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DEVELOPMENT OF SSR MARKERS FOR THE GENUS *PATELLIFOLIA* (CHENOPODIACEAE)**1**

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**Primer Note**

The genus *Patellifolia* A. J. Scott, Ford-Lloyd & J. T. Williams (Chenopodiaceae) is considered a valuable source of resistance traits for sugar beet breeding (Frese, 2002). It is composed of the tetraploid self-fertile species *P. patellaris* (Moq.) A. J. Scott, Ford-Lloyd & J. T. Williams and the two diploid self-sterile species *P. procumbens* (Chr. Sm.) A. J. Scott, Ford-Lloyd & J. T. Williams and *P. webbiana* (Moq.) A. J. Scott, Ford-Lloyd & J. T. Williams. Szota (1964, 1971; cited in Jassem, 1992) observed that the diploid species hybridize spontaneously, form fertile offspring, and should be considered distinct variants of the same species. Despite later attempts at clarification, this taxonomic question still remains unresolved. *Patellifolia* species are found primarily on the Canary Islands, Madeira, Cape Verde, Morocco, and the Iberian Peninsula. The species occur in dynamic habitats such as roadsides or abandoned agricultural fields. Their natural habitats and populations seem to be threatened (El Bahloul et al., 2009; Monteiro et al., 2013), which may cause loss of genetic diversity within the species. Therefore, it was necessary to develop a larger set of new SSR markers to investigate the distribution of genetic diversity in the genus *Patellifolia*.

**METHODS AND RESULTS**

Microsatellite marker development—Five hundred forty-three mega base pairs representing 72,453 single sequences with an average size of 7499 nucleotides of the unpublished genome assembly Papiro-1.0 from the *P. procumbens* accession BGRC 35335 (renamed by the genebank of the Institute of Plant Genetics and Crop Plant Research [IPK], Gatersleben, Germany, as BETA 951) were screened for SSRs using SciRoKo version 3.4 software (Kofler et al., 2007) and default search parameters. A study of barley sequences revealed a positive correlation between the length of di-, tri-, and tetranucleotide perfect repeats and degree of polymorphism (Thiel et al., 2003). Therefore, a Perl script was developed to filter SSRs for di-, tri-, and tetranucleotide perfect repeats and for SSRs of minimum lengths (18 nucleotides for di-, 21 nucleotides for tri-, 24 nucleotides for tetranucleotide repeats). Replication slippage events are the major cause of SSR mutations, and because a higher GC content favors replication slippage (Zhou et al., 2011), GC-rich SSRs may exhibit a higher degree of polymorphism. On the other hand, a high GC content can make PCR amplification difficult, so SSRs composed of solely A/T or G/C nucleotides were removed from the set of SSRs using the same Perl script, resulting in a total of 3648 SSRs. SciRoKo was used to extract the 200 nucleotides upstream and downstream flanking genomic sequences of the SSRs, and corresponding primers were designed with Primer3 (Rozen and Skaletsky, 1999). Primers were 20 nucleotides in length, had a fairly high melting temperature of 60°C, and the size of the PCR products was approximately 200 bp (Table 1). Validation of 53 SSRs was conducted using a capillary

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Table 1. Characteristics of 25 polymorphic microsatellite markers developed from Patellifolia procumbens genomic sequences.

| Locus       | Primer sequences (5’−3’) | Repeat motif | Allele size range (bp) | T_{m} (°C) | GenBank accession no. |
|-------------|--------------------------|--------------|------------------------|------------|-----------------------|
| JKIPat01    | F: AGAGTACCCCTGGAGAAAAGGGG | (GA)_3       | 170–191                | 50         | KU888809              |
|             | R: CTTTAATAGATAGGCGGCCCGG  | (CA)_8       | 196–227                | 50         | KU888810              |
| JKIPat02    | F: AAGTGCCACACGCAGCAGCAGC | (TG)$_{19}$  | 198–231                | 52         | KU888811              |
|             | R: AAGTTCACTCATTCTCAGG     | (CT)$_{26}$  | 216–228                | 52         | KU888812              |
| JKIPat03    | F: TTGCCTTGCTTACCGCCCGCC  | (GT)$_{16}$  | 181–232                | 50         | KU888813              |
|             | R: ACAGTTGTGGCGTGTGGCAGG   | (AC)$_{6}$   | 164–169                | 52         | KU888817              |
| JKIPat04    | F: TTCCTCTCCTTACCTCAGC    | (CT)$_{11}$  | 157–184                | 54         | KU888818              |
|             | R: TTTGCTTGTAGTGGTGGCGG   | (AG)$_{2}$   | 165–191                | 54         | KU888819              |
| JKIPat05    | F: TACCTCTTGGGAGTCAGG     | (CA)$_{2}$   | 151–179                | 48         | KU888820              |
|             | R: ACAGTTGTGGCGTGTGGCAGG   | (CA)$_{4}$   | 164–169                | 52         | KU888817              |
| JKIPat06    | F: TACCTCTTGGGAGTCAGG     | (AG)$_{8}$   | 219–223                | 54         | KU888821              |
|             | R: AAGCTGGCGTACCTACGGC    | (GC)$_{2}$   | 174–192                | 52         | KU888822              |
| JKIPat07    | F: TACCTCTTGGGAGTCAGG     | (CA)$_{2}$   | 224–256                | 48         | KU888823              |
|             | R: AAGCTGGCGTACCTACGGC    | (CT)$_{14}$  | 188–202                | 48         | KU888824              |
| JKIPat08    | F: TACCTCTTGGGAGTCAGG     | (AG)$_{8}$   | 177–191                | 48         | KU888825              |
|             | R: AAGCTGGCGTACCTACGGC    | (GT)$_{10}$  | 203–258                | 52         | KU888826              |
| JKIPat10    | F: TACCTCTTGGGAGTCAGG     | (TC)$_{2}$   | 231–256                | 50         | KU888827              |
|             | R: AAGCTGGCGTACCTACGGC    | (TG)$_{19}$  | 175–195                | 50         | KU888828              |
| JKIPat11    | F: TACCTCTTGGGAGTCAGG     | (GA)$_{2}$   | 130–148                | 50         | KU888829              |
|             | R: AAGCTGGCGTACCTACGGC    | (GT)$_{10}$  | 185–224                | 50         | KU888830              |
| JKIPat12    | F: TACCTCTTGGGAGTCAGG     | (TA)$_{6}$   | 182–214                | 48         | KU888831              |
|             | R: AAGCTGGCGTACCTACGGC    | (AT)$_{10}$  | 179–195                | 50         | KU888832              |
| JKIPat13    | F: TACCTCTTGGGAGTCAGG     | (GA)$_{3}$   | 159–191                | 48         | KU888833              |

Note: T_{m} = annealing temperature.

*Refers to the resequenced PCR products from genomic P. procumbens (BETA 951) DNA (except JKIPat16: resequenced from genomic P. webbiana DNA).

Touchdown PCR profile: 5 min at 94°C; followed by 12 cycles of 30 s at 94°C, 45 s at 60–54°C (decreasing by 0.5°C/cycle), 45 s at 72°C; followed by 30 cycles of 30 s at 94°C, 45 s at 54°C, 45 s at 72°C; followed by a final extension at 72°C for 10 min.

Plant material and PCR protocol—Three P. patellaris populations originating from Murcia (AZO), Balena (BAL), and Alicante (MOR), as well as one population each of P. procumbens (Tenerife) and P. webbiana (Gran Canaria), were included within the analysis (Appendix 1). The collectors photographed the plants of the five occurrences for documentation, collected voucher specimens of the three P. patellaris populations, sampled a maximum of 1 g of fresh leaf material from 20 to 40 individuals per species (Appendix 1), desiccated the material using silica gel within 24 h until brittle (Chase and Hills, 1991), and stored it at room temperature before further processing. Genomic DNA was prepared from dried (20 mg) leaf material after vigorous homogenization in a mixer-mill disruptor according to a modified cetyltrimethylammonium bromide (CTAB) protocol (Saghai-Maroof et al., 1984). DNA amplification was carried out in a total volume of 10 μL. The PCR mix contained 25 ng of template DNA, 1.5 mM MgCl₂, 200 μM of each dNTP, 0.25 μM of each primer, and 0.5 units Taq DNA polymerase. A touchdown PCR profile was generally used (Table 1).

Microsatellite marker data analysis—Numbers of SSR alleles, polymorphism information content (PIC), observed heterozygosity (H_{o}), and gene diversity or expected heterozygosity (H_{e}) were calculated using the ALLELE procedure of SAS (version 9.3; SAS Institute, Cary, North Carolina, USA).

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Along with the 25 polymorphic SSR loci yielded 85, 187, and 202 alleles in P. patellaris, P. procumbens, and P. webbiana, respectively. Most of the 25 SSR markers showed polymorphism in all three species. JKIPat16 constituted an exception as it amplified specifically in P. webbiana (Appendix S1). The number of alleles per locus within a species ranged from one to seven (P. patellaris), two to 15 (P. procumbens), and two to 14 (P. webbiana) (Table 2, Appendix S1). Of the individuals examined in the tetraploid species P. patellaris, each proved to carry a maximum of two alleles per SSR, possibly indicating allotetraploidy of this species.

The PIC values were lowest in P. patellaris (0.0730), followed by P. webbiana (0.040–0.878), and highest in P. procumbens (0.317–0.883). H_o and H_e were lowest in P. patellaris (H_o = 0.000–1.000, H_e = 0.000–0.766), slightly higher in P. webbiana (H_o = 0.042–0.917, H_e = 0.041–0.888), and highest in P. procumbens (H_o = 0.208–0.958, H_e = 0.353–0.893) (Table 2, Appendix S1). Apart from phenotypic variation due to environmental effects, the three P. patellaris populations showed no apparent morphological differences. However, at the genetic level (Table 2), population BAL showed the highest genetic diversity and polymorphism among the three species, and may also be useful for investigations of the species’ mating systems and seed dispersal mechanisms.

The data presented here underline the field observations. The plant stand of P. procumbens sampled at Punta del Hidalgo showed large morphological variation that cannot be solely explained by a higher phenotypic plasticity or environmental factors. The high phenotypic variation at the natural site corresponds well with the high genetic diversity observed in P. procumbens. Self-fertile P. patellaris used in this study showed less SSR marker variation than the self-sterile species P. procumbens, which is likely due to a limited gene flow between occurrences of a self-fertile species that, in addition, is distributed in spatially isolated patches. These observations need to be investigated in detail in further studies.

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APPENDIX 1. Voucher information for *Patellifolia* species used in this study.

| Species | Population ID | Voucher specimen accession no.* | Collection locality | Collector | Geographic coordinates | n |
|---------|---------------|---------------------------------|---------------------|-----------|------------------------|---|
| *P. patellaris* (Moq.) A. J. Scott, Ford-Lloyd & J. T. Williams | BAL2104150900 | GeDiPa-project-17, GeDiPa-project-18 | Balerma, Playa de Balerma, Spain | Maria Luisa Rubio Teso & Linney Duarte | 36.723517°N, 2.88011°W | 40 |
| *P. patellaris* | MOR090315100 | GeDiPa-project-1, GeDiPa-project-2, GeDiPa-project-3 | Alicante, Cap de Moraira, Cova de les Cendres, Spain | P. Pablo Ferrer Gallego & Inmaculada Ferrando | 38.68559°N, 0.152064°E | 20 |
| *P. patellaris* | AZO2403151630 | GeDiPa-project-12 | Murcia, La Azohia, Playa de la Azohia, Spain | Maria Luisa Rubio Teso | 37.55742°N, 1.168407°W | 24 |
| *P. procumbens* (Chr. Sm.) A. J. Scott, Ford-Lloyd & J. T. Williams | TPH0604151144 | — | Tenerife, Punta del Hidalgo, Spain | Lothar Frese | 28.573109°N, 16.318080°W | 24 |
| *P. webbiana* (Moq.) A. J. Scott, Ford-Lloyd & J. T. Williams | Graisl1 | — | La Isleta, Gran Canaria, Spain | Arnoldo Santos Guerra | 28.165702°N, 15.437347°W | 24 |

*Note: n = number of individuals.*

*Vouchers deposited at the Herbarium of the Instituto de Investigação Científica Tropical (LISC), Lisbon, Portugal. For TPH0604151144, several plants were photographed to document the phenotypic variation. For Graisl1, *P. webbiana* is a highly endangered species; a photo was taken.*