Microsatellite Primers for a Species of South African Everlasting Daisy (Helichrysum odoratissimum; Gnaphalieae, Asteraceae)

Authors: Glennon, Kelsey L., and Cron, Glynis V.

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Primer Note

Microsatellite primers for a species of South African everlasting daisy (*Helichrysum odoratissimum*; Gnaphalieae, Asteraceae)[1]

Kelsey L. Glennon[2,3] and Glynis V. Cron[2]

[2]School of Animal, Plant, and Environmental Sciences, University of the Witwatersrand, Private Bag 3, WITS 2050 Johannesburg, South Africa

- *Premise of the study:* Microsatellites were developed for the widespread *Helichrysum odoratissimum* (Asteraceae) to estimate gene flow across diploid populations and to test if gene flow occurs among other closely related lineages within this genus.
- *Methods and Results:* Ten primer pairs were developed and tested using populations across South Africa; however, only seven primer pairs were polymorphic for the target species. The seven polymorphic primers amplified di- and trinucleotide repeats with up to 16 alleles per locus among 125 diploid individuals used for analyses.
- *Conclusions:* These markers can be used to estimate gene flow among populations of known ploidy level of *H. odoratissimum* to test evolutionary hypotheses. Furthermore, these markers amplify successfully in other *Helichrysum* species, including the other three taxonomic Group 4 species, and therefore can be used to inform taxonomic work on these species.

Key words: Asteraceae; gene flow; *Helichrysum odoratissimum*; polyploidy; southern Africa; speciation.

South Africa's plant diversity is remarkable; however, the actual number of species that comprise this diversity is unknown. Polyploidy plays a prominent role in plant diversity in that ~15% of speciation events occur via polyploidy and ~35% of plant genera contain polyploids (Wood et al., 2009). Recent work highlights the gap in polyploid research in South Africa and the need to assess the presence of polyploid species (Krejčíkova et al., 2013). *Helichrysum odoratissimum* (L.) Sweet is a native South African perennial herb that belongs to tribe Gnaphalieae in the Asteraceae (daisy family). The species is also part of Hilliard's taxonomic Group 4 in the genus, which includes three additional species, *H. gymnocomum* DC., *H. infaustum* J. M. Wood & M. S. Evans, and *H. griseolanatum* Hilliard (Hilliard, 1983). *Helichrysum odoratissimum* contains three different ploidy levels: diploid, tetraploid, and hexaploid ($2n$ = 14, 28, 42, respectively; Galbany-Casals and Romo, 2008). Given that there are three ploidy levels in *H. odoratissimum*, polyploidy may contribute to the morphological and ecological diversity in this species, and subsequently to South Africa's remarkable floral diversity. We developed microsatellites for future work that will examine gene flow patterns among *H. odoratissimum* populations of known ploidies.

METHODS AND RESULTS

DNA was extracted from 125 *H. odoratissimum* diploid individuals that were collected from across South Africa (Appendix 1) using QIAGEN's DNeasy Extraction kits following the manufacturer's protocol (QIAGEN, Valencia, California, USA). Extracted DNA was visualized on a 1% agarose gel. Four DNA samples from four representative populations (Appendix 1) were sent to Inqaba Biotec (Pretoria, South Africa) for initial microsatellite enrichment. At Inqaba, a Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) approach was used to identify microsatellite regions (Zane et al., 2002). Briefly, DNA is digested using *Mse*I, resulting amplicons are enriched for repeat regions using biotinylated beads, and identified enriched regions are spliced into vectors to separate out and amplify repeat-enriched regions. Inqaba then sequenced 10 vectors that ranged approximately 250–600 bp in size, to ensure that the colonies contained repeat regions and no regions were over-represented. Once enrichment was confirmed, the microsatellite library was sequenced using next-generation sequencing (NGS) techniques. Once compiled, NGS data were received from Inqaba and MSATCOMMANDER version 2.8 (Faircloth, 2008) was used to identify unique microsatellite repeat regions. From these data, we identified 30 potential regions (10 di-, 10 tri-, and 10 tetranucleotide repeats) and developed primer pairs for these regions using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012; Table 1).

We tested these primer pairs for amplification success and possible polymorphism across 10 geographically distinct *H. odoratissimum* populations (10 individuals total; Appendix 1). Primers were diluted to 20 µM and tested using the following PCR protocols. Amplifications were performed at a 10-µL final volume of DreamTaq 2× Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA) with 0.2 µM of labeled forward and reverse primers, and 0.5 µL of 1 µg/mL bovine serum albumin (BSA). Two primers (Ho12, Ho13) were amplified using the above recipe with the addition of 0.1 µM 5′ M13 forward primer labeled with 6-FAM dye, and touchdown PCR conditions were followed according to Schuelke (2000). All reactions included 1 µL DNA of varying concentrations dependent on the sample (5–20 ng estimated from agarose gel visualization). For the other eight primers, we followed the DreamTaq PCR program outline: 95°C for 3 min; 95°C for 40 s; a specified annealing

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[3]Author for correspondence: kelsey.glennon@wits.ac.za

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TABLE 1. Characteristics of 10 microsatellite loci for *Helichrysum odoratissimum*.[a]

Locus	Primer sequences (5′–3′)[b]	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
Ho1	F: ACCACCTCCTCACCTTTCAC R: GCCGTGGTTCTTTATTCCCG	$(AG)_8$	144–206	50.9	Pr032756436
Ho2	F: GGATGGCGATTCCCAATTGG R: CCTCCTCACTCCTAAGCACC	$(AG)_{12}$	155–243	50.9	Pr032756441
Ho4	F: CAAGATGTGCAGCGAGTGAG R: GTCACGAGAGGACCAAGGAG	$(AC)_{18}$	159–196	50.9	Pr032756442
Ho7	F: ATGCTGATGGTGGCTAGGTC R: ACCCCTTTCTAACCGACACC	$(TGG)_{11}$	169–203	50.9	Pr032756444
Ho8	F: GACACCTAGCCAAGGACTGC R: GCGTCCCCTGAGTTCATTAC	$(CAA)_8$	198–249	50.9	Pr032756445
Ho12	F: [M13F]GAGATTGGGATTTCTTCTTG R: CCACACCTTCACTACCACTAC	$(GTG)_{18}$	153–210	TD[c]	Pr032756437
Ho13	F: [M13F]TATTTCAAGAAAACTGCCCTAA R: CAGATAAGTTGTTGAGGAGGA	$(ACC)_{19}$	149–203	TD[c]	Pr032756438
Ho5*	F: AGATGGTGGTCACTACTGCC R: GCCCGTTCCTCTCAACAAAG	$(AC)_{15}$	195	58	Pr032756443
Ho17*	F: CGACTGATGAGTCCTGAGTAA R: TCATTTCCTTCACTCTCACAC	$(AG)_{18}$	135	55	Pr032756439
Ho18*	F: TTCCTTATCAAACATTTACCG R: TCGTTGTTTTGTGTTGAGTATT	$(CCA)_{21}$	304	58	Pr032756440

Note: T_a = annealing temperature.

[a]The seven polymorphic markers of the 10 markers were tested on 125 samples (from 10 populations across South Africa), representing populations found across South Africa (see Appendix 1).

[b]Two primers (Ho12, Ho13) were amplified using an M13F tag primer and a touchdown (TD) protocol from Schuelke (2000). [M13F] = Additional sequence added to forward primer for labeled tagging: TGTAAAACGACGGCCAG.

[c]Annealing temperatures for this PCR program are 56°C and then a subsequent 53°C, following the touchdown thermocycling program outlined in Schuelke (2000).

* Indicates that the primer was monomorphic for the species and was not used in further genetic analyses.

temperature (Table 1) for 40 s; 72°C for 30 s; 72°C for 4 min. Gel electrophoresis was used to assess polymorphism of the primer pairs using a 4% MetaPhor agarose gel (Lonza, Sigma-Aldrich Pty. Ltd., Johannesburg, South Africa) with a 50-bp ladder (ThermoFisher Scientific). Genetic analyses only included microsatellites that were deemed to be polymorphic across the 10 populations from agarose gels. Of the 30 pairs, we identified 10 primer sets that amplified successfully and repeatedly. Of those 10 primer sets, seven were polymorphic for our target species. We then cloned putative polymorphic amplicons from 2–5 individuals using a QIAGEN PCR Cloning*plus* Kit (QIAGEN, Whitehead Scientific (Pty.) Ltd., Johannesburg, South Africa) per the manufacturer's instructions. Amplified clone inserts from three individuals per primer pair (1–5 inserts per individual depending on cloning success) were sent to the DNA Sequencing Unit at the Central Analytical Facility (CAF), Stellenbosch University (Stellenbosch, South Africa), to verify that the amplicons contained the correct repeat region. Once verified, the remaining 114 individuals were amplified. Each forward primer was given a different dye label (VIC, NED, FAM) to allow for pooling PCR products for fragment analysis at CAF. Fragment analysis was carried out on an ABI 3130xl (Applied Biosystems, Foster City, California, USA) using a GeneScan 500 LIZ Size Standard (Applied Biosystems). Chromatographs were scored manually using PeakScanner version 1.0 (Applied Biosystems). Data were then formatted for population genetic analyses.

TABLE 2. Genetic diversity indices across populations for the seven polymorphic microsatellite loci.

Population[a]	A	H_o	H_e	F_{IS}
Mike's Pass, KZN (n = 5)	4.0	0.543	0.614	0.224
Cathkin, KZN (n = 5)	3.0	0.429	0.458	0.065
Monk's Cowl, KZN (n = 5)	2.571	0.714	0.488	–0.463
Buffleskloof Nature Reserve, MP (n = 17)	7.286	0.795	0.773	–0.054
Steepside, EC (n = 10)	4.857	0.706	0.710	0.005
Elliot, EC (n =30)	8.429	0.561	0.755	0.258*
Naude's Nek, EC (n = 6)	5.0	0.538	0.762	0.294
Circle Forest, WC (n = 13)	5.286	0.529	0.602	0.122
Swartberg, WC (n = 9)	3.286	0.411	0.415	0.010
Piketberg, WC (n = 25)	5.714	0.534	0.592	0.120

Note: A = average number of alleles identified for each locus; F_{IS} = fixation index; H_e = expected heterozygosity within populations; H_o = observed heterozygosity; n = number of individuals representing each population.

[a]South African provinces: EC = Eastern Cape; KZN = KwaZulu-Natal; MP = Mpumalanga; WC = Western Cape. See Appendix 1 for locality and voucher information.

* Indicates that population was not in Hardy–Weinberg equilibrium after a Bonferroni correction of multiple comparisons (adjusted P = 0.001).

We used 125 individuals from 10 populations from across South Africa to establish the usefulness of the seven polymorphic primers in population genetic analyses. These individuals are known diploids from flow cytometry data (Glennon et al., in prep.) and published chromosome counts (Galbany-Casals and Romo, 2008). Seven of the 10 loci were polymorphic within diploid *H. odoratissimum*. The number of alleles (A) and observed (H_o) and expected heterozygosity (H_e) were estimated for each locus using GenoDive (Meirmans and Van Tienderen, 2004; Table 2). Furthermore, deviation from Hardy–Weinberg equilibrium (HWE) was tested for each population in GenoDive using a fixation index (F_{IS}) estimate that is equivalent to Weir and Cockerham's (1984) F-statistics. We conducted a Bonferroni correction for HWE significance levels due to multiple comparisons of populations across loci. Linkage disequilibrium was tested using GenePop (Raymond and Rousset, 1995; Rousset, 2008).

The mean A per locus was 15.42 (Table 2). H_o values ranged from 0.172 to 0.848, and H_e values ranged from 0.367 to 0.870 across loci. Only one population deviated from HWE (Elliot, Table 2), and F_{IS} values ranged from –0.463 to 0.294. This deviation may be due to potential substructuring of the population, as it spanned a large geographic area. Genetic diversity estimates across the 10 populations showed that the average A per population was 4.9 and that H_o values ranged from 0.411 to 0.795 (Table 2). There was linkage disequilibrium between three pairs of the primers (Ho1/Ho2, Ho2/Ho7, Ho1/Ho12), but only in three of the 10 populations (Buffleskloof, Elliot, and Piketberg). Lastly, we tested a total of 10 additional *Helichrysum* Mill. species found throughout South Africa to test for cross-species amplification (Appendix 1). PCR amplicons were run on a 4% MetaPhor agarose gel to determine if the loci were polymorphic among species. We found that the primers amplified across all of the taxa, but bands were relatively faint for *H. dasycephalum* O. Hoffm., *H. glomeratum* Klatt, and *H. psilolepis* Harv. The amplicons differed in size across all species ranging from approximately 150 to 350 bp. We also successfully amplified DNA from additional polyploid *H. odoratissimum* individuals (data not shown).

CONCLUSIONS

These seven loci will be useful in determining patterns of gene flow as well as other population genetic parameters for future studies of population divergence within *H. odoratissimum*. The polymorphic microsatellites amplified will be useful in studies regarding speciation patterns in the taxonomic Group 4 of *Helichrysum*, and together with the monomorphic markers may also be potentially useful for future taxonomic revisions of closely related *Helichrysum* species in South Africa.

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Appendix 1. Species used, voucher, collection locality, and GPS coordinates of all populations that represent individuals used in this study.

Species	Voucher[a]	Collection locality[b]	GPS coordinates	*n*
Helichrysum odoratissimum (L.) Sweet	KG01	Cathkin, KZN[†]	29°0′45.35″S, 29°25′9.76″E	5
	KG02	Mike's Pass, KZN	28°58.106′S, 29°14.073′E	5
	KG03	Monk's Cowl, KZN[†]	29°03.348′S, 29°23.907′E	5
	KG06	Buffleskloof Nature Reserve, MP	25°17.339′S, 30°31.732′E	17
	KG15	Steepside, EC	30°50′57.91″S, 27°48′3.45″E	10
	KG17	Elliot, EC[†]	31°13′10.77″S, 27°50′47.39″E	30
	KG23	Naude's Nek, EC	30°43′56.71″S, 28°8′33.86″E	6
	KG43	Circle Forest, WC	33°58′59.3″S, 22°58′21.35″E	13
	KG47	Piketberg, WC	32°45′41.29″S, 23°10′3.44″E	25
	KG44	Swartberg, WC[†]	33°21′30.65″S, 22°3′15.6″E	9
H. griseolanatum Hilliard	KG25	Woodcliff, EC	30°59′13.19″S, 28°16′1.19″E	3[c]
H. gymnocomum DC.	Cron s.n.	Monk's Cowl, KZN	28°56′24.53″S, 28°56′24.53″E	1[c]
H. infaustum J. M. Wood & M. S. Evans	Cron 1135	Witsieshoek, KZN	28°31′59.88″S, 28°49′0.12″E	5[c]
H. appendiculatum (L. f.) Less.	Cron 1091	Monk's Cowl, KZN	28°56′24.53″S, 28°56′24.53″E	1[c]
H. aureonitens Sch. Bip.	Cron 1110	Cathedral Peak, KZN	28°55′23.88″S, 29°8′2.04″E	1[c]
H. glomeratum Klatt	KG38	Elliot, EC	31°13′10.77″S, 27°50′47.39″E	1[c]
H. nudifolium (L.) Less. var. *piloselloides* (L. f.) Beentje	Cron 1026	Barberton, MP	25°47′9.96″S, 31°3′11.16″E	1[c]
H. splendidum (Thunb.) Less.	Cron 1092	Buffleskloof Nature Reserve, MP	25°17′20.33″S, 30°31′43.91″E	1[c]
H. psilolepis Harv.	KG34	Skilderkraans, EC	31°10′7.57″S, 27°11′29.62″E	1[c]
H. dasycephalum O. Hoffm.	KG18	Elliot, EC	31°13′10.77″S, 27°50′47.39″E	1[c]

Note: *n* = number of individuals.

[a] Voucher specimens deposited at C. E. Moss Herbarium, University of the Witwatersrand, Johannesburg, South Africa.

[b] South African provinces: EC = Eastern Cape; KZN = KwaZulu-Natal; MP = Mpumalanga; WC = Western Cape.

[c] Samples used to test cross-amplification success of *Helichrysum* species.

[†] Populations for which an individual was sent for microsatellite development.