Polymorphic Microsatellite Loci for *Haumania danckelmaniana* and Transferability to *H. liebrechtsiana* (Marantaceae)

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Primer Note

Polymorphic microsatellite loci for *Haumania danckelmaniana* and transferability to *H. liebrechtsiana* (Marantaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed for the species *Haumania danckelmaniana* (Marantaceae) from central tropical Africa.
- *Methods and Results:* Microsatellite isolation was performed simultaneously on three different species of Marantaceae through a procedure that combines multiplex microsatellite enrichment and next-generation sequencing. From 80 primers selected for initial screening, 20 markers positively amplified in *H. danckelmaniana*, of which 10 presented unambiguous amplification products within the expected size range and eight were polymorphic with four to nine alleles per locus. Positive transferability with the related species *H. liebrechtsiana* was observed for the same 10 markers.
- *Conclusions:* The polymorphic microsatellite markers are suitable for studies in genetic diversity and structure, mating system, and gene flow in *H. danckelmaniana* and the closely related species *H. liebrechtsiana*.

Key words: Africa; *Haumania*; Marantaceae; phylogeography; rainforest; simple sequence repeat (SSR).

Haumania J. Léonard is a plant genus of the family Marantaceae endemic to central tropical Africa (Dhetchuvi, 1996). The genus comprises three species of climbers confined to the understory, gaps, and forest edges of lowland tropical rainforest. *Haumania danckelmaniana* (J. Braun & K. Schum.) Milne-Redh. is distributed from Cameroon and Equatorial Guinea to Gabon and northern Republic of the Congo. *Haumania liebrechtsiana* (De Wild. & T. Durand) J. Léonard can be found from Gabon to the Republic of the Congo and the Democratic Republic of the Congo. *Haumania leonardiana* G. Evrard & Bamps is distributed in northern Democratic Republic of the Congo. With their parapatric distribution pattern across phytogeographic domains in central Africa, species of the genus *Haumania* are of great interest in the field of phylogeography to extend our knowledge of the patterns and processes of speciation and intraspecific diversification in this tropical region. In this study, we isolated polymorphic microsatellite loci from *H. danckelmaniana* and showed that they can be transferred to the closely related species *H. liebrechtsiana*, allowing for further phylogeographic investigations in tropical central Africa.

METHODS AND RESULTS

Microsatellite primers were isolated in *H. danckelmaniana* at Genoscreen (Lille, France) using the 454 GS-FLX Titanium platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) through a procedure that combines multiplex microsatellite enrichment and pyrosequencing (Malausa et al., 2011; Micheneau et al., 2011). Total DNA was extracted from silica gel–dried leaves using the NucleoSpin Plant kit (Macherey-Nagel, Düren, Germany). A mixture of ca. 5 µg of DNA of three species to simultaneously recover microsatellite primers in a single next-generation sequencing run (*H. danckelmaniana*, *Hypselodelphys poggeana* (K. Schum.) Milne-Redh., *Marantochloa incertifolia* Dhetchuvi, including multiple samples from the same locality per species; Appendix 1) was sent to Genoscreen, and was used to isolate microsatellite loci using a 1/32nd GS-FLX plate of the Roche Sequencer (454 Life Sciences, a Roche Company), following the protocol of Malausa et al. (2011).

We used Primer3 (Rozen and Skaletsky, 1999) implemented in the QDD bioinformatics pipeline (Meglécz et al., 2010) with the criteria described in Malausa et al. (2011) to automatically design 5657 primer pairs targeting 1285 microsatellite loci. From these, we selected 80 primer pairs representing the longest di- and trinucleotide repeats (≥8 repeats), and having more than 10 flanking nucleotides between microsatellite motifs and designed primers. The 80 designed primer pairs were first tested individually to verify amplification in *H. danckelmaniana*, with the following PCR conditions: 1 µL buffer (10×), 0.4 µL $MgCl_2$ (25 mM), 0.3 µL dNTPs (10 mM each), 0.2 µL of each primer (0.01 mM), 0.05 µL *Taq* polymerase (TopTaq DNA Polymerase, 5 U/µL; QIAGEN, Venlo, The Netherlands), 1 µL of template DNA (of ca. 10–50 ng/µL, specimen ACL0711), and H_2O to make a final volume of 10 µL. Amplifications were performed as follows: 94°C (4 min), followed by 40 cycles of 94°C (30 s), 56°C (45 s), 72°C (1 min), and a final extension at 72°C for 10 min. Twenty markers generated products as indicated on a 1% agarose gel stained with SYBR Safe (Invitrogen, Merelbeke, Belgium).

For these 20 markers, unambiguous amplification and levels of polymorphism were tested on up to seven individuals chosen across the entire species' distribution range (Appendix 1) using a modified protocol of Schuelke (2000), which incorporates three primers (M13-like protocol) (for further details see Micheneau et al., 2011). Ten markers presented unambiguous amplification products within the expected size range, and eight of these were polymorphic (for primers see Table 1). These latter 10 loci also amplified in the closely related species *H. liebrechtsiana* using the same protocol as described for *H. danckelmaniana.*

With the eight polymorphic markers, preliminary population genetic analyses were carried out in two populations per species (*H. danckelmaniana*: Ivindo

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Table 1. Characterization of 10 microsatellite markers isolated from *Haumania danckelmaniana*.

Locus	Primer sequences (5′–3′)	Fluorescent label[a]	Repeat motif	Allele size range (bp)	*A*	T_a (°C)	GenBank accession no.
F2†	F: TGAAATGGAGGCACAGCTAA R: AAAATTGGCATGGAGAGGTG	Q4-PET	$(TTC)_{16}$	123–155	6	59.93	KT806120
F28†	F: CATGGTGGTCTCCACTTTGC R: CCGACCACGTCTTACCAAGT	Q1-6-FAM	$(TCG)_9$	184–206	8	60.02	KT806121
F41‡	F: GCAATTCTTTCCTGCTCACC R: TGGGGACTTCGCAAGATAAA	Q1-6-FAM	$(TG)_9$	184	1	60.57	KT806122
F62†	F: ATTTCTTATCGCCTGCCTCA R: TCTTCATGTGGTGTCTGGTCTC	Q3-VIC	$(AG)_8$	256–263	5	60.15	KT806123
F65†	F: TCCTCTTCATTCACTCGCTAGA R: TCTGCAAAGACAAACACAATCA	Q1-6-FAM	$(TCT)_8$	235–244	4	60.54	KT806124
F66†	F: GAAGCAAGGCAATCACCATT R: GCATAAATTCTCCACTAGCATGG	Q2-NED	$(AAG)_8$	179–200	8	60.00	KT806125
F67‡	F: TTTTAACGGAATCTTGCAGGG R: TTTTGCAGGAACCACCTACC	Q4-PET	$(CT)_8$	123	1	59.97	KT806126
F70†	F: GCAATGCAGGCCATAGGTAA R: CGCTTTACGAGGGCATTTTA	Q4-PET	$(ACG)_8$	198–211	5	60.21	KT806127
F74†	F: ACGCCTAGCAGCCAAGTAGA R: CGCAACCCCATACTCCATAC	Q3-VIC	$(AGA)_8$	175–185	5	60.21	KT806128
F80‡	F: GGACTCAAAAGGAATCCTAAGACA R: TGATCAAGGTGGGATGATGA	Q1-6-FAM	$(CT)_8$	256–305	9	59.85	KT806129

Note: *A* = number of alleles; T_a = annealing temperature.

[a]Q1 = TGTAAAACGACGGCCAGT (Schuelke, 2000); Q2 = TAGGAGTGCAGCAAGCAT; Q3 = CACTGCTTAGAGCGATGC; Q4 = CTAGTTATTGCTCAGCGGT (Q2–Q4, after Culley et al., 2008).

†Locus included in multiplex.

‡Locus amplified in simplex.

[Gabon, *N* = 22, coordinates: 0°11′21.84″S, 12°33′32.04″E] and Pallisco [Cameroon, *N* = 96, coordinates: 3°28′52.32″N, 13°34′41.88″E]; *H. liebrechtsiana*: East Mont de Cristal A and B [Gabon, *N* = 27, coordinates A: 0°35′48.84″N, 11°12′16.92″E and *N* = 72, coordinates B: 0°33′16.20″N, 11°6′20.16″E]). Seven of these loci were amplified in one multiplex reaction (see Table 1) using the QIAGEN Multiplex kit in a final 15-µL reaction volume. PCRs were carried out as follows: 7.5 µL Multiplex PCR Master Mix, 1.5 µL primer mix (with Q-tailed forward primers at 0.7 µM and reverse primers at 2 µM), 0.15 µL of each fluorescent Q1–Q4 primers (10 µM), 1.5 µL DNA, and 3.9 µL H_2O. The multiplex PCR program consisted of 95°C for 15 min; followed by 23–30 cycles each of 94°C (30 s), 57°C (90 s), and 72°C (90 s); followed by 10 cycles each of 94°C (30 s), 53°C (45 s), and 72°C (45 s); and a final extension at 60°C for 30 min. The remaining locus (F80) was amplified in a separate PCR with a *Taq* DNA polymerase (QIAGEN) under the following conditions: 94°C (4 min); followed by 20 cycles each of 94°C (30 s), 56°C (45 s), and 72°C (1 min); plus 10 cycles each of 94°C (30 s), 53°C (45 s), and 72°C (45 s); and a final extension at 72°C for 10 min. One microliter of each PCR product was directly added to 12 µL HiDi formamide and 0.2 µL GeneScan 500 LIZ Size Standard and run on an ABI 3730 sequencer (Applied Biosystems, Lennik, The Netherlands). Alleles were defined using Peak Scanner software (Applied Biosystems). In a few individuals, some primer pairs did not yield amplification. Expected (H_e) and observed heterozygosities (H_o) and tests for deviation from Hardy–Weinberg equilibrium (HWE) were estimated in SPAGeDi version 1.3 (Hardy and Vekemans, 2002). Results were adjusted for multiple comparisons using a sequential Bonferroni correction.

H_e and H_o ranged from 0.354 to 0.720 and 0.318 to 0.810 (*H. danckelmaniana* Ivindo population), from 0.081 to 0.683 and 0.083 to 0.625 (*H. danckelmaniana* Pallisco population), from 0.073 to 0.866 and 0 to 0.818 (*H. liebrechtsiana* East Mont de Cristal A population), and from 0.135 to 0.859 and 0.085 to 0.775 (*H. liebrechtsiana* East Mont de Cristal B population), respectively (Table 2). Two loci showed a deviation from HWE in the Cameroon population of *H. danckelmaniana* and two and five loci showed the same in *H. liebrechtsiana* in the populations from Gabon and Cameroon, respectively. To check whether departure from HWE at a given locus might be explained by the presence of null alleles, we used the software INEST (Chybicki and Burczyk, 2009), which jointly estimates inbreeding and null allele frequencies under the individual inbreeding model. Null allele frequency estimates were significantly different from zero at all except two loci that deviated from HWE, indicating the presence of null alleles. In all cases, the estimated frequency of null alleles was <23%.

CONCLUSIONS

The simple sequence repeat markers herein described are the first developed for *H. danckelmaniana*. These microsatellites are important tools for genetic studies in *H. danckelmaniana* and may be used to evaluate the genetic variability of the related species *H. liebrechtsiana*, aiming to elucidate questions regarding genetic diversity, spatial genetic structure, mating system, and gene flow.

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Table 2. Characterization of eight polymorphic microsatellite loci from two populations of *Haumania danckelmaniana* and *H. liebrechtsiana*.[a]

	H. danckelmaniana									*H. liebrechtsiana*								
	Ivindo (Gabon)					Pallisco (Cameroon)				East Mont de Cristal, A (Gabon)					East Mont de Cristal, B (Gabon)				
Locus	*N*	*A*	H_o	H_e	r^b	*N*	*A*	H_o	H_e^c	r^b	*N*	*A*	H_o	H_e^c	r^b	*N*	*A*	H_o	H_e^c	r^b
F2	22	4	0.409	0.614	0.143 ± 0.156	96	5	0.625	0.586	0.017 ± 0.030	27	7	0.148	0.142	0.083 ± 0.132	72	7	0.431	0.452	0.027 ± 0.046
F28	22	5	0.524	0.538	0.071 ± 0.112	92	5	0.511	0.491	0.027 ± 0.044	27	7	0.381	0.780**	0.230 ± 0.160[d]	65	7	0.492	0.787**	0.167 ± 0.084[d]
F62	22	3	0.682	0.622	0.053 ± 0.080	93	5	0.355	0.591**	0.149 ± 0.084[d]	27	3	0.593	0.654	0.073 ± 0.102	72	4	0.500	0.703**	0.118 ± 0.086[d]
F65	16	4	0.686	0.720	0.0635 ± 0.132	42	2	0.083	0.081	0.0634 ± 0.104	13	5	0.308	0.662**	0.107 ± 0.170	34	10	0.441	0.750**	0.063 ± 0.112
F66	21	2	0.409	0.485	0.110 ± 0.142	89	7	0.472	0.624**	0.101 ± 0.070[d]	21	6	0.333	0.357	0.080 ± 0.116	72	8	0.361	0.365	0.039 ± 0.060
F70	21	4	0.810	0.650	0.041 ± 0.070	93	5	0.591	0.683	0.055 ± 0.062	27	4	0.444	0.556	0.107 ± 0.124	71	8	0.775	0.797	0.026 ± 0.040
F74	22	3	0.318	0.354	0.083 ± 0.130	97	3	0.113	0.127	0.065 ± 0.078	27	4	0.000	0.073	0.157 ± 0.180	71	5	0.085	0.135**	0.103 ± 0.104[d]
F80	20	6	0.450	0.533	0.097 ± 0.134	87	7	0.402	0.480	0.082 ± 0.070	22	6	0.818	0.866	0.064 ± 0.086	57	11	0.509	0.859**	0.185 ± 0.088[d]

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; *N* = sample size; *r* = null allele frequency.

[a] For population details refer to Appendix 1.

[b] pij ± (2*SE).

[c] Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Note that there were no deviations at the $P < 0.05$ or $P < 0.001$ level.

[d] Indication for presence of null alleles based on INEST after 10,000 iterations.

Appendix 1. Voucher information for Marantaceae specimens used in this study.

Species	DNA voucher	Country	Locality	Latitude	Longitude	Herbarium specimen	Collector	Collection date[a]
Haumania danckelmaniana (J. Braun & K. Schum.) Milne-Redh.	ACL0041[b]	Gabon	Ivindo	0°11′21.84″N	12°33′32.04″E	AL944	Ley, A.C.	27.11.2008
Haumania danckelmaniana	ACL0045[b]	Gabon	Mont de Cristal—east	0°36′42.48″N	11°12′48.96″E	AL710	Ley, A.C.	19.11.2008
Haumania danckelmaniana	ACL0051[b]	Gabon	Matote	0°52′01.20″N	13°31′09.12″E	AL1158	Ley, A.C.	05.12.2008
Haumania danckelmaniana	ACL0711[b]	Gabon	Bonzadie	0°46′40.44″N	13°08′13.56″E	AL511	Ley, A.C.	08.12.2008
Haumania danckelmaniana	ACL0724[b]	Gabon	Doussala	2°19′49.80″S	10°36′39.96″E	AL382	Ley, A.C.	04.11.2008
Haumania danckelmaniana	ACL0736[b]	Gabon	Mandji	1°40′50.52″S	10°21′11.16″E	AL549	Ley, A.C.	13.11.2008
Haumania danckelmaniana	ACL0776[b]	Gabon	Ivindo	0°10′06.60″N	12°29′57.48″E	AL899	Ley, A.C.	26.11.2008
Haumania danckelmaniana	ACL0846[b]	Republic of the Congo	CIB Pokola	2°09′01.80″N	16°10′37.92″E	JFG 62	Gillet, J.-F.	19.03.2009
Haumania danckelmaniana	ACL0992[b]	Cameroon	Mt. Elefant	2°48′09.36″N	10°01′42.24″E	AL491	Ley, A.C.	20.01.2009
Haumania danckelmaniana	ACL0994[b]	Cameroon	Mt. Elefant	2°48′06.84″N	10°01′28.20″E	AL504	Ley, A.C.	20.01.2009
Haumania danckelmaniana	ACL1003[b]	Cameroon	Mt. Elefant	2°47′54.96″N	10°01′11.28″E	AL523	Ley, A.C.	20.01.2009
Haumania danckelmaniana	ACL0878[c]	Cameroon	Bibondi	3°18′10.80″N	10°38′58.20″E	AL755	Ley, A.C.	06.02.2009
Haumania danckelmaniana	ACL0880[c]	Cameroon	Bibondi	3°18′10.80″N	10°38′58.20″E	AL755	Ley, A.C.	06.02.2009
Haumania danckelmaniana	ACL0882[c]	Cameroon	Bibondi	3°18′10.80″N	10°38′58.20″E	AL755	Ley, A.C.	06.02.2009
Haumania danckelmaniana	ACL0883[c]	Cameroon	Bibondi	3°18′10.80″N	10°38′58.20″E	AL755	Ley, A.C.	06.02.2009
Haumania danckelmaniana								
Marantochloa incertifolia Dhetchuvi	ACL4317[c]	Gabon	Makokou	0°30′45.00″N	12°48′13.68″E	AL179	Ley, A.C.	23.09.2005
Hypselodelphys poggeana (K. Schum.) Miln.-Redh.	ACL1495[c]	Gabon	Mbigou, Mikoandza	2°01′20.64″S	12°3′11.88″E	AL413	Ley, A.C.	07.11.2008
Hypselodelphys poggeana	ACL1499[c]	Gabon	Mbigou, Mikoandza	2°01′20.64″S	12°3′11.88″E	AL413	Ley, A.C.	07.11.2008

[a] Dates are presented in the format day.month.year.
[b] Specimens used to test primer amplification and variability.
[c] Specimens used to isolate microsatellite loci through multiplex microsatellite enrichment and next-generation sequencing.