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Primer Note

Development of microsatellite markers for the neotropical vine *Dalechampia scandens* (Euphorbiaceae)[1]

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- *Premise of the study:* Microsatellite markers were developed to assess polymorphism and level of genetic diversity in four Mexican populations of the neotropical vine *Dalechampia scandens* (Euphorbiaceae).
- *Methods and Results:* Thirty-seven microsatellite markers representing bi-, tri-, tetra-, and pentanucleotide microsatellite repeats were developed. In total, 166 alleles were identified across 54 individuals. The number of alleles varied from one to 11 with an average of 4.49 alleles per locus. All loci except one were highly polymorphic between populations, whereas considerably less variation was detected within populations for most loci. The average observed and expected heterozygosities across study populations ranged from 0 to 0.63 and 0 to 0.59, respectively, for individual loci, and a deviation from Hardy–Weinberg equilibrium was observed for most loci.
- *Conclusions:* The developed markers may be useful for studying genetic structure, parentage analysis, mapping, phylogeography, and cross-amplification in other closely related species of *Dalechampia*.

Key words: *Dalechampia scandens*; Euphorbiaceae; genetic diversity; microsatellite loci.

Dalechampia scandens L. (Euphorbiaceae) is a neotropical twining vine native to Mexico, Central America, and South America (Webster and Armbruster, 1991). The species presents a complex hermaphroditic pseudanthial blossom (clusters of female and male flowers forming flowerlike structures). Attached to the male flowers is a resin-producing gland, which secretes resin for pollinator reward (Armbruster, 1984, 1985). Blossoms are self-compatible and often self-pollinate during a bisexual phase. The main floral visitors are species of resin-collecting bees from the Apidae or Megachilidae family. Because of the attractive floral complex and specialized pollination system, *Dalechampia* L. species have been used to study the evolution and selection of pollination systems and floral characters (e.g., Armbruster, 1985; Armbruster et al., 2009; Bolstad et al., 2010; Pélabon et al., 2012).

Molecular data provide invaluable information to address many ecological and evolutionary questions. Nuclear ribosomal DNA and cpDNA have previously been used to investigate the phylogenetic relationships among *Dalechampia* species (Armbruster et al., 2009). Inter-simple sequence repeat (ISSR) markers have also been used to analyze the effects of genetic distances between parents on progeny fitness-related traits in *D. scandens* interpopulation crosses (Pélabon et al., 2005). DNA sequence data may be difficult to apply on intraspecific studies in plants due to low levels of polymorphisms, and ISSR markers have sometimes been shown to yield low reproducibility. Microsatellites (simple sequence repeats [SSRs]) are highly reproducible genetic markers often showing appreciable levels of polymorphisms that have been successfully applied to study a broad spectrum of biological questions. The aim of this study is to identify genomic regions harboring microsatellite loci and to develop appropriate sequence-tagged site markers in *D. scandens*.

METHODS AND RESULTS

Four natural populations of *D. scandens*—Ciudad del Carmen (CC: $n = 20$, 18°56′29″N, 91°18′01″W), Cozumel (CO: $n = 10$, 20°22′10″N, 86°59′40″W), Puerto Morelos (PM: $n = 11$, 20°51′11″N, 86°53′43″W), and Valladolid (V: $n = 13$, 20°42′31″N, 88°15′06″W)—covering large parts of the species range in Mexico were used in this study (see Appendix 1 for voucher information). The Ciudad del Carmen and Puerto Morelos populations differ from the Cozumel

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TABLE 1. Characteristics of 37 microsatellite loci in *Dalechampia scandens*.

Locus[a]	Primer sequences (5′–3′)[b] Forward	Reverse	Fluorescent dye[c]	Repeat motif	T_m (°C)	Allele size range (bp)	*A*	GenBank accession no.
CCdi4	CAATTTCGCCGGATTGTTGT	CTATGAATCGGATGCAAACCT	PET	$(GA)_{20}$	61.21	190–218	8	JX668765
CCdi9	TCTTCCTGTTCGGTCCTACCTTT	GAGAATTCGGATTAGATCGTAGAGAGA	6-FAM	$(TAT)_5$	63.10	119–126	3	JX668777
CCdi10	CATTGCTTCCATCGACGTTCC	CTAGCCTCCTCCCCCAATCAA	6-FAM	$(TC)_{25}$	65.08	199–247	10	JX668754
CCdi11	GCTGACAACGGAATTCAAAAGGA	GAGAACACGCAAAGGGAAGTGAA	HEX	$(CT)_8$	63.88	306–336	5	JX668755
CCdi13	AGGTTGCCATTTCCCCACATC	TCAACTGGACAAGTAAAACACGACTAC	6-FAM	$(TC)_9$	64.93	98–113	4	JX668756
CCdi23	TCTTTCCTTCACTTTCTCCTCTCC	AAACCATGAGACACGATGCCAAA	6-FAM	$(CT)_7$	63.78	151–179	5	JX668757
CCdi24	TTGCATTCCTTCACTGACAA	TTCAAACCATGAGCCAACAA	HEX	$(AC)_8$	58.81	136–148	3	JX668758
CCdi25	TATCCACCCTGCCGTTAATCATAG	CATCAGTACCACACCCTCGAAACA	HEX	$(CT)_{12}$	64.02	220–243	5	JX668759
CCdi27	AACAAACCTGAAGAAAAAGGGAGGA	GGCTCTCACTTTTAGAACCCACA	6-FAM	$(GAG)_7$	64.54	68–81	5	JX668760
CCdi29	GAAAGAAGGAGGCCCCACCA	GCAAAAGCATGAGGATTGAGG	HEX	$(CT)_{14}$	63.21	53–78	6	JX668761
CCdi33	TTCCAAGAGGTCATGTTGATTGATG	GACTGCGTGTGTGTATGTGTTTGTG	HEX	$(AC)_8$	63.37	180–196	3	JX668762
CCdi38	CGTCCCGTCACATCACTCA	AAAGGGACAGGAGTGGAAA	HEX	$(CT)_{11}$	60.50	110–112	2	JX668763
CCdi39-1	GACATGCAGAGGAGGAAGGGAAA	GAGGAGAAGAGAATTTAAGGAGAAGGA	6-FAM	$(CAATC)_5$	63.60	328–374	8	JX668764
CCdi39-2	CCCAACCTCTCTTCTTTCACCTT	TCTTCGACGTCCAGCATTT	6-FAM	$(TCT)_6$	61.28	93–96	2	JX668764
CCdi41	TGGGTACCTGAAACTTGTGATGATGG	TCGCTTTGTTTCTATCGCTTGCT	6-FAM	$(TG)_{10}$	64.87	200–225	4	JX668766
CCdi45	GGTACGAAGTAAAGTAATGCAAGGA	CCTGCAAAACTACAATAATGACCTG	PET	$(AC)_{10}$	62.78	117–132	6	JX668767
CCdi47	GAAGAGAAGCGGCATTGTATGAG	GCAATTTCCCACATCTTCTTTG	HEX	$(AG)_{11}$	61.57	216–232	2	JX668768
CCdi50	GCTTGCGGGAGCAGGACAACATAC	CCCTTCAAGCTTTCTCGAACATTACA	6-FAM	$(AG)_{13}$	65.30	285–315	11	JX668769
CCdi52	TGCAAACCATTCATTTTAATTCC	AAGTCAACGGTCCACTTACCA	6-FAM	$(TG)_8$	58.56	74–78	3	JX668770
CCdi53	CAATAGAAATGCCAGGAACAC	GCATAATGCACAGTGAAAC	PET	$(TC)_{11}$	58.35	104–128	8	JX668771
CCdi54	CAACCGAAGAAACTCCATGACAAC	TACCTTGACCTTCCTTCCAACAC	HEX	$(GA)_{17}$	64.39	272–297	8	JX668772
CCdi63	TTTCGATATCATTTATCTTCCTTTTTC	CTCTCTGGGAACCTTCCACTT	HEX	$(CT)_{12}$	58.29	130–132	2	JX668773
CCdi67	CTGTTTGCGAAAGGCAGGAGGTG	TTGAGATCCCTCACCAAGAACATAGA	PET	$(GA)_{18}$	64.19	156–181	5	JX668774
CCdi71	GTGGAGGCGACCAAGACCAACC	TGCGACCATGTAAAGTGTTAGGAAAGA	HEX	$(TC)_8$	65.38	194–198	2	JX668775
CCdi74	TTATGACTCCTTCGCAACAATCC	CATACCAAAGACCTGCATCTTCCT	PET	$(CT)_{14}$	62.99	144–173	5	JX668776
CCtri1	CATTGAGAACCAACACCCCACA	GGAGGATTCAAGAAAGAGGGAAGG	HEX	$(ATC)_5$	65.35	168	1	JX668778
CCtri2	TTGCGTAAGAAGCCAACCAAACA	CAAAGATCAATCATGCCTTTCCCTTC	6-FAM	$(AGA)_6$	64.60	77–83	2	JX668784
CCtri3	GCGGTTGCTTAGTCAAAACTCCTACA	GGGTATTTATAGAAGGAGAGGAGGAAAG	6-FAM	$(TC)_7$	64.85	88–96	2	JX668786
CCtri6	GGAACGGAGTCATGACAAGTAAG	CTCATCATCCATTTTTCCTCCA	HEX	$(TG)_8$	60.42	97–133	6	JX668787
CCtri8	TGGCAATTGGGACTTTCCTCTTC	GAGGCCATTGTTGTGGACTGGTT	PET	$(CTT)_5$	65.28	165–198	3	JX668788
CCtri10	CACTTCCCTCTCAGTCTTGTTTTGG	CTGAAGCTGTTTGCTGGCTGT	6-FAM	$(TCC)_6$	66.52	181–187	3	JX668779
CCtri13	TGGAGACATAAGGCAAGGATGG	CCATGTGGATGAATGAGTAAGTGG	6-FAM	$(TTC)_9$	64.26	216–222	6	JX668780
CCtri14-1	ACAATCTCACCCAACCAATCA	GGCTGAGGTCAGAAGTCATTTT	PET	$(TTA)_{10}$	61.74	104–116	3	JX668781
CCtri14-2	CCACTGCTCCTTCTTCTCCTC	CATTAAATGTGGTGAAGATAATG	NED	$(CTT)_8$	56.72	78–82	3	JX668781
CCtri15	CAAATAAAGACTGCAGCACAAAG	TCCATAGAAAGATCACATTAAGCAA	PET	$(GTT)_6$	59.42	126–127	2	JX668782
CCtri17	AAAGAAAGTGATCTGGTGAAGG	CATGAAAGGCAAGAGGAAAGAAG	6-FAM	$(GAT)_{14}$	61.30	220–248	7	JX668783
CCtri21	GAAACAGAGTATTGGAGAAAGAGG	CAGAATTCTTCTGCTTTTGG	6-FAM	$(ATG)_{10}$	58.15	138–154	3	JX668785

Note: *A* = number of different alleles across all populations; T_m = melting temperature.

[a]Two different markers were identified for clones CCtri14 and CCdi39; these are tagged with the suffixes -1 and -2 for each clone.

[b]The forward and reverse sequence of flanking region.

[c]Fluorescent dye for labeling the 5′ end of the forward primer.

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Table 2. Allelic diversity and observed and expected heterozygosities in 37 microsatellite loci in four Mexican populations of *Dalechampia scandens.*

	Ciudad del Carmen (n = 20)				Puerto Morelos (n = 11)				Cozumel (n = 10)				Valladolid (n = 13)			
Locus	A	H_o	H_e	P	A	H_o	H_e	P	A	H_o	H_e	P	A	H_o	H_e	P
CCdi4	3	0.05	0.59	<0.001	2	0.30	0.39	0.481	3	1	0.57	0.007	3	1	0.56	0.002
CCdi9	1	0	0		1	0	0		3	1	0.57	0.007	2	1	0.52	0.001
CCdi10	4	0.25	0.57	0.001	3	0.18	0.60	0.002	3	1	0.57	0.007	3	1	0.62	0.004
CCdi11	2	0.10	0.43	0.001	1	0	0		3	1	0.57	0.007	3	1	0.59	0.003
CCdi13	2	0.05	0.51	<0.001	1	0	0		3	1	0.57	0.007	3	1	0.65	0.004
CCdi23	1	0	0		2	0.09	0.09	1	1	0	0		2	0.08	0.08	1
CCdi24	1	0	0		1	0	0		1	0	0		1	0	0	
CCdi25	1	0	0		2	0.18	0.48	0.06	2	1	0.53	0.007	3	1	0.56	0.001
CCdi27	2	0.05	0.05	1	3	0	0.69	<0.001	2	1	0.53	0.007	2	1	0.52	0.001
CCdi29	2	0.15	0.22	0.246	2	0.27	0.45	0.233	1	0	0		1	0	0	
CCdi33	2	0.05	0.30	0.002	1	0	0		1	0	0		1	0	0	
CCdi38	1	0	0		1	0	0		1	0	0		2	0.08	0.08	1
CCdi39-1	4	0.15	0.23	0.249	3	0	0.33	0.002	2	0.10	0.10	1	3	0.50	0.45	0.342
CCdi39-2	2	0.05	0.05	1	1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
CCdi41	1	0	0		1	0	0		4	1	0.62	<0.001	3	1	0.56	0.001
CCdi45	1	0	0		2	0.10	0.27	0.158	2	0.90	0.52	0.046	5	0.77	0.78	0.018
CCdi47	1	0	0		1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
CCdi50	3	0.10	0.50	<0.001	3	0.09	0.26	0.048	3	1	0.57	0.007	7	1	0.78	<0.001
CCdi52	2	0.20	0.43	0.027	1	0	0		1	0	0		2	0.08	0.08	1
CCdi53	2	0	0.10	0.026	1	0	0		4	1	0.62	0.001	4	0.92	0.66	0.042
CCdi54	3	0	0.35	<0.001	3	0.50	0.43	1	2	1	0.53	0.007	2	1	0.52	0.002
CCdi63	2	0.30	0.51	0.084	1	0	0		1	0	0		2	0.08	0.47	0.004
CCdi67	2	0.15	0.41	0.01	2	0.18	0.52	0.061	1	0	0		1	0	0	
CCdi71	1	0	0		1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
CCdi74	2	0.10	0.18	0.153	1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
CCtri1	1	0	0		1	0	0		1	0	0		1	0	0	
CCtri2	1	0	0		1	0	0		1	0	0		1	0	0	
CCtri3	1	0	0		1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
CCtri6	2	0.10	0.43	0.001	1	0	0		1	0	0		2	0	0.52	<0.001
CCtri8	2	0.05	0.05	1	1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
CCtri10	1	0	0		1	0	0		2	0	0.19	0.053	2	0.08	0.08	1
CCtri13	2	0.25	0.36	0.214	2	0	0.17	0.047	2	0	0.19	0.053	4	0.23	0.59	0.001
CCtri14-1	2	0	0.51	<0.001	1	0	0		2	1	0.53	0.007	2	0.92	0.52	0.006
CCtri14-2	1	0	0		2	0.09	0.09	1	0	0	0		0	0	0	
CCtri15	1	0	0		1	0	0		2	0	0.19	0.053	2	0.08	0.32	0.026
CCtri17	4	0.25	0.66	0.001	2	0.20	0.34	0.306	3	1	0.57	0.007	3	1	0.56	0.001
CCtri21	1	0	0		1	0	0		2	1	0.53	0.007	2	1	0.52	0.001
Mean	1.81	0.06	0.20		1.51	0.06	0.14		1.95	0.57	0.33		2.32	0.59	0.40	
SD	0.91	0.09	0.23		0.73	0.11	0.21		0.91	0.50	0.26		1.25	0.46	0.26	

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; P = P value of exact test for Hardy–Weinberg equilibrium.

and Valladolid in blossom size, the former populations being characterized by large blossoms compared to the latter. Leaf tissue from the Ciudad del Carmen population was used to identify microsatellite loci and to develop primers for the amplification of these loci. The library construction was performed using restriction enzymes *Bsa*AI and *Hinc*II following Hamilton et al. (1999). A double-stranded SNX linker was simultaneously ligated to the ends of these fragments with *Xmn*I. The enrichment of DNA fragments containing microsatellite loci was conducted by different 3′-biotinylated oligonucleotides (dimers, trimers, and tetramers), and streptavidin-coated magnetic beads were used to capture the enriched fragments. PCR of genomic DNA fragments enriched for microsatellites was conducted using SNX forward primer (5′-CTAAGGCCTTGCTAGCAGAAGC-3′). PCR reactions were performed in a total volume of 50 µL containing Platinum *Taq* polymerase (Invitrogen, Carlsbad, California, USA), 10× PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, and 10 µM SNX primer. The thermal profile was set at 94°C for 50 s, 55°C for 45 s, and 72°C for 1 min, for 35 cycles. The PCR products were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and were digested with *Nhe*I and ligated to plasmid pUC19 (New England Biolabs, Ipswich, Massachusetts, USA). The purified ligation was transformed to Electromax DH5α-E cells (Invitrogen) using an Eppendorf Electroporator 2510 (Eppendorf AG, Hamburg, Germany). After electroporation, 300 µL S.O.C. medium (Invitrogen) at room temperature was added to the mixture and incubated in a 37°C water bath for 1 h, followed by incubation at 4°C for 30 min. Two microliters of each library was mixed with 98 µL S.O.C. medium, and 30 and 60 µL of each mixture were spread on the 90-mm Luria–Bertani (LB)/ampicillin/X-gal plates followed by incubation at 37°C overnight. A total of 700–900 colonies were plated onto 150-mm LB/agar plates with 50 µg/mL of ampicillin and left to grow at 37°C overnight. Hybridization was performed by placing a Magna Lift (137-mm) nylon transfer membrane (Osmonics, Westboro, Massachusetts, USA) on a 150-mm LB/agar plate using 5× saline sodium citrate (SSC)/0.5% sodium dodecyl sulfate (SDS)/1 mM EDTA/0.1% bovine serum albumin (BSA) buffer and incubated at 50°C for at least 2 h. The radiolabeled probes were hybridized to a membrane, incubated at 50°C, and rotated overnight. The membrane was exposed to Kodak BioMax MR35 × 43 cm single-emulsion film (Carestream Health Inc., Rochester, New York, USA), and the developed autoradiograph was aligned to an LB plate to localize the positive colonies. In total, 109 positive clones containing microsatellite repeats were sequenced with an ABI 3730xl DNA Analyzer using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA).

Primer pairs (n = 42) for flanking regions of microsatellite repeats were designed by BatchPrimer3 (You et al., 2008). Two individuals from each population were used to test for primer amplification. Microsatellite loci were amplified in a 10-µL reaction containing 2× Type-it Microsatellite PCR Kit (containing HotStarTaq *Plus* DNA Polymerase, $MgCl_2$, and dNTPs) from QIAGEN, 0.2 µM of each primer, and 10 ng DNA. PCR amplification was performed on an ABI 9600 thermal cycler (Applied Biosystems) at 95°C for 5 min; 10 initial cycles as touchdown at 94°C for 30 s, 60–50°C for 45 s, 72°C for 45 s;

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25 cycles at 94°C for 30 s, 50°C for 45 s, 72°C for 45 s; and final extension at 72°C for 10 min. For successful markers (n = 39), the 5′ end of the forward primer was fluorescently labeled with different fluorophores (6-FAM, HEX, NED, and PET; Table 1) and fragment analysis was conducted with an ABI 3130xl Genetic Analyzer (Applied Biosystems). Nucleotide sequences for microsatellite clones were deposited in GenBank (accession no.: JX668754–JX668790). Two markers, CCtri5 and CCtri12, were removed from data analyses (data not shown). The estimation of the number of alleles (A), observed (H_o) and expected (H_e) heterozygosities, and test for Hardy–Weinberg equilibrium were performed using Arlequin version 3.5.1.3 (Excoffier and Lischer, 2010).

In total, 37 microsatellite loci were successfully screened for variability across the four populations of *D. scandens* (Table 1). Locus CCtri14-2 amplified only in CC and PM populations, and CCtri1 was monomorphic across all individuals/populations. Overall, 166 alleles were identified across all loci/individuals, with an average of 4.49 (SD = 2.47, range 1–11) alleles per locus (Table 1). The proportion of polymorphic loci varied from 0.38 (for PM) to 0.81 (for V). The average number of alleles across all loci varied between 1.51 (SD = 0.73, for PM) and 2.32 (SD = 1.25, for V; Table 2). The average H_o over all loci ranged from 0.06 (for PM) to 0.59 (for V; Table 2). Similarly, the average H_e across all loci ranged from 0.14 (SD = 0.21, for PM) to 0.40 (SD = 0.26, for V). Overall, lower levels of genetic variation were found in CC and PM compared with CO and V populations.

CONCLUSIONS

The microsatellite markers reported here provide a valuable tool for various kinds of genetic and ecological studies in *D. scandens*. The present set of markers will be useful in addressing questions about causes of genetic structure, demographic history, phylogeography, and mating systems of natural populations of *D. scandens*. These markers will be attempted for cross-amplification in closely related species of *Dalechampia*.

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Appendix 1. Information for voucher specimens of *Dalechampia scandens* deposited at the herbarium of the Norwegian University of Science and Technology (NTNU) Museum (TRH), NTNU, Trondheim, Norway.

Voucher information	TRH/V-7479	TRH/V-7481	TRH/V-7480	TRH/V-7482
Sample	CC2303	PM0901	CO0403	V0101
Country	Mexico	Mexico	Mexico	Mexico
Collection locality	Ciudad del Carmen	Puerto Morelos	Cozumel	Valladolid
Ecology	Roadside open habitat close to beach	Roadside by forest	Roadside by forest	Roadside by forest
Collector name	Geir H. Bolstad	Geir H. Bolstad	Geir H. Bolstad	Geir H. Bolstad
Collection date	October 2007	October 2007	October 2007	October 2007
Determinator name	Geir H. Bolstad	Geir H. Bolstad	Geir H. Bolstad	Geir H. Bolstad
Determination date	October 2007	October 2007	October 2007	October 2007
Geographic coordinates	18°56′29″N, 91°18′01″W	20°51′11″N, 86°53′43″W	20°22′10″N, 86°59′40″W	20°42′31″N, 88°15′06″W
Altitude	2 m	7 m	13 m	31 m

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