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A set of plastid microsatellite loci for the genus *Dyckia* (Bromeliaceae) derived from 454 pyrosequencing[1]

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- *Premise of the study:* Phylogeographical analyses of *Dyckia* (Bromeliaceae) suffer from low levels of sequence variation. Plastid microsatellite markers were developed to achieve a better-resolved genus-wide plastid genealogy of *Dyckia*.
- *Methods and Results:* Approximately 84% of the *D. marnier-lapostollei* plastome was sequenced using 454 technology. Flanking primer pairs were designed for 34 out of 36 chloroplast simple sequence repeats (cpSSRs) detected, and 12 loci were further characterized by genotyping *Dyckia* samples at the level of populations and species. Three, five, and six cpSSRs were polymorphic among four individuals of *D. limae*, 12 individuals of *D. dissitiflora*, and 12 of *D. pernambucana*, respectively, with two to three alleles per locus and species. All loci were polymorphic among 19 different *Dyckia* species, with three to 10 alleles per locus. Ten primer pairs cross-amplified with bromeliad genera from five subfamilies.
- *Conclusions:* The set of 12 cpSSR markers provides a toolbox to analyze phylogeographical patterns of *Dyckia* species.

Key words: Bromeliaceae; cpSSR; *Dyckia*; plastome; population genetics.

The genus *Dyckia* Schult. f. (Bromeliaceae) currently comprises 147 described species of xerophytic, terrestrial, or epilithic rosette plants with showy yellow, red, or orange flowers (Smith and Downs, 1974). The genus is distributed across eastern South America, with a center of diversity in the cerrado biome of Brazil and adjacent countries. Species of *Dyckia* and of its closest relative *Encholirium* Mart. ex Schult. f. typically inhabit azonal, arid, or rupicolous habitats that are characterized by poor soil, little water supply, high temperatures, and strong sun exposure. Pollination is mainly by hummingbirds and insects. Fruits are capsules that release winged, wind-dispersed seeds upon maturity (Smith and Downs, 1974).

Little is known about infrageneric relationships within *Dyckia*, the genetic structure and variation within its species, and the mechanisms of speciation. This paucity of information is in part due to the fact that many *Dyckia* species are rare and narrow endemics, which are barely represented in herbaria and living collections. Some species are even known from their type locality only. Another problem is the high degree of intraspecific morphological plasticity, which makes species delimitation in *Dyckia* notoriously difficult. We have initiated a genus-wide molecular phylogenetic study of *Dyckia*, based on plastid and nuclear DNA sequences. Our preliminary results indicate very low levels of plastid sequence divergence, suggesting a young age of the genus (Krapp, unpublished data). Whereas chloroplast haplotypes are often shared between species, haplotype networks based on plastid DNA show a pronounced geographical pattern across the distributional range of the genus. Chloroplast simple sequence repeats (cpSSRs), also called chloroplast microsatellites, are among the most sensitive tools for evaluating plastid DNA variation (Ebert and Peakall, 2009). To achieve a better-resolved genus-wide plastid phylogeography of *Dyckia*, we developed a set of 12 polymorphic cpSSR markers based on de novo 454 sequencing.

METHODS AND RESULTS

Total genomic DNA was isolated from one individual plant of *Dyckia marnier-lapostollei* L. B. Sm. var. *estevesii* Rauh from Goiania, central Brazil (see Appendix 1), using a modified cetyltrimethylammonium bromide (CTAB) procedure (Tel-Zur et al., 1999). This species was chosen because its plastid haplotype takes a central position in a statistical parsimony network, suggesting an ancestral state within the genus (Krapp, unpublished results). Fragmentation of a 5-µg DNA aliquot by nebulization, preparation of barcoded libraries, and shotgun sequencing on a Roche 454 GS-FLX with the Titanium Sequencing Kit XLR70 and the Titanium PicoTiterPlate Kit (Roche Diagnostics, Penzberg, Germany) were performed as described previously (Wöhrmann et al., 2012a). Altogether, 59 624 reads were obtained from three independent runs. The proportion of a single 454 sequencing lane devoted to *D. marnier-lapostollei* was 4.2%, 2.1%, and 4.1% in the first, second, and third run, respectively. Sequences of plastid origin were identified using the BLAST function of the software package Geneious (Drummond et al., 2010). The fully sequenced plastome of *Typha latifolia* L. (Guisinger et al., 2010)

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Table 1. Characteristics of 12 chloroplast microsatellite primer pairs developed in *Dyckia marnier-lapostollei* var. *estevesii*.

Locus	Primer sequences (5′–3′)	Position	Repeat motif	Size (bp)	GenBank accession no.[a]
DSSR-L01	F: GTCAATTTTCAAGTTCAGCC	*atp*B-*rbc*L	$(T)_{13}C(A)_{10}$	75	JQ743912
	R: TCACGATTTCATCTACTTGC	intergenic			
DSSR-L04	F: AAAGGATGAGATCAATTCGG	*ndh*A	$(T)_9$*	94	JQ743913
	R: AAGATACATCGGAAAGTCCC	intronic			
DSSR-L06	F: ATTGATTGAATAAACCGGGG	*trn*K-UUU-*rps*16	$(T)_{13}$	77	JQ743914
	R: TAAATAAGAAATTGGAATGG	intergenic			
DSSR-N01	F: GTTCCCAGTAAGAACCAACC	*rpo*C1	$(T)_{14}$	102	JQ743915
	R: CTCAATAATTTCACATTTCC	intronic			
DSSR-N04	F: GAAATCAATGTGTCGATTCC	*clp*P	$(T)_{11}$	87	JQ743916
	R: TTTNAATAGAAAGAAGACCC	intronic			
DSSR-N05	F: TGAGATGAGTTTTGGCTCCC	*clp*P	$(A)_{12}$	85	JQ743917
	R: AACAATACATCAATGATAGG	intronic			
DSSR-N07	F: ATTATATACATCTGAAACCC	*trn*P-UGG-*psa*J	$(A)_{13}$	74	JQ743918
	R: CTTCCTCCTGAGCATTACGG	intergenic			
DSSR-N10	F: TNAATCAATATGGCGAAGGC	*clp*P	$(T)_{10}$	79	JQ743919
	R: ATTCCCTCACGCTTGGCGCC	intronic			
DSSR-N11	F: ATAGATAAAATTATCGGGCC	*ndh*G-*ndh*I	$(A)_{18}$	100	JQ743920
	R: AAATTTAAACTACATATTCC	intergenic			
DSSR-N15	F: CTTCCATTTATCCATATCCC	*rpl*16	$(T)_{11}$	64	JQ743921
	R: AAAATAAATCTGATTATGGG	intronic			
DSSR-N16	F: TTATACCAAATGATCAATCG	*rpl*16-*rps*3	$(T)_{13}$	90	JQ743922
	R: ACTCTTTCATTCTTTTTCCG	intergenic			
DSSR-N18	F: AAATAGGTAATCTATTCCCC	*psb*K-*psb*I	$(A)_{15}$	63	JQ743923
	R: GTAAGCATTACACAATCTCC	intergenic			

[a] GenBank accession numbers of the sequences on which the primers are based.

* The SSR motif at DSSR-L04 had only nine T residues in *D. marnier-lapostollei*, but had up to 14 T residues in other *Dyckia* species for which sequence data were available for primer design.

was taken as a reference. A total of 3826 plastid reads were assembled into 77 contigs and 12 singletons, which together represent 113 449 bases of the *D. marnier-lapostollei* plastome (counting the two inverted repeats only once). This corresponds to an overall coverage of ~84%, when compared to the *T. latifolia* plastome. A total of 36 mononucleotide repeats with ≥10 bases were detected (181 with ≥7 bases) using the FIND function of PhyDE (Müller et al., 2010). Besides two short dinucleotide repeats, each with an $(AT)_5$ motif, no other types of SSRs were observed. Flanking primer pairs were designed by eye for 34 loci, with a default length of 20 nucleotides and a GC clamp of up to three nucleotides at the 3′ end. For three loci (DSSR-L01, DSSR-L04, and DSSR-L06; Table 1), consensus primers were derived from alignments of the *D. marnier-lapostollei* sequence with sequence data previously generated by Sanger sequencing of the same loci in other *Dyckia* species (Krapp, unpublished data).

Primer functionality was initially tested on a single accession each of *D. marnier-lapostollei*, *D. dissitiflora* Schult. f., and *D. pernambucana* L. B. Sm. PCRs were carried out in 10-μL volumes using a Biometra T1-cycler or a Biometra T-Gradient cycler (Biometra GmbH, Göttingen, Germany), using

Table 2. Observed allele sizes at 12 chloroplast microsatellite loci in three populations of *D. dissitiflora* and *D. pernambucana* and one population of *D. limae*, allele numbers and size range in 19 different *Dyckia* species (one individual each), and cross-amplification in eight additional genera of Bromeliaceae (see Appendix 1).

	Allele sizes									Cross-amplification in other bromeliad genera[†]							
	D. dissitiflora			*D. pernambucana*			*D. limae*	19 *Dyckia* species									
Locus	Cachoeira* (*N* = 4)	Lajes* (*N* = 4)	Morrão* (*N* = 4)	Aldeia* (*N* = 4)	Brejo* (*N* = 4)	Papagaio* (*N* = 4)	Jerusalém* (*N* = 4)	No. of alleles	Size range (bp)	En	De	Fo	Pi	Pu	An	He	Ti
DSSR-L01	75	75, 76	75, 76	77	75, 76	77	76, 77	9	72–80	+	+	+	+	+	+	+	—
DSSR-L04	98	98	98	99	97	98	99	6	94–99	+	+	+	+	+	+	+	+
DSSR-L06	79	79, 80	79, 80	78	78	78	78	8	73–82	+	+	+	+	+	+	+	+
DSSR-N01	102	102	102	102	102	102	102	8	98–109	+	+	+	+	+	+	+	+
DSSR-N04	91	91	91	91	92	91	91	8	87–98	+	+	—	—	—	—	—	—
DSSR-N05	86	86	86	85, 86	85, 86	86	85, 86	4	84–87	+	+	+	+	+	+	+	+
DSSR-N07	74	74	74	74	74	74	74	5	71–75	+	+	—	+	+	+	+	+
DSSR-N10	81	79	79, 81	79	79	79	79	3	79–81	+	+	+	+	+	+	+	+
DSSR-N11	99	96 100	99	97	98	98	98	10	94–104	+	+	+	—	—	—	+	—
DSSR-N15	65	65	65	65	65	65	65	3	64–66	+	+	+	+	+	+	+	+
DSSR-N16	90	90	90	90	90	90	90	5	87–91	+	+	+	+	+	+	+	+
DSSR-N18	68	72, 73	68, 72	66	66	67	62, 66	9	62–73	+	+	+	+	+	+	+	+

Note: + = amplification; — = no amplification; An = *Ananas* (Bromelioideae); De = *Deuterocohnia*; En = *Encholirium*; Fo = *Fosterella*; Pi = *Pitcairnia* (all Pitcairnioideae); He = Hechtia (Hechtioideae); Pu = *Puya* (Puyoideae); Ti = *Tillandsia* (Tillandsoideae).

* Locality information for the populations is provided in Appendix 1.

[†] Single PCR product in the expected size range.

the indirect fluorescence labeling procedure described by Schuelke (2000). Each assay contained approximately 1 ng of template DNA, 1× Mango-*Taq* reaction buffer (Bioline, Taunton, Massachusetts, USA), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.04 μM forward primer carrying a 5′-M13 tail, 0.16 μM of M13 forward primer with fluorescent 5′-IRD700 modification, 0.16 μM unlabeled reverse primer, 0.5 μg/μL BSA, and 0.05 U Mango-*Taq* DNA polymerase (Bioline). All loci were amplified using a standard PCR program with an initial denaturation at 80°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 52°C for 1 min, and elongation at 65°C for 2 min. Final extension was performed at 65°C for 10 min. Samples were electrophoresed on denaturing 6% polyacrylamide gels in 1× TBE buffer, using an automated sequencer (Li-Cor 4200 IR^2; Li-Cor Biosciences, Bad Homburg, Germany). Fragment sizes were determined by visual examination with the help an external size standard, as outlined by Wöhrmann et al. (2012a). Allele sizes were validated by repeated PCRs of subsets of samples using either Mango-*Taq* polymerase or a set of proofreading polymerases (Long High Fidelity Enzyme Mix; Rovalab, Teltow, Germany), following the protocol supplied by the manufacturer.

The 12 most polymorphic cpSSR loci were used to genotype (1) population samples from *D. limae* L. B. Sm., *D. dissitiflora*, and *D. pernambucana*; (2) single accessions from 16 additional *Dyckia* species; and (3) one or two species each of eight bromeliad genera belonging to five subfamilies. *Dyckia dissitiflora* was chosen as an example of a *Dyckia* species with a relatively large distribution range across Brazil, whereas *D. limae* and *D. pernambucana* were taken as a typical example of two species that are not clearly distinguishable by morphological characters. Locus characteristics, primer sequences, and GenBank accession numbers of these 12 markers are summarized in Table 1, fragment sizes for all samples and loci are compiled in Table 2, and all plant materials used in this study are listed in Appendix 1.

Three, five, and six cpSSR loci were polymorphic among four individuals of *D. limae*, 12 individuals from three populations of *D. dissitiflora*, and 12 individuals from three populations of *D. pernambucana*, respectively (Table 2). Two to three alleles were observed per locus and species. All loci were highly polymorphic at the species level, with three to 10 alleles per locus across 19 *Dyckia* species (Table 2). Allele size distributions were generally compatible, with a variable number of mononucleotide repeats being the molecular basis for size variation. Overall, only six out of 540 individual PCRs performed with any *Dyckia* species failed. All loci produced single PCR fragments within the expected size range in the closely related genera *Encholirium* and *Deuterocohnia* Mez, and nine of the 12 primer pairs successfully cross-amplified in six other genera from five subfamilies of Bromeliaceae (Table 2).

CONCLUSIONS

The set of 12 novel cpSSR markers presented here provides a promising toolbox for reconstructing plastid genealogies and elucidating phylogeographical patterns within *Dyckia*. In conjunction with nuclear SSR markers that are currently being developed in our group (Wöhrmann et al., 2012b), the cpSSRs are also promising candidates for population genetic analyses in *D. dissitiflora*, *D. limae*, *D. pernambucana*, and probably many other *Dyckia* species. Primer binding sites appear to be well-conserved among Bromeliaceae, suggesting that the 12 cpSSR markers may be applicable for genetic studies throughout the family.

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Appendix 1. Plant material used for this study.

Species	Collector (Herbarium)[a]	Location[b]	GPS coordinates
Dyckia dissitiflora Schult. f. Pop. Cachoeira (*N* = 4)	*A. M. Iseppon, Pinangé, D. & Cruz, G. 1605* (UFP)	Cachoeira "Ferro Doido," Bahia (BR)	−11.6279; −41.0005
Dyckia dissitiflora Schult. f. Pop. Lajes (*N* = 4)	*A. M. Iseppon, Pinangé, D. & Cruz, G. 1598* (UFP)	Lajes, Bahia (BR)	−11.6010; −41.1645
Dyckia dissitiflora Schult. f. Pop. Morrão (*N* = 4)	*A. M. Iseppon, Pinangé, D. & Cruz, G. 1562* (UFP)	Morrão, Bahia (BR)	−11.5901; −41.2072
Dyckia limae L. B. Sm. Pop. Jerusalém (*N* = 4)	*A. M. Wanderley s.n.* (UFP)	Serra de Jerusalém, Pernambuco (BR)	−8.5837; −37.2384
Dyckia pernambucana L. B. Sm. Pop. Aldeia (*N* = 4)	*D. Pinangé et al. DCKB/09.2009* (UFP)	Aldeia Couro d'Anta, Pernambuco (BR)	−8.3254; −36.7562
Dyckia pernambucana L. B. Sm. Pop. Brejo (*N* = 4)	*D. Pinangé et al. DKCA/09.2009* (UFP)	Brejo da Madre de Deus, Pernambuco (BR)	−8.1894; −36.3931
Dyckia pernambucana L. B. Sm. Pop. Papagaio (*N* = 4)	*A. M. Wanderley s.n.* (UFP)	Pico do Papagaio, Pernambuco (BR)	−7.8228; −38.0554
Dyckia aff. *brevifolia* Baker	*P. Braun 840* (HD)	Itacambira, Minas Gerais (BR)	−17.0667; −43.3000
Dyckia estevesii Rauh	*P. Braun s.n.* (HD)	BR	NA
Dyckia ferox Mez	*W. Rauh 64237* (HD)	Cerro Colorado, Cordoba (RA)	−30.1000; −63.9333
Dyckia goehringii E. Gross & Rauh	*W. Rauh 67622* (HD)	Diamantina, Minas Gerais (BR)	−18.2500; −43.6000
Dyckia granmogulensis Rauh	*W. Rauh 56484* (HD)	Grão Mogol, Minas Gerais (BR)	−16.5667; −42.9000
Dyckia aff. *ibiramensis* Reitz	*L. Horst 1287* (HD)	Diamantina, Minas Gerais (BR)	−18.2500; −43.6000
Dyckia leptostachya Baker	*H. Amerhauser s.n.* (WU)	Caacupé, Cordillera (PY)	−25.3833; −57.1500
Dyckia lindevaldae Rauh	*P. Braun BR 691* (HD)	Alto Paraiso, Goiás (BR)	−14.1167; −47.5167
Dyckia macedoi L. B. Sm.	*R. B. Louzada, Pinangé, D. & Medeiros, M. 151* (SP)	Santana do Riacho, Minas Gerais (BR)	−19.3539; −43.6237
Dyckia marnier-lapostollei var. *estevesii* Rauh	*L. Horst 5* (HD)	Goiania, Goiás (BR)	−16.6667; −49.2667
Dyckia marnier-lapostollei L. B. Sm.	*L. Horst 4* (HD)	Cristalina, Goiás (BR)	−16.7500; −47.6000
Dyckia microcalyx Baker	*W. Till 6020* (WU)	Cerros Acahay, Paraguari (PY)	−25.9167; −57.1500
Dyckia aff. *pumila* L. B. Sm.	*P. Braun BR 696* (HD)	Ponte Branca, Mato Grosso (BR)	−16.4500; −52.6667
Dyckia remotiflora var. indet. Otto & A. Dietr.	*L. Horst s.n.* (HD)	BR	NA
Dyckia tobatiensis Hassl.	*W. & S. Till 6050* (WU)	Tobati, Cordillera (PY)	−25.2500; −57.0667
Dyckia velascana Mez	*W. & S. Till 5012* (WU)	Ascochinga, Cordoba (RA)	−30.9500; −64.2667
Dyckia vestita Hassl.	*W. & S. Till 6018* (WU)	Paraguari, Paraguari (PY)	−25.6333; −57.1500
Encholirium horridum L. B. Sm.	*W. Schindhelm s.n.* (HD)	Pedra Azul, Minas Gerais (BR)	−15.9867; −41.4069
Encholirium magalhaesii L. B. Sm.	*s.n.* (BONN)	BR	NA
Deuterocohnia brevispicata Rauh & L. Hrom.	*N. Schütz 06/028* (FR)	Florida, Santa Cruz (BOL)	−18.0154; −64.1001
Deuterocohnia glandulosa E. Gross	*N. Schütz 06/019* (FR)	Ipati, Santa Cruz (BOL)	−19.7063; −63.6521
Fosterella villosula (Harms) L. B. Sm.	*J. Peters 06.0105* (HD)	Cochabamba, Cochabamba (BOL)	−17.0611; −65.6444
Fosterella weddelliana (Brongn. ex Baker) L. B. Sm.	*M. Miyagawa s.n.* (HD)	Solacana (BOL)	NA
Pitcairnia feliciana (A. Chev.) Harms & Mildbr.	*I. Ebert & D. Bangoura s.n. ex coll. P. Bak* (WU)	RG	NA
Pitcairnia heterophylla (Lindl.) Beer	*K. Senghas O-11230* (HD)	Cruz de Ocotte, Guerrero (MEX)	17.5500; 99.8833
Puya ferruginea (Ruiz & Pav.) L. B. Sm.	*W. Rauh s.n.* (HD)	Rio Marañon (PE)	NA
Puya herzogii Wittm.	*T. Krömer 6581* (HD)	Carrasco, Cochabamba (BOL)	−17.1933; −64.9731
Ananas ananassoides (Baker) L. B. Sm.	*P. Maas s.n.* (HD)	Est. Amazonas (BR)	NA
Hechtia caerulea (Matuda) L. B. Sm.	*W. Rauh s.n.* (HD)	Est. Mexico (MEX)	NA
Tillandsia usneoides (L.) L.	*W. Rauh s.n.* (HD)	Yungas Cachi (RA)	NA

Note: *N* = population size; NA = not available.

[a] Herbaria: BONN = University of Bonn; FR = Senckenberg Research Institute, Frankfurt; HD = Botanical Garden of Heidelberg; SP = Instituto de Botânica; São Paulo; UFP = Universidade Federal de Pernambuco; WU = University of Vienna.

[b] BOL = Bolivia; BR = Brazil; MEX = Mexico; PE = Peru; PY = Paraguay; RA = Argentina; RG = Guinea.