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PRIMER NOTE

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

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- *Premise of the study:* Microsatellite primers were developed to promote studies on the patterns of genetic diversity within *Patellifolia patellaris* (Chenopodiaceae) and the relationship between the three species of the genus *Patellifolia*.
- *Methods and Results:* The genomic sequence from *P. procumbens* was screened for simple sequence repeats (SSRs), and 3648 SSRs were identified. A subset of 53 SSR markers was validated, of which 25 proved to be polymorphic in the three species except for the *P. webbiana*–specific marker JKIPat16. The number of alleles ranged from 85 in *P. patellaris*, 187 in *P. procumbens*, and 202 in *P. webbiana*.
- *Conclusions:* The set of 25 new markers will facilitate studies of the relationships between the three *Patellifolia* species and of the spatial and temporal distribution of genetic diversity within the species.

Key words: Chenopodiaceae; genetic diversity; microsatellite marker; *Patellifolia*; polymorphism.

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. Assessing genetic diversity and the extent of genetic erosion within species is essential for planning and implementation of effective conservation management and utilization programs.

Molecular markers like simple sequence repeats (SSRs) or microsatellites often exhibit a high allelic diversity and are able to detect polymorphisms (Wan et al., 2004), even between individuals (Jarne and Lagoda, 1996). SSRs represent sets of repeated small sequences found throughout the genome (Morgante and Olivieri, 1993). SSR markers developed in *Beta vulgaris* L. (McGrath et al., 2007) proved to be unsuitable for genetic diversity studies in *Patellifolia*. Furthermore, in our analysis of six SSR markers (Bv2, Bv3, Bv6, Bv7, BvMS67, and BvMS86) provided by El Bahloul and Gaboun (2013), we found that only two (Bv3, BvMS86) produced polymorphic PCR products in *Patellifolia*. 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 Papro-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 Primerfox (http://www.primerfox.com/) and 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[a]	Allele size range (bp)	T_a (°C)[b]	GenBank accession no.
JKIPat01	F: AGAGTACCTTGGAGGAATGG	$(GA)_8$	170–191	50	KU888809
	R: CTTTAATAGATAGGGCCGCG				
JKIPat02	F: AACGTCAACAAGCCCAATCG	$(CA)_8$	196–227	50	KU888810
	R: AGGGAAAGTTGTAGTCCTGC				
JKIPat03	F: TTGCTTCATTCAACAGCCGC	$(TG)_{19}$	198–231	52	KU888811
	R: AAGTTCCATCATCCTGCAGG				
JKIPat04	F: TCTCTATTGGCCGGAAATGG	$(CT)_8$	216–228	52	KU888812
	R: AGAAAGAAAGCAGAGCAGGG				
JKIPat05	F: TTCTATCCTGCTGCTTCTGG	$(GT)_8(GA)_{16}$	181–232	50	KU888813
	R: GCTCAAAGTCTGCATTTCCC				
JKIPat06	F: AAGAAGAGAGAGCAGACAGC	$(AG)_{24}$	161–197	48	KU888814
	R: TCTCTGGTCCTCAAACAACC				
JKIPat07	F: CTTCTTGCCTCTCATCTTGG	$(TC)_8$	173–179	48	KU888815
	R: GGGTACACATTCATGTGTCC				
JKIPat08	F: TCGAAATTGGGAAAGGGTGG	$(AG)_8$	183–193	48	KU888816
	R: CGGTCTCTGAAAGTTCATCC				
JKIPat10	F: ACCATGTTGGAGTTTCGAGG	$(CA)_6$	164–169	52	KU888817
	R: AGAACCCTTGTTTGGGAACG				
JKIPat11	F: CTCTTCTCACTTCTCACACG	$(TC)_{11}$	157–184	54	KU888818
	R: TTTGGTTGATGTGGTTGGGC				
JKIPat12	F: GCAAGGAATTTGCAGTGAGG	$(AG)_9$	165–191	54	KU888819
	R: CGGCAAACAAACTCAATCCG				
JKIPat13	F: TACCTTGTGGTGACTTCTGG	$(CA)_2(GA)_8$	151–179	48	KU888820
	R: ACAAGTATTCAGCAGGCAGC				
JKIPat14	F: TTTCCTTGCTCATGTGTGGC	$(AC)_8$	219–223	54	KU888821
	R: AAACGCTTGGCATGACTTGC				
JKIPat15	F: GACCATGTGACGTCTAAACC	$(GT)_{10}$	174–192	52	KU888822
	R: TTGCCTCAATCATCACCACC				
JKIPat16	F: TTATACACACACACACGCGC	$(CA)_{29}$	224–256	48	KU888823
	R: CTTACTGGCGTTCTCTTTCC				
JKIPat17	F: TCCCTCATTAACAAAGCCGC	$(CT)_{14}$	188–202	48	KU888824
	R: AGTTCAGCTACTTCATGCCG				
JKIPat18	F: CTGGCAAGGTTAACGTTACC	$(TG)_8(AG)_4$	177–191	48	KU888825
	R: GGATCAGCATTAGTCAACGG				
JKIPat19	F: AACGCAAGCATAGTCAGTGG	$(GGAT)_8$	203–258	52	KU888826
	R: TGCGAATTGCGTTGTTCAGC				
JKIPat20	F: TGTCTTAATCCGCTTGTCCG	$(TCTT)_9$	231–256	50	KU888827
	R: ATCAGTCAATCAGGATGCCG				
JKIPat21	F: GCTGAAGCACTAATTTGGGC	$(GGAA)_7$	175–195	50	KU888828
	R: ATGCAACCTCACTCTTCTCG				
JKIPat22	F: AATGGAAGAAGTTGAGGGCC	$(GAAA)_3$	130–148	50	KU888829
	R: GTCTTCTTCTCCTCTCTTCC				
JKIPat23	F: AAAGATAACGACACGTGGCG	$(TA)_8(GATA)_5$	185–224	50	KU888830
	R: CAATGAATGGTGGAAGGAGG				
JKIPat24	F: TGCTCAGCAAATCACTGAGG	$(ATTC)_7$	182–214	48	KU888831
	R: GGTATTCAGACTCAACCTGG				
JKIPat25	F: TTTGAAATCCTGGTTCCGCC	$(GTGA)_9$	179–195	50	KU888832
	R: AGTCCAACCACCTTAGTACC				
JKIPat26	F: GTAGTCTGGTTCAAGACTCG	$(GA)_9(TAGA)_3$	159–191	48	KU888833
	R: GGAGGCTTCTTTGAAGATCC				

Note: T_a = annealing temperature.

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

[b]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.

electrophoresis Genome Laboratory GeXP Genetic Analysis System (Beckman Coulter, Brea, California, USA), resulting in 25 polymorphic markers, as well as by cloning and resequencing of the PCR products (Table 1).

***Plant material and PCR protocol*—**Three *P. patellaris* populations originating from Murcia (AZO), Balerma (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 $MgCl_2$, 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).

TABLE 2. Genetic key data of newly developed SSR markers in three different *Patellifolia patellaris* populations.

	BAL2104150900 (n = 40)				MOR0903151000 (n = 20)				AZO2403151630 (n = 24)			
Locus	A	PIC	H_o	H_e	A	PIC	H_o	H_e	A	PIC	H_o	H_e
JKIPat01	3	0.174	0.000	0.184	2	0.091	0.000	0.095	1	0.000	0.000	0.000
JKIPat02	5	0.445	0.975	0.548	1	0.000	0.000	0.000	4	0.653	1.000	0.707
JKIPat03	3	0.371	0.725	0.471	6	0.697	0.700	0.733	2	0.364	0.792	0.478
JKIPat04	4	0.593	0.950	0.654	3	0.442	0.950	0.546	2	0.375	1.000	0.500
JKIPat05	3	0.411	0.975	0.524	1	0.000	0.000	0.000	1	0.000	0.000	0.000
JKIPat06	5	0.428	0.975	0.536	3	0.442	0.800	0.545	1	0.000	0.000	0.000
JKIPat07	6	0.446	0.975	0.548	2	0.091	0.000	0.095	1	0.000	0.000	0.000
JKIPat08	3	0.416	0.000	0.501	1	0.000	0.000	0.000	1	0.000	0.000	0.000
JKIPat10	4	0.368	0.000	0.423	2	0.223	0.000	0.255	1	0.000	0.000	0.000
JKIPat11	2	0.048	0.000	0.049	5	0.703	0.750	0.744	6	0.725	0.083	0.761
JKIPat12	4	0.428	0.975	0.536	2	0.375	1.000	0.500	3	0.525	1.000	0.603
JKIPat13	2	0.375	1.000	0.500	4	0.554	1.000	0.628	4	0.570	0.958	0.636
JKIPat14	4	0.475	0.925	0.569	1	0.000	0.000	0.000	2	0.328	0.000	0.413
JKIPat15	4	0.677	1.000	0.728	5	0.530	1.000	0.608	6	0.730	1.000	0.766
JKIPat17	2	0.129	0.000	0.139	2	0.091	0.000	0.095	2	0.239	0.250	0.278
JKIPat18	2	0.048	0.000	0.049	2	0.091	0.000	0.095	4	0.219	0.042	0.228
JKIPat19	5	0.618	1.000	0.677	5	0.644	0.950	0.696	3	0.525	1.000	0.603
JKIPat20	2	0.091	0.000	0.095	1	0.000	0.000	0.000	1	0.000	0.000	0.000
JKIPat21	7	0.524	0.950	0.603	2	0.375	1.000	0.500	2	0.375	1.000	0.500
JKIPat22	2	0.180	0.225	0.200	3	0.347	0.400	0.395	2	0.359	0.667	0.469
JKIPat23	4	0.280	0.000	0.303	1	0.000	0.000	0.000	1	0.000	0.000	0.000
JKIPat24	2	0.375	1.000	0.500	2	0.223	0.000	0.255	1	0.000	0.000	0.000
JKIPat25	3	0.421	0.675	0.508	2	0.269	0.400	0.320	1	0.000	0.000	0.000
JKIPat26	4	0.476	1.000	0.569	4	0.525	0.900	0.606	3	0.525	1.000	0.603
Total	85				62				55			

Note: A = number of observed alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled; PIC = polymorphism information content.

Altogether, 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* (0–0.730), 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 with a total of 85 different alleles and all markers exhibiting polymorphisms, followed by population MOR (62 alleles) and population AZO (55 alleles). All markers except one (JKIPat17) yielded different numbers of alleles in the three populations (Table 2), reflecting high resolution of the marker set and its suitability for the analysis of genetic variation within and between *Patellifolia* populations.

CONCLUSIONS

Since the second half of the 19th century, taxonomists and geneticists have worked on the small genus *Patellifolia*. However, a reliable key to the species still does not exist and information on the evolutionary relationships between the three species is scarce. The new set of highly polymorphic SSR markers may prove useful to fill existing knowledge gaps. For instance, the 25 SSRs reported here may be used for studying the large-scale spatial distribution pattern of genetic diversity within the genus *Patellifolia*, the pattern of fine-scale spatial genetic structure at the population level, and evolutionary relationships 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.[a]	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	37.557442°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, 15.437437°W	24

Note: *n* = number of individuals.

[a]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.