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Development and characterization of microsatellite loci in dioecious figs (*Ficus*, Moraceae)[1]

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- *Premise of the study:* Microsatellite markers for dioecious fig species, *Ficus bernaysii* King (section *Sycocarpus*), *Ficus pachyrrhachis* K. Schum. & Lauterb. (section *Sycocarpus*), and *Ficus copiosa* Steud. (section *Sycidium*) were developed to estimate gene flow among closely related fig species.
- *Methods and Results:* Loci were developed from tri- and tetranucleotide-enriched genomic libraries. The six most repeatable and scorable loci for *F. bernaysii* and *F. pachyrrhachis* were characterized in 50 individuals each of five species from section *Sycocarpus*. Four primer pairs from *F. copiosa* were screened in 50 individuals each of two species from section *Sycidium*. Observed heterozygosity ranged from 0.53 to 0.93 in loci developed from *F. bernaysii*, 0.25 to 0.86 in loci developed from *F. pachyrrhachis,* and 0.68 to 0.87 in loci developed from *F. copiosa*.
- *Conclusions:* Amplification across the two *Ficus* sections was observed in seven of the ten total primer pairs.

Key words: *Ficus*; microsatellites; New Guinea; *Sycidium*; *Sycocarpus*.

The genus *Ficus* (Moraceae), which contains over 750 species of tropical woody plants, is perhaps best known for its obligate mutualism with agaonid wasp pollinators. Molecular phylogenetic analyses have provided insight into evolutionary relationships among major *Ficus* lineages (Ronsted et al., 2008), but relationships among recently diverged species remain obscure due to low sequence variation among close relatives (Silvieus et al., 2008) and questions regarding hybridization among figs (Machado et al., 2005). The development and characterization of rapidly evolving markers such as microsatellites is needed to provide information on population genetics and species-level phylogeny in *Ficus*.

Here, we report the development and characterization of six microsatellite loci from *Ficus bernaysii* and *Ficus pachyrrhachis* (section *Sycocarpus*) and four microsatellite loci from *Ficus copiosa* (section *Sycidium*). These two sections represent the most diverse lineages of dioecious figs (Weiblen, 2000).

METHODS AND RESULTS

Leaf tissue for the study was collected from 50 individuals each of five species in section *Sycocarpus* (*F. bernaysii* King, *F. congesta* Roxb., *F. hispidioides* S. Moore, *F. morobensis* C. C. Berg, and *F. pachyrrhachis* Laut. & K. Schum.) and two species in section *Sycidium* (*F. copiosa* Steud. and *F. wassa* Roxb.) from populations located in a 4 km^2 area of lowland rainforest surrounding Ohu village in Madang Province, Papua New Guinea (5°13′38″S, 145°40′44″E). Leaf samples were dried in silica gel immediately upon collection and stored at −20°C until extraction. Specimen vouchers were deposited at the University of Minnesota Herbarium (Appendix 1). Genomic DNA was extracted from dried leaf tissue using the DNeasy Plant Mini Kit (QIAGEN, Washington, D.C., USA). Microsatellite loci were isolated from a subset of the focal species, *F. copiosa*, *F. bernaysii,* and *F. pachyrrhachis*, using the Dynabeads 2003 enrichment protocol of Glenn and Schable (2005). Genomic DNA of one individual per species was enriched using a set of five microsatellite motifs. *Ficus copiosa* and *F. pachyrrhachis* enrichments used Motif Set 1 [$AAAT_8$, $AACT_8$, $AAGT_8$, $ACAT_8$, $AGAT_8$] and the *F. bernaysii* enrichment used Motif Set 2 [AAC_6, ACT_{12}, AAT_{12}, ATC_8, AAG_8]. Enriched fragments were used as a template for amplification in a polymerase chain reaction (PCR) and PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocol. Bacterial colonies were amplified directly using M13 primers and screened on agarose gel. PCR products of positive clones were purified with Exonuclease I (New England Biolabs, Ipswich, Massachusetts, USA) and Shrimp Alkaline Phosphatase (SAP; Affyemetrix, Santa Clara, California, USA) according to the Glenn and Schable (2005) protocol. DNA sequencing was performed with M13 primers using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, California, USA). Sequencing was performed on an ABI PRISM 3730 DNA Analyzer.

Based on 95 sequenced clones from *F. bernaysii*, primers were developed for seven loci containing eight or more perfect repeats imbedded in sufficient flanking sequence to allow for primer design. These were screened for scorability in 16 individuals of *F. bernaysii*. The three that amplified and could be consistently scored are reported here. Based on 150 sequenced clones from *F. pachyrrhachis*, primers were designed for seven loci containing eight or more perfect repeats imbedded in enough flanking sequence to allow for primer design. These were screened for scorability in 16 individuals of *F. pachyrrhachis,* and the three that amplified are reported here. Of 215 sequenced clones from *F. copiosa*, primers were developed for seven loci containing eight or more perfect repeats imbedded in enough flanking sequence to allow for primer design. These were screened for scorability in 16 individuals of *F. copiosa,* and the four that amplified are reported here. Primers were developed using Primer3 (Rozen and Skaletsky, 2000).

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TABLE 1. Microsatellite loci developed in *Ficus bernaysii* (B30, B47, and B83), *Ficus pachyrrhachis* (P164, P211, and P215), and *F. copiosa* (C244, C246, C281, and C410), primer sequences, repeat motifs, size ranges, PCR conditions, annealing temperatures, and GenBank accession numbers.

Primer	Primer sequence (5′–3′)	Repeat motif	Size	PCR	T_a	GenBank Accession No.
B30	F: TTAATTTGGCCCTGACCTTG R: CGGCGCAAATGATTCTTAAT	$(TCT)_{15}$	215–347	3	60	HQ323652
B47	F: TTTTTGTCTGGTTTTGGGTGT R: CACAATCCCCACATGATGAA	$(GAA)_{13}$	171–219	1	53	HQ323653
B83	F: CCCACCTAAAGCTGCCAATA R: TCTCCCCTTTACCCCTTTTT	$(AG)_{15}$	165–195	1	53	HQ323654
P164	F: ATCAAATCCCCACATTCCAA R: GTAGCTTGGGAGTGGAAGCA	$(CT)_{11}$	227–288	3	60	HQ323655
P211	F: CCCGTTGGAGAAATTCAAAA R: AGAATCACCGCCTTCGATTT	$(GA)_4…(GA)_{15}$	99–127	1	53	HQ323656
P215	F: ACCCCCATCATCTACTCGTG R: AACCCCATCAACAAAGAAGC	$(ATGT)_{10}$	212–244	1	53	HQ323657
C244	F: GAAGGGATTGCTCAGGCATA R: TGGGACCCACTCTTACTTGTG	$(GA)_{12}$	219–249	3	60	HQ323658
C246	F: TATCGGGAGATGGAGAGTGG R: CAAAAAGCTTCTTGAGAAACA	$(TA)_3(CATA)_{12}(TA)_3$	222–270	1	53	HQ323659
C281	F: ACTGTCAACTTTGAATAGAGA R: GTGACGGGTCATGCTATCCT	$(GA)_{13}$	239–261	2	54	HQ323660
C410	F: CAGCGGTTGAGATTCTAGGC R: TTCCTCCACTAACTTTTCATGTG	$(GAA)_{12}$	218–269	2	54	HQ323661

Loci were screened and scored in 50 individuals of each of five species in section *Sycocarpus* (*F. bernaysii, F. congesta, F. hispidioides, F. morobensis,* and *F. pachyrrhachis*) and two species in section *Sycidium* (*F. copiosa* and *F. wassa*). Amplification of microsatellite loci was performed on an Eppendorf Mastercycler in a total volume of 10 μL using 0.2 mM fluorescent end-labeled forward primer and unlabeled reverse primer, 0.2 mM buffer solution, 0.2 mM of each dNTP, 0.8 mM BSA, 0.3 units of TaKaRa Ex Taq polymerase (TAKARA BIO, Otsu, Shiga, Japan) and 20–50 ng template DNA. One of three sets of PCR conditions was used for each microsatellite locus. The first PCR condition included initial denaturing at 94°C (4 min) followed by 30 cycles of 94°C (30 s), 53°C (30 s), 72°C (45 s), and a final elongation step of 72°C (10 min). Condition 2 was a touchdown protocol with initial denaturing at 95°C (5 min) followed by a 10 cycle touchdown of 95°C (15 s), 64°C–54°C (30 s) and 72°C (30 s), 20 additional cycles at a 54°C annealing temperature, followed by a final elongation step of 72°C (10 min). Condition 3 was a touchdown protocol with a higher beginning with an annealing temperature initial denaturing at 95°C (5 min) followed by a 15 cycle touchdown of 95°C (15 s), 75°C–60°C (30 s) and 72°C (30 s), 20 additional cycles at a 60°C annealing temperature, followed by a final elongation step of 72°C (10 min). Microsatellite alleles were visualized using an ABI 377 Sequencer along with a ROX 500 (Applied Biosystems) size standard and scored using Genotyper 2.5 (Applied Biosystems).

GENEPOP on the web (Raymond and Rousset, 1995) was used to calculate number of alleles (N_a), observed and expected heterozygosity (H_o and H_E), and to test for Hardy–Weinberg equilibrium and linkage disequilibrium. The presence of sibling groups was tested using kinship analysis implemented in Kinalyzer (Ashley et al., 2009) using a broader data set of 13–14 microsatellite loci including those reported here and previously published loci (Zavodna et al., 2005; Crozier et al., 2007). Results reported in Tables 1 and 2 are based on samples of 50 individuals from single populations of each species.

Primers developed in *F. bernaysii* amplified 6–21 alleles per species in *Sycocarpus,* and expected heterozygosity ranged from 0.53 to 0.93. In contrast, primers developed in *F. pachyrrhachis* amplified 4–13 alleles per species from section *Sycocarpus*, and expected heterozygosity ranged from 0.25 to 0.86 (Table 2). Primers developed in *F. copiosa* amplified 5–15 alleles per species from section *Sycidium* and expected heterozygosity ranged from 0.68 to 0.87 (Table 2). The low heterozygosity observed in all species was due mainly to sampling of sibling groups identified in kinship analysis (Moe, unpublished data). After the removal of siblings from the analysis, populations were in Hardy–Weinberg equilibrium at most loci and had no significant evidence of linkage disequilibrium. Exceptions to Hardy–Weinberg equilibrium were most likely due to null alleles and are marked with asterisks in Table 2. Microsatellite loci B30, B47, and P164 also amplified in *Sycidium* species and all loci developed in *F. copiosa* (C244, C246, C281, C410) amplified in *Sycocarpus* species, but loci had low or no variability in these cross-amplifications.

CONCLUSIONS

These new microsatellite loci are highly variable within two dioecious *Ficus* sections and cross-amplify in closely related species. Several loci amplified across *Ficus* sections, but

TABLE 2. Results of initial primer screening based on a sample of 50 individuals from each species. The number of observed alleles (N_a), expected heterozygosity (H_E), and observed heterozygosity (H_o) for five species of *Ficus* section *Sycocarpus* and two species of *Ficus* section *Sycidium* are shown. Observed heterozygosity marked with an asterisk indicates significant deviation from H-W equilibrium after sibling groups were removed.

	$N_a/H_E/H_o$						
Primer	*F. bernaysii*	*F. congesta*	*F. hispidioides*	*F. morobensis*	*F. pachyrrhachis*	*F. copiosa*	*F. wassa*
B30	17/0.81/0.31*	14/0.91/0.50	21/0.93/0.36*	10/0.74/0.33	10/0.62/0.38		
B47	6/0.53/0.22*	8/0.76/0.74	6/0.73/0.72	7/0.81/0.81	8/0.76/0.79		
B83	11/0.90/0.70	12/0.80/0.74	10/0.68/0.48	10/0.84/0.77	10/0.74/0.40*		
P164	12/0.86/0.82	6/0.77/0.54*	7/0.48/0.46	7/0.66/0.79	4/0.66/0.75		
P211	10/0.66/0.66	6/0.61/0.50	13/0.82/0.68*	8/0.73/0.69	5/0.25/0.12		
P215	9/0.75/0.62	9/0.80/0.76	8/0.75/0.70	9/0.83/0.85	10/0.82/0.79		
C244						11/0.81/0.76	15/0.87/0.86
C246						13/0.86/0.90	9/0.74/0.53
C281						10/0.79/0.65	11/0.87/0.82
C410						5/0.68/0.37	15/0.76/0.37*

variability was reduced. Therefore, these markers may be useful for examining genetic structure within species, detecting gene flow among closely related species, and determining evolutionary relationships among recently diverged species in this ecologically important genus.

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APPENDIX 1. Voucher information for taxa used in this study. All voucher specimens are deposited in MIN.

Species – Country and Locality, Accession number

***Ficus bernaysii* King** – Papua New Guinea, Madang Province, Ohu, 920562

***Ficus congesta* Roxb.** – Papua New Guinea, Madang Province, Ohu, 920560

***Ficus copiosa* Steud.** – Papua New Guinea, Madang Province, Ohu, 920559

***Ficus hispidioides* Moore** – Papua New Guinea, Madang Province, Ohu, 920557

***Ficus morobensis* C.C. Berg** – Papua New Guinea, Madang Province, Ohu, 920561

***Ficus pachyrrhachis* Laut. et K. Schum** – Papua New Guinea, Madang Province, Ohu, 920556

***Ficus wassa* Roxb.** – Papua New Guinea, Madang Province, Ohu, 920558