taxa of the native range. Allele size range (bp) and number of alleles ($A$) are given.

| | *P. bajeensis* (n = 18) | | *P. inflata* (n = 42) | | *P. integrifolia* subsp. *integrifolia* (n = 30) | | *P. interior* (n = 42) | |
|---|---|---|---|---|---|---|---|---|
| Primer | Size range (bp) | $A$ | Size range (bp) | $A$ | Size range (bp) | $A$ | Size range (bp) | $A$ |
| PID1D6 | 263–272 | 2 | 257–278 | 7 | 260–278 | 7 | 257–278 | 7 |
| PID1F1 | 161–175 | 3 | 171–181 | 5 | 169–175 | 3 | 169–189 | 11 |
| PID1G6 | 216 | 1 | 212–218 | 4 | 216–218 | 2 | 212–218 | 3 |
| PID2F2 | NA | NA | NA | NA | NA | NA | NA | NA |
| PID3C4 | 247–263 | 4 | 247–267 | 8 | 251–263 | 5 | 249–265 | 7 |
| PID3G3 | NA | NA | 198 | 1 | NA | NA | 234–238 | 2 |
| PID3G5 | 170–173 | 2 | 170 | 1 | 170–176 | 2 | 170 | 1 |
| PID3G7 | NA | NA | 172 | 1 | NA | NA | NA | NA |
| PID3H7 | 129–132 | 2 | 129–135 | 3 | 129–135 | 3 | 126–132 | 3 |
| PID4C6 | 173–176 | 2 | 176–179 | 2 | 176 | 1 | 176 | 1 |
| PID4G8 | NA | NA | 228–232 | 2 | 228–230 | 2 | 230–268 | 3 |

*Note*: NA = no amplification.

short-distance seed dispersal, which may cause populations to be constituted of genetically close individuals (Stehmann et al., 2009). The results could alternatively be related to null alleles, with MICRO-CHECKER suggesting the presence of null alleles in deviating loci ($P < 0.004$). One pair of loci showed significant linkage disequilibrium for one population after Bonferroni correction ($P < 0.0009$). However, with no additional information, physical linkage of loci cannot be distinguished from disequilibrium due to population processes such as nonrandom mating (Hedrick, 2005).

Amplification and variability were also tested in *P. inflata*, *P. interior*, *P. integrifolia* subsp. *integrifolia*, and *P. bajeensis*. Of the 11 primer pairs tested, seven successfully amplified PCR products in these species, 10 amplified in at least one of the species, and one did not result in any PCR product (Table 2). For *P. inflata*, 10 loci were amplified in at least 10 individuals, while for the other species the transferability success was lower. Of these 10 loci, eight exhibited equal or higher levels of polymorphism in the transferred species than in the source species (except for *P. bajeensis*). The latter was not expected based on studies that compare the behavior for homologous SSRs in plant species (Jarne and Lagoda, 1996).

## CONCLUSIONS

This is the first study to report SSR markers for the *P. integrifolia* complex. Our results showed better transferability of the tested markers to *P. inflata* and *P. interior* than to other species. Most of the loci developed here might prove to be useful to address a range of questions on genetic diversity and structure, speciation, and migration, especially within *P. inflata*, *P. interior*, and *P. integrifolia* subsp. *integrifolia*. The development of these markers may contribute to different areas of study in *Petunia.* Also, information on population dynamics of the species may help establish strategies for a conservation priority of population groups that best represent the history of these species.

## LITERATURE CITED


BEHEREGARAY, L. B., L. M. MÖLLER, T. S. SCHWARTZ, N. L. CHAO, AND G. CACCONE. 2004. Microsatellite markers for the cardinal tetra *Paracheirodon axelrodi*, a commercially important fish from central Amazonia. *Molecular Ecology Notes* 4: 330–332.

CHEN, S., K. MATSUBARA, T. OMORI, H. KOKUBUN, H. KODAMA, H. WATANABE, G. HASHIMOTO, ET AL. 2007. Phylogenetic analysis of the genus *Petunia* (Solanaceae) based on the sequence of the HF1 gene. *Journal of Plant Research* 120: 385–397.

GERATS, T., AND M. VANDENBUSSCHE. 2005. A model system for comparative research: *Petunia. Trends in Plant Science* 10: 251–256.

HEDRICK, P. W. 2005. Genetics of populations. Jones and Bartlett Publishers, Boston, Massachusetts, USA.

JARNE, P., AND P. J. L. LAGODA. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution* 11: 424–429.

KULCHESKI, F. R., V. C. MUSCHNER, A. P. LORENZ-LEMKE, J. R. STEHMANN, S. L. BONATTO, F. M. SALZANO, AND L. B. FREITAS. 2006. Molecular phylogenetic analysis of *Petunia* Juss. (Solanaceae). *Genetica* 126: 3–14.

RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *The Journal of Heredity* 86: 248–249.

ROZEN, S., AND H. J. SKALETSKY. 2000. Primer3 on the WWW for general users and for biologist programmers. *In* S. Krawetz and S. Misener [eds.], Bioinformatics methods and protocols: Methods in molecular biology, 365–386. Humana Press, Totowa, New Jersey, USA.

STEHMANN, J. R., A. P. LORENZ-LEMKE, L. B. FREITAS, AND J. SEMIR. 2009. The genus *Petunia. In* T. Gerats and J. Strommer [eds.], *Petunia*: Evolutionary, developmental and physiological genetics, 1–28. Springer, New York, New York, USA.

VAN OOSTERHOUT, C., W. F. HUTCHINSON, D. P. M. WILLS, AND P. SHIPLEY. 2004. Micro-checker: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.


APPENDIX 1. Information on voucher specimens deposited in the herbarium of Universidade Federal de Minas Gerais, Belo Horizonte, Brazil (BHCB).

| Taxon | Voucher specimens | Locality in Brazil |
|---|---|---|
| *Petunia bajeensis* T. Ando & Hashim. | 102127 | Bagé, Rio Grande do Sul, 31°24′35″S, 54°38′03″W |
| *Petunia inflata* R. E. Fr. | 114603 | São Luiz Gonzaga, Rio Grande do Sul, 28°27′24″S, 55°07′24″W |
| | 114610 | Santo Cristo, Rio Grande do Sul, 27°50′16″S, 54°38′03″W |
| *Petunia integrifolia* (Hook.) Schinz & Thell. subsp. | 104901 | Taim, Rio Grande do Sul, 30°32′32″S, 52°34′34″W |
| *depauperata* (R. E. Fr.) Stehmann | 104857 | Garopaba, Santa Catarina, 28°01′26″S, 48°36′50″W |
| *Petunia integrifolia* (Hook.) Schinz & Thell. subsp. | 75139 | Cachoeira do Sul, Rio Grande do Sul, 30°27′11″S, 52°56′08″W |
| *integrifolia* | 102115 | Quaraí, Rio Grande do Sul, 30°26′14″S, 56°20′06″W |
| *Petunia interior* T. Ando & Hashim. | 114612 | Dois Irmãos das Missões, Rio Grande do Sul, 27°37′40″S, 53°33′53″W |
| | 114598 | Panambi, Rio Grande do Sul, 28°20′14″S, 53°34′02″W |
| | 114602 | São Luiz Gonzaga, Rio Grande do Sul, 28°24′22″S, 54°41′28″W |