Novel microsatellite markers for *Dalechampia scandens* (Euphorbiaceae) and closely related taxa: application to studying a species complex

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ABSTRACT

We developed novel microsatellite markers for *Dalechampia scandens* L. (Euphorbiaceae). The target plants belong to a distinct, but undescribed, species in the *D. scandens* species complex, characterized by small resin-producing glands. In total, 110 alleles over 36 novel markers were identified across 39 individuals from three populations. The number of alleles varied from one to seven, with an average of 3.06 ± 0.26 alleles per locus. The developed markers, along with previously developed ones for large-glanded *D. scandens* species, were tested for amplification in 11 additional species of the genus *Dalechampia*. Four markers did not produce any detectable allele in 37 individuals from two populations of the large-glanded species. Average expected heterozygosity for small- and large-glanded populations was 0.34 and 0.19, respectively. Cross-species amplification showed that 89% of all markers were successfully amplified in at least one of the eleven other *Dalechampia* species. These microsatellite markers may be useful for detecting undescribed species in the *D. scandens* species complex, and can be used for comparative analyses of genetic structure, mating system, and phylogeography of other *Dalechampia* species.

Key words: cryptic biodiversity, cross-species amplification, *Dalechampia*, Euphorbiaceae, microsatellite loci, species complex.

Introduction

Dalechampia scandens L. (Euphorbiaceae) is a twining vine native to Mexico, Central and South America (Webster and Armbruster, 1991). It is typical of the genus in producing hermaphroditic pseudanthial blossoms. Upon initial blossom opening, only the female flowers are receptive; male flowers open 1-3 days later. Plants are self-compatible, and blossoms can self-pollinate during the bisexual phase, when male flowers are open and female flowers are still receptive. The subinflorescence that bears the male flowers also bears a gland-like cluster of bractlets that secretes a terpenoid resin collected by female euglossine, megachilid and/or meliponine bees (Armbruster, 1984). Populations of *D. scandens* vary greatly in blossom size, particularly in the size of the resin gland (offering the pollinator reward), which tends to correlate with the size of the most common pollinators (Armbruster, 1984, 1988; Hansen et al., 2000).

Studies including both inter-population hybridization (Pélabon et al., 2004; Pélabon et al., 2005) and genetic analyses (Bolstad et al., 2014) suggest that *D. scandens* comprises at least two species that differ in the morphology and size of their blossom. These two putative species are primarily characterized by the size of their resin gland and have been referred to as "large-glanded" and "small-glanded" *D. scandens* (Armbruster et al., 2009; Bolstad et al., 2014). Recently, Falahati-Anbaran et al. (2013) developed 39 microsatellite markers for populations of large-glanded *D. scandens*, hereafter "large-gland specific markers" (Falahati-Anbaran et al., 2013). These markers have been used to estimate the phylogenetic relationships among populations (Bolstad et al., 2014) and for paternity analysis in an experimental study of the effect of multiple paternity on seed mass (Pélabon et al., 2015).

Cross amplification of the large-gland specific markers previously developed with individuals from populations of the small-glanded species showed a high degree of variation in terms of expected heterozygosity as compared to the large-glanded *D. scandens* (Falahati-Anbaran et al., 2013). Although differences in heterozygosity between the small and large-glanded species may result from differences in mating system and the frequency of self-pollination (Opedal et al., 2015), these different levels of heterozygosity may also result from the combination of different alleles by natural hybridization between two or more distinct genomes in the small-glanded species. Microsatellite markers have proven to be useful in studies of natural hybridization events and polyploidization of various plant species (e.g. Duminil et al., 2012; Rai et al., 2013; Rijal et al., 2015). Such markers have also been applied to detect genetic structure of populations and to study relationships among closely related species (e.g.

Barkley et al., 2009; Rai et al., 2013). Chromosome numbers in *Dalechampia* vary from 2n = 36 to 2n = 196 (Vanzela et al., 1997). Because of the large number of chromosomes, additional informative markers beyond those developed by Falahati-Anbaran et al. (2013) would improve our ability to study the evolutionary history of these undescribed species as well as other *Dalechampia* species within the genus.

Here, we report the development of novel microsatellite markers from small-glanded individuals of *D. scandens*, markers that should allow investigation of patterns of variation across members of the species complex. We also test cross amplification of all microsatellite markers in 11 additional species of *Dalechampia*.

Materials and Methods

Plant materials

Microsatellite markers were developed from plants of *D. scandens* collected in the population El Limón (E) located in Veracruz State, Mexico (9.685°N, -96.497°W). The novel markers were tested for variation across two other small-glanded populations and two large-glanded ones (Table 1). In addition, cross-amplification of the developed markers was tested in 11 other species of *Dalechampia* (Table 1). To test for polyploidy we crossed several small- and large-glanded individuals from various populations and checked the allelic patterns in 10 F_1 hybrids.

Microsatellite library construction, cloning and sequencing

We used a protocol similar to Hamilton et al. (1999) to construct an enriched microsatellite library. Genomic DNA was digested using two 6-base cutter *BsaA I* and *HincII* enzymes (NEB), generating blunt-ended fragments each ligated to a double-stranded SNX linker by T4 DNA ligase. The oligonucleotide linker also contained a restriction site for *Nhe I* enzyme. To enrich fragments of microsatellite repeats, the digestion/ligation reactions were mixed with several biotinylated oligonucleotides containing di, tri and tetra repetitive motives (GT, TC, TA, TTA, GTT, TTC, GCT, GAT, GTA, GTG, GTC, TCC, TTTA, TTTG, TTC, GATA, GTAT, GAAT, GATT, GTTA, TTAC, GATG, GGTT, GCTT, GTAG, GTCA, GTTC, TCAC, TTCC). The enriched fragments were captured with streptavidin-coated magnetic beads (NEB), and PCR was performed on these fragments using

SNX forward primer. The thermal profile for PCR reactions was 94 °C for 50 s, 55 °C for 45 s, 72 °C for 90 s for 35 cycles. PCR reactions that produced smears without discrete bands were purified with a Qiaquick PCR purification kit (Qiagen). The purified PCR products were digested with *Nhe I* and inserted in an *Xba I*-digested plasmid, pUC 19 (NEB). The ligated vector was transformed into *E. coli* DH5-α cells (Invitrogen, Carlsbad, CA, USA). Positive clones were screened with radiolabeled probes containing microsatellite oligonucleotides. PCR was performed on the extracted plasmid DNA with a universal M13 forward primer using BigDye V.3.1 kit (Applied Biosystems, Forster City, Ca, USA), and fragments were sequenced with ABI 3730xl DNA Analyzers. The di-, tri-, and tetra-nucleotide microsatellite repeats were identified and the forward and reverse primers were designed for each sequence using BatchPrimer 3 (You et al., 2008).

In total, 37 primer pairs were designed from 88 sequenced clones with appropriate nucleotide length in the flanking region of microsatellite motives, of which 36 produced amplified products. Simple primers were ordered from Sigma-Aldrich (St. Louis, MO, USA) and the amplification was tested on one small sample per population to check for functionality. PCR reactions were performed in a 5 µl volume containing 2X Type-it Microsatellite PCR (Qiagen), 0.2 µM of forward and reverse primers and approximately 10 ng of extracted DNA. Amplification was carried out at 95 °C for 5 min (1 cycle), 94 °C for 30 s, 60 -50 °C for 45 s, 72 °C for 45 s (for 10 cycles as touchdown with decreasing the annealing temperature 1 degree at each cycle), followed by 25 cycles at 94 °C for 30 s, 50 °C for 45 s, 72 °C for 45 s and a final extension at 72 °C for 10 min on an ABI 9600 thermal cycler. After ensuring the amplification of 36 primer pairs on 2% agarose gel, the 5' end of forward primer for successful markers were fluorescently labeled with different fluorophores (6-FAM and HEX from Sigma-Aldrich, NED and PET from Applied Biosystems). The microsatellite alleles were detected using GeneMapper V 4. (Applied Biosystems). The sequences of successfully amplified microsatellite clones were submitted to GenBank with accession numbers KP708537- KP708570 (Table 2). Two microsatellite clones E_di2 and E_tri14 were each consists of two microsatellite regions indicated by extensions 1 and 2.

Data analyses

Some markers deviated from biallelic patterns in some individuals from the small-glanded populations, and in the genetic analyses we used the R-package Polysat v 1.3-3 (Clark and Jasieniuk, 2011; RCoreTeam, 2015),

which allows the presence of more than two alleles in individuals with different ploidy levels. Expected heterozygosity, H_E, within population corrected for sample size was estimated using Nei statistics (Nei, 1978) in SPAGeDi (Hardy and Vekemans, 2002). The frequency of null alleles within population was estimated for each marker based on EM algorithm implemented in FreeNA program (Chapuis and Estoup, 2007). The presence of linkage disequilibrium (LD) between loci was tested and the *P*-value for statistical significance of LD was computed using an EM algorithm with 10000 permutations. Proportion of loci in linkage disequilibrium (P_D) was calculated by a method described in Stenøien and Såstad (1999). Hardy-Weinberg equilibrium was examined with an exact test implemented in Arlequin 3.5 (Excoffier and Lischer, 2010).

Results

Microsatellite polymorphisms within Dalechampia scandens *species complex*

In total 36 novel microsatellite markers were developed for individuals from the small-glanded species (hereafter small-gland specific markers) of *Dalechampia scandens* (Table 3). All these markers were successfully amplified in 39 individuals from three small-glanded populations. Thirty-one out of 36 loci (86%) expressed more than one allele over all small-glanded individuals, while five loci, Edi7, Etri5, Etri17, Etet7, Etri4, were monomorphic. In total, we observed 110 alleles, with an average of 3.06 ± 0.26 alleles per locus (range 1 to 7) in all individuals. The expected heterozygosity, H_E, was 0.30, 0.29 and 0.43 in El Limon, Cozumel and Chamela populations, respectively. The heterozygosity varied from zero to 0.71 among loci in El Limon population. Several loci (22%) expressed more than two alleles in at least one small-glanded individual. Deviation from Hardy-Weinberg equilibrium and the frequency of null alleles are presented in Table S1. The average frequency of null alleles in the small-glanded populations was low, with an average (±SE) of 0.03 ± 0.01. The proportion of loci in linkage disequilibrium for large-gland (CC 0.09, PM 0.15) and small-glanded (Cham 0.49, CO 0.80, E 0.02) populations were 0.12 ± 0.03 and 0.44± 0.22, respectively.

The cross-amplifications of developed markers showed that most markers were successfully amplified in large-glanded individuals. However, four loci (Etet12, Edi20, Etri16 and Etet4) did not produce any bands in 37 individuals from two large-glanded populations. The expected heterozygosity, H_E, was 0.17 and 0.22 in Ciudad del Carmen and Puerto Morelos populations, respectively. Overall, 74 alleles were observed in all large-glanded populations with an average of 2.31 ±0.21 (range 1-5, Table 3). Combining the small-gland- (n = 36) and large-gland- (n = 39) specific loci gave 227 and 177 alleles, with an average 3.03± 0.18 and 2.49± 0.18 alleles per locus, in small- and large-glanded individuals, respectively. Fifty-two of the 352 alleles (15%) were similar across both small- and large-glanded individuals. These results reveal a clear separation between individuals from large- and small-glanded populations.

Polymorphism in species related to Dalechampia scandens

The new markers reported here for the small-glanded populations, as well as those previously developed for the large-glanded populations, were examined for amplification in 11 other species of *Dalechampia*. The results presented in Table S2 show that 69 out of 75 markers were successfully amplified in at least one of these eleven species. Fourteen out of 75 loci were amplified in all species. The number of alleles detected over 39 individuals from the 11 species was 443 with an average of 6.42 ± 0.32 (range 1-14) alleles per locus. The proportion of amplified loci varied from 0.41 in *D. schottii* to 0.81 in *D. pernambucensis*. The proportion of polymorphic loci varied from 2% in *D. brownsbergensis*, *D. hildebrandtii*, *D. parvifolia*, and *D. pernambucensis* to 50% in *D.magnistipulata*. The average heterozygosity in four species (with at least three individuals analyzed, i.e., *D. dioscoreifolia* var. *pubescens*, *D. heteromorpha*, *D.magnistipulata*, and *D. schottii*) was higher than 0.15 (Table S2). Most of these species contained individuals with more than two alleles per locus as described below (Table S3).

Individuals with more than two alleles per locus were identified in seven species, and the proportion of loci with more than two alleles per individual was variable, with the following proportion: *D. scandens* (Chamela): 21%; *D. scandens* (Cozumel): 1%; *D. scandens* (El Limon): 1%; *D. dioscoreifolia* var. *pubescens*: 9%; *D. heteromorpha*: 16%; *D.juruana*: 3%; *D.magnistipulata*: 3%; *D.stipulacea*: 5%; and *D. pernambucensis*: 2%. Genotyping of F_1 hybrids obtained from crosses between small- and large-glanded individuals showed presence of three alleles at 23-

29 loci in 10 different hybrids. Because in diploid species a maximum of two alleles is expected at any given locus, these results suggest higher ploidy levels across these species, where the extra alleles represent extra copies in the genome.

Discussion

We report novel microsatellite markers that successfully amplified in individuals from two distinct species in the *Dalechampia scandens* species complex. Most of these markers could also be applied in several other species of *Dalechampia.* One fifth of the markers represent evolutionarily conserved regions across all studied species. The developed microsatellite markers clearly differentiated the morphologically divergent small- and large-glanded populations of the *D. scandens* complex. This supports the hypothesis of the existence of two (or more) distinct species. Our results also suggest that there is different ploidy levels among the species studied.

Microsatellite markers are codominantly inherited and are thus useful for distinguishing heterozygous from homozygous individual in diploids. Individuals with more than two alleles for a specific marker could be the result of duplications events at given loci in diploid species resulting in amplification of several paralogous genomic regions. However, in our study, the presence of individuals with more than two alleles at a high number of loci suggests a polyploidization event associated with the origin of the small-glanded species and several of the other species studied. This hypothesis is partly supported by crosses between some small- and large-glanded individuals where infertile F_1 hybrids express up to three alleles at one third of microsatellite loci that are polymorphic between parents. A triploid hybrid is expected from crossing between diploid and tetraploid parents. Furthermore, preliminary data on chromosome counts show differences in chromosome numbers between the small- and large-glanded individuals (Escudero M, and Hansen T. F. unpublished results).

Microsatellite markers have been useful in species delimitation in taxonomically complex plant groups due to their much higher amounts of variation than chloroplast or other nuclear markers (Duminil et al., 2012). Microsatellites have also been useful in systematics studies of complex species in non-plant organisms (Griffiths et al., 2010; Simpson et al., 2013; Wang et al., 2014). While four out of 36 markers for the small-glanded species failed

to amplify microsatellite regions in the genomes of large-glanded individuals, all 39 markers specific to the large-glanded populations were present in both large and small-glanded individuals. This suggests that the orthologue primer binding sites in flanking regions of the microsatellite motives have diverged in the genome of the large-glanded species. Alternatively, this could also be due to the presence of unique regions or chromosomes in the genome of the small-glanded species that are not present in the genome of the large-glanded species. The small-gland specific marker, *Etet4*, which failed to amplify in large-glanded individuals, was amplified in nine other *Dalechampia* species, suggesting a loss of primer complementary region in the large-glanded individuals of *D. scandens*.

Previous investigations using nuclear ITS, ETS and chloroplast data have indicated that *D. pernambucensis* is sister to *D. scandens* (Armbruster et al., 2009). The higher percentage of cross-species amplified markers (81%) seen here supports this relationship. Conservatism of microsatellite regions among congeneric species has also been found in *Capsicum* sp. (Rai et al., 2013). Hence, microsatellite markers may perform well in studying relationships among closely related species.

The set of microsatellite loci reported here also provides a valuable tool for other kinds of studies in ecology and evolution of *Dalechampia*, including questions about the causes of genetic structure, demographic history, phylogeography, and mating-system evolution.

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References

Armbruster, W. S. (1984) The role of resin in angiosperm pollination: ecological and chemical considerations. *American Journal of Botany,* **71:** 1149-1160.

Armbruster, W. S. (1988) Multilevel Comparative Analysis of the Morphology, Function, and Evolution of Dalechampia Blossoms. *Ecology,* **69:** 1746-1761.

Armbruster, W. S., Lee, J. & Baldwin, B. G. (2009) Macroevolutionary patterns of defense and pollination in *Dalechampia* vines: Adaptation, exaptation, and evolutionary novelty. *Proceedings of the National Academy of Sciences,* **106:** 18085-18090.

Barkley, N. A., Krueger, R. R., Federici, C. T. & Roose, M. L. (2009) What phylogeny and gene genealogy analyses reveal about homoplasy in citrus microsatellite alleles. *Plant Systematics and Evolution,* **282**.

Bolstad, G. H., Hansen, T. F., Pélabon, C., Falahati-Anbaran, M., Pérez-Barrales, R. & Armbruster, W. S. (2014) Genetic constraints predict evolutionary divergence in Dalechampia blossoms. *Philosophical Transactions of the Royal Society of London B Biological Sciences,* **369**.

Chapuis, M.-P. & Estoup, A. (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution,* **24:** 621-631.

Clark, L. V. & Jasieniuk, M. (2011) polysat: an R package for polyploid microsatellite analysis. *Molecular Ecology Resources,* **11:** 562-566.

Duminil, J., Kenfack, D., Viscosi, V., Grumiau, L. & Hardy, O. J. (2012) Testing species delimitation in sympatric species complexes: the case of an African tropical tree, Carapa spp. (Meliaceae). *Molecular phylogenetics and evolution,* **62:** 275-285.

Excoffier, L. & Lischer, H. E. L. (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources,* **10:** 564-567.

Falahati-Anbaran, M., Stenøien, H. K., Pélabon, C., Bolstad, G. H., Perez-Barrales, R., Hansen, T. F. & Armbruster, W. S. (2013) Development of microsatellite markers for the neotropical vine *Dalechampia scandens* (Euphorbiaceae). *Applications in Plant Sciences,* **1:** 1200492.

Griffiths, A. M., Sims, D. W., Cotterell, S. P., El Nagar, A., Ellis, J. R., Lynghammar, A., McHugh, M., Neat, F. C., Pade, N. G., Queiroz, N., Serra-Pereira, B., Rapp, T., Wearmouth, V. J. & Genner, M. J. (2010) Molecular markers reveal spatially segregated cryptic species in a critically endangered fish, the common skate (Dipturus batis). *Proceedings of the Royal Society B: Biological Sciences,* **277:** 1497-1503.

Hamilton, M. B., Pincus, E. L., Di Fiore, A. & Fleischer, R. C. (1999) Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. *Biotechniques,* **27:** 500-507.

Hansen, T. F., Armbruster, W. S. & Antonsen, L. (2000) Comparative analysis of character displacement and spatial adaptations as illustrated by the evolution of *Dalechampia* blossoms. *The American Naturalist,* **156:** S17-S34.

Hardy, O. J. & Vekemans, X. (2002) spagedi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes,* **2:** 618-620.

Nei, M. (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics,* **89:** 583-590.

Opedal, Ø. H., Armbruster, W. S. & Pélabon, C. (2015) Inbreeding effects in a mixed-mating vine: effects of mating history, pollen competition and stress on the cost of inbreeding. *AoB Plants,* **7**.

Pélabon, C., Albertsen, E., Falahati-Anbaran, M., Wright, J. & Armbruster, W. S. (2015) Does multiple paternity affect seed mass in angiosperms? An experimental test in Dalechampia scandens. *Journal of Evolutionary Biology,* **28:** 1719–1733.

Pélabon, C., Carlson, M. L., Hansen, T. F. & Armbruster, W. S. (2005) Effects of crossing distance on offspring fitness and developmental stability in *Dalechampia scandens* (Euphorbiaceae). *American Journal of Botany,* **92:** 842-851.

Pélabon, C., Hansen, T. F., Carlson, M. L. & Armbruster, W. S. (2004) Variational and Genetic Properties of Developmental Stability in Dalechampia scandens. *Evolution,* **58:** 504-514.

Rai, V. P., Kumar, R., Kumar, S., Rai, A., Kumar, S., Singh, M., Singh, S. P., Rai, A. B. & Paliwal, R. (2013) Genetic diversity in Capsicum germplasm based on microsatellite and random amplified microsatellite polymorphism markers. *Physiology and Molecular Biology of Plants,* **19:** 575-586.

RCoreTeam (2015) R: A language and environment for statistical computing. , Version. R Foundation for Statistical Computing, Vienna, Austria. Available from URL: http://www.R-project.org/.

Rijal, D., Falahati-Anbaran, M., Alm, T. & Alsos, I. (2015) Microsatellite Markers for Heracleum persicum (Apiaceae) and Allied Taxa: Application of Next-Generation Sequencing to Develop Genetic Resources for Invasive Species Management. *Plant Molecular Biology Reporter,* **33:** 1381-1390.

Simpson, M. C., Wilken, P. M., Coetzee, M. P. A., Wingfield, M. J. & Wingfield, B. D. (2013) Analysis of microsatellite markers in the genome of the plant pathogen Ceratocystis fimbriata. *Fungal Biology,* **117:** 545-555.

Stenøien, H. K. & Såstad, S. M. (1999) Genetic structure in three haploid peat mosses (*Sphagnum*). *Heredity,* **82:** 391-400.

Vanzela, A. L. L., Ruas, P. M. & Marin-Morales, M. A. (1997) Karyotype studies of some species of *Dalechampia* Plum. (Euphorbiaceae). *Botanical Journal of the Linnean Society,* **125:** 25-33.

Wang, H.-L., Yang, J., Boykin, L. M., Zhao, Q.-Y., Wang, Y.-J., Liu, S.-S. & Wang, X.-W. (2014) Developing conversed microsatellite markers and their implications in evolutionary analysis of the *Bemisia tabaci* complex. *Scientific Reports,* **4:** 6351.

Webster, G. L. & Armbruster, W. S. (1991) A synopsis of the neotropical species of Dalechampia (Euphorbiaceae). *Botanical Journal of the Linnean Society,* **105:** 137-177.

You, F., Huo, N., Gu, Y., Luo, M.-c., Ma, Y., Hane, D., Lazo, G., Dvorak, J. & Anderson, O. (2008) BatchPrimer3: A high throughput web application for PCR and sequencing primer design. *BMC Bioinformatics,* **9:** 1-13.

Tables

Table 1. Geographical information and sample size, N, for different populations/species of *Dalechampia* used in the development of microsatellite markers.

Species/population*	Abbreviation	location/ country	latitude (°)	longitude (°)	altitude (m)	N
D. scandens L./ Ciudad del Carmen [a]	CC	Tabasco, Mexico	18.941	-91.300	2	20
D. scandens/ Puerto Morelos [a]	PM	Quintana Roo, Mexico	20.853	-86.895	7	17
D. scandens/ Chamela [b]	Cham	Jalisco, Mexico	19.498	-105.045	85	25
D. scandens/ Cozumel [b]	CO	Quinatana Roo, Mexico	20.369	-86.994	13	9
D. scandens/ El Limon [b]	E	Veracruz, Mexico	19.685	-96.497	133	5
D. parvifolia Lam.	Dpar	Dar es Salaam, Tanzania	-6.779	39.203	88	1
D. stipulacea Müll. Arg.	Dsti	Merida, Venezuela	8.462	-71.519	495	1
D. pernambucensis Baillon	Dper	Amazonas, Brazil	-2.737	-59.769	120	1
D. juruana Ule	Djur	Barinitas, Venezuela	8.755	-70.402	510	2
D. schottii Greenm.	Dsch	Quintana Roo, Mexico	20.210	-87.451	7	3
D. brownsbergensis GL Webster & Armbr.	Dbro	Bolivar, Venezuela	6.445	-61.510	166	3
D. magnistipulata GL Webster & Armbr.	Dmag	Veracruz, Mexico	18.534	-95.063	95	3
D. hildebrandtii Pax	Dhil	Bunju, Tanzania	-6.830	39.067	107	3
D. dioscoreifolia Poepp. *var. pubescens* Müll. Arg.	Ddio	Tarapoto, Peru	-6588	-76.306	204	5
D. aristolochiifolia H.B.K.	Dari	Cajamarca,Peru	-6.857	-78.048	1430	6
D. heteromorpha Pax & Hoffm.	Dhet	P.N. Soberanía, Panama	9.134	-79.721	57	11

***** a and b indicate small- and large-glanded populations of *D. scandens*, respectively

Table 2. Characteristic features of 36 novel microsatellite loci developed for small-glanded *Dalechampia scandens*. The allele sizes are based on the expected size from the sequences. Na represents number of different alleles over 39 individuals from 3 small-glanded populations. E_di2 and E_tri_14 clones are each represented by two markers.

Locus	Primer sequences (5′–3′)		Fluorescent dye	Repeat motif	Concentration (Mm)	T_m (ºC)	Allele size (bp)	N_a	GenBan accessio no.
	Forward	Reverse							
di2-1	CGCAATCGAATTACCACGCTTT	AACTTAATCCTGTCTGTCTGCTTCC	HEX	(TC)11	0.15	65.31	148	2	KP70853
di2-2	AATTGGAAGCAGACAGACAGGA	TCCACAGCGAATCTCTCTACGATG	NED	(CT)11	0.25	61.18	271	5	KP70853
di5	GCAAGTGGAAATCTAGGGTGGAA	CCCATGTCTCTACGCTGGTCAAA	PET	(AG)14	0.15	63.63	133	4	KP70853
di6	CGGTTGTTATTCATGCCATTGTTG	CCGACAGCCTGCATTTAGTTGTT	6-FAM	(AC)7	0.15	65.29	186	2	KP70853
di7	TTTTCCATACATGAATATGC	GGAAGAATCTAGCAACCCTGAAG	HEX	(AC)9	0.25	51.11	192	1	KP70854
di8	CAGCCAAATAAGTCCTGCAACCA	TCTAACAAATTGATCCCTCGCAAC	6-FAM	(TG)7	0.25	65.45	280	2	KP70854
di9	GGCAGATTATCCATCCTTTGTTTC	AAAGTCCCACCACAAATCC	PET	(TG)8	0.225	62.1	244	3	KP70854
di10	CAAACCGATACACATAACACATGAACC	ACAGAGCAGAGACTCCCGTCAAA	HEX	(AG)14	0.2	64.4	267	5	KP70854
di11	TTGAGGGGCTTTTTGCTCCTGT	CAACACCAATCAATTCCACAACTC	NED	(GT)6	0.1	66.34	212	2	KP70854
di13	AAAAGGACAGTTCCACACACATAG	TGGGGAATTATAGAGTGCGATTAGA	NED	(TC)15	0.125	59.49	120	4	KP70854
di14	CACCTCGCACTACTTCCACTCCT	ATCGGCGCTTCATTTCTTTCTGT	NED	(CA)11	0.125	64.11	164	3	KP70854
di15	AACGGTCAAAAGCGAACCT	TCAAAGCCGTCCATTACTTTTTCTC	NED	(CT)15	0.125	59.73	164	6	KP70854
di_17	GCCCATACAGCCATCACCTGCAAA	ACCCAGCCAACCAAATAATCCCACA	6-FAM	(CT)10	0.2	70.96	285	5	KP70854
di_20	AACCAATCATTCTGTCTGTCTG	CGACTGATCCTACAAGAACAAGAGA	HEX	(TC)9	0.15	56.77	100	2	KP70854
di_22	CTTCCCGCGTCCTAATTGT	GCCTTTTATTGGTTTCGTGGA	6-FAM	(CT)16	0.15	60.08	107	4	KP70855
di_23	CGTGGTCAATTTCTTAACCTGCAAA	AGGTACGAAGAAAGTAATGCAAGGA	6-FAM	(TG)12	0.15	65.25	147	5	KP70855
di_30	GATCTCGTTGCGGAACCCTCT	CGCATACAATACAACGAAACCCTAAC	HEX	(CT)11	0.2	65.01	162	7	KP70855
di_33	CCAAATCAATCGACTACGCTCCTCAAA	GGATTAATCAACACCAGTTCTGCAACGA	6-FAM	(CT)13	0.175	68.88	292	2	KP70855
di_34	CCCATCATGAGATCAACAGCCATTAC	CCATTCCTCTCAACTTTCTCGCCTCTC	PET	(GAA)6	0.15	66.67	100	2	KP70855
di_41	GGAACATGGCATTGCGTAGAGGA	CGCTGCCTCGCAGGATTACA	HEX	(CT)18	0.15	67.45	244	4	KP70855
tri1	GCAAGTCGTTTAAGGGATTGAT	AGCAATCACGACAAGGATGAAGA	HEX	(TTG)7	0.2	59.51	181	4	KP70855
tri2	GCCCGAGGAGGGTTTGATTT	GAGCAGCCATGGGAGAGTAG	6-FAM	(GAA)14	0.2	64.62	284	3	KP70855
tri4	GGCAACTCTTCTATGAAGTTGTGATTG	GAAGGAAGAAGCAGATCATTTGACAC	6-FAM	(GAT)5	0.15	63.43	218	1	KP70855
tri5	CGTTGAATGCAAGAACCTGACAAA	CGTTCATCTCGAGACTTCCCATC	6-FAM	(AAC)7	0.15	65.44	155	1	KP70855
tri9	TGAGTAGTGGTGACGAAGTTATGGA	GGCGTCACAGGCCAGTAGTAAA	6-FAM	(AGA)11	0.2	62.1	225	6	KP70856
tri11	CCAGTCTTTCACTTTCACTATCACC	CATCACCACCACAACTAATTTCAC	PET	(ATC)9	0.2	60.43	263	2	KP70856
tri12	CAACGACGGGGAGGTGATTT	GGCAGACGTAATTAGTTGTTGGGTAG	NED	(ATT)10	0.2	64.38	162	3	KP70856
tri_13	AAAAGGGTTAGATGTAAATAAATA	CTATTTCACGCACAATTTAGG	6-FAM	(ATG)8	0.35	50.91	152	2	KP70856
tri_14-1	TCTGTGGGTCAAGCGAAGGAGAAGA	TAACACCACGTCAGCGCTAACACCA	HEX	(TGT)6	0.125	69.43	134	3	KP70856
tri_14-2	GCGACGAAGAACCTAGCAAGAACAA	TCCTATCATGTCTGCATTCTCCACCA	NED	(GAA)9	0.125	66.81	195	3	KP70856
tri_15	GACACTCACTCCTTTCCGCTTCACT	TGGAAGAAGAAGACAACGACGACAA	NED	(TTC)7	0.15	66.28	145	2	KP70856
tri_16	GGGTGGTGACGTCCTATAAAGCAGAA	TGGTCGCAGAAATATGCATGACGAAG	6-FAM	(TGT)5	0.15	67.24	164	2	KP70856
tri_17	GGTCTCCATCGTAGGGTTTGT	CAAAAATAATTTCGCCTTTT	HEX	(ATG)6	0.25	60.24	92	1	KP70856
tet4	CAACTCCTACGAGACCTACGAGCAA	CCCTCTCGAGATTGAAAGAAACTAAACC	6-FAM	(AGAA)6	0.25	65.03	353	1	KP70856
tet7	TATCGGCTGCACCTCATTTTC	GCATCATGCAGTCGTCAA	6-FAM	(TTTA)8	0.3	62.37	132	4	KP70856
tet12	CTTGGCATGAGCGTATGATGGAA	TACTAGGCACCCGAAACAAAGCA	HEX	(GGTT)5	0.125	66.35	132	2	KP70857

Table 3. Number of alleles (N_a), expected heterozygosity corrected for sample size (H_E) and allele-size range (*A*s, in base pairs) in thirty six microsatellite loci in two small-gland (CC and PM) and three large-glanded (Cham, CO, E) populations of *Dalechampia scandens.*

	Ciudad del Carmen , CC (20 inds)			Puerto Morelos, PM (17 inds)			Chamela, Cham (25 inds)			Cozumel, CO (9 inds)			El Limon, E (5 inds)		
Locus	N_a	H_E	*A*s	N_a	H_E	*A*s	N_a	H_E	*A*s	N_a	H_E	*A*s	N_a	H_E	*A*s
Edi2-1	3	0.55	134-140	2	0.48	134-138	2	0.51	136-145	2	0.53	136-145	2	0.56	136-145
Edi2-2	1	0	280	2	0.46	280-283	3	0.63	267-270	2	0.52	265-274	2	0.57	265-270
Edi5R	1	0.00	130	1	0.00	128	3	0.59	118-134	2	0.53	118-137	2	0.56	118-132
Edi6	2	0.35	179-181	2	0.48	179-183	2	0.51	179-181	2	0.53	179-181	2	0.56	179-181
Edi7	-	-	-	1	0	186	1	0.00	186	-	-	-	1	0	186
Edi8	1	0.00	280	1	0.00	280	2	0.51	251-280	2	0.53	251-280	2	0.56	251-280
Edi9R	1	0.00	228	1	0.00	228	3	0.68	228-244	2	0.53	228-239	3	0.71	228-244
Edi10	2	0.48	248-252	3	0.48	248-256	4	0.56	256-264	2	0.53	248-262	2	0.56	256-264
Edi11	1	0	214	1	0	214	2	0.08	212-213	1	0.00	212	1	0	212
Edi13	2	0.50	112-120	2	0.45	112-114	3	0.65	110-122	1	0.00	118	1	0.00	116
di14	1	0.00	138	1	0.00	138	3	0.62	138-160	2	0.53	138-160	2	0.56	138-160
Edi15	1	0	146	4	0.68	146-159	3	0.52	148-157	2	0.53	146-159	2	0.56	134-159
Edi17	2	0.46	294-296	3	0.28	300-307	5	0.67	281-288	2	0.53	282-286	2	0.56	282-286
Edi20	-	-	-	-	-	-	2	0.38	97-99	1	0.00	97	1	0	97
Edi23	1	0.00	140	2	0.16	140-151	3	0.58	137-142	2	0.53	133-142	2	0.56	142-144
Edi30	1	0	169	4	0.60	165-187	4	0.61	157-181	2	0.53	161-183	3	0.64	157-183
Edi22	1	0	98	1	0	98	3	0.53	100-104	2	0.53	100-106	1	0.00	102
Edi33	1	0	288	1	0	290	2	0.32	286-290	1	0.00	290	1	0.00	290
Edi34	2	0.32	96-100	2	0.40	94-104	2	0.39	100-102	1	0.00	100	1	0.00	100
Edi41	2	0.10	194-208	3	0.43	184-210	4	0.39	196-212	1	0.00	206	1	0.00	206
Etri1	-	-	-	1	0.00	174	4	0.62	172-177	2	0.52	172-177	2	0.56	172-177
Etri2	1	0.00	282	2	0.11	270-276	2	0.50	276-279	1	0.00	279	1	0.00	282
Etri4	1	0	216	1	0.00	216	1	0.00	216	1	0.00	216	1	0.00	216
Etri5	2	0.26	146-149	1	0	149	1	0.00	149	1	0	149	1	0	149
Etri9	3	0.64	217-237	2	0.40	228-231	5	0.66	210-234	2	0.53	219-231	3	0.64	219-231
Etri11	1	0.00	280	2	0.50	271-277	1	0.00	283	1	0	283	1	0.00	262
Etri12	1	0.00	153	2	0.50	150-153	2	0.51	144-156	2	0.53	144-156	2	0.52	144-159
Etri13	-	-	-	1	0.00	157	2	0.42	147-150	1	0.00	150	1	0.00	150
Etri14-1	1	0.00	126	1	0.00	126	3	0.58	126-132	2	0.53	129-132	2	0.56	129-132
Etri14-2	3	0.52	197-203	1	0.00	197	3	0.62	183-195	2	0.53	183-191	2	0.56	183-191
Etri15	2	0.26	117-190	2	0.16	117-190	2	0.51	141-153	2	0.53	141-153	2	0.56	141-153
Etri16	-	-	-	-	-	-	2	0.28	163-166	1	0.00	163	2	0.34	163-166
Etri17	1	0.00	88	1	0.00	88	1	0.00	88	1	0.00	88	1	0.00	88
Etet4	-	-	-	-	-	-	1	0.00	348	1	0.00	348	1	0.00	348
Etet7	2	0.35	121-125	3	0.49	116-125	3	0.40	121-137	1	0.00	121	1	0	129
Etet12	-	-	-	-	-	-	2	0.51	126-130	1	0	130	1	0	130

Supplementary Materials:

Table S1. Deviations from Hardy-Weinberg (HW) equilibrium and null allele frequencies for the developed loci in *Dalechampia scandens, A,* H_O, H_E , *P* and F_{NULL} represents allele number, observed heterozygosity, expected heterozygosity, *P* value to test HW equilibrium and frequency of null alleles for each microsatellite loci, respectively. Genotypes with more than 2 alleles per locus were excluded from the analysis.

Table S2. Number of alleles (N_a), expected heterozygosity corrected for sample size (H_E) and allele size range (*A*s, in base pairs) in thirty six microsatellite loci developed in this study and 39 markers developed by Falahati Anbaran et al (2013) in 11 species of *Dalechampia.*

Table S3. Number of loci (N_L) and genotypes (N_G) with more than two alleles observed in *Dalechampia scandens* and other related species.