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PRIMER NOTE

DEVELOPMENT OF 14 POLYMORPHIC MICROSATELLITE LOCI FOR *FICUS TIKOUA* (MORACEAE)[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed to facilitate studies on the fine-scale population genetic structure of *Ficus tikoua* (Moraceae), a prostrate shrub known to have highly restricted gene flow.
- *Methods and Results:* Microsatellite primers were developed using the biotin-streptavidin capture method and scanned for polymorphism within 76 individuals sampled from three natural *F. tikoua* populations. Fourteen loci were shown to be polymorphic, with allele numbers ranging from three to 16. The observed and expected heterozygosity in the three populations ranged from 0 to 1 and from 0 to 0.87, respectively. Substantial divergence was found among the populations at some loci. All loci can be successfully amplified in at least eight other *Ficus* species, indicating good transferability within the genus.
- *Conclusions:* The 14 microsatellite loci will be a helpful tool for assessing the fine-scale genetic structure of *F. tikoua*.

Key words: *Ficus* subgenus *Sycomorus*; *Ficus tikoua*; fig tree; gene flow; microsatellite; Moraceae.

Fig trees (*Ficus* L., Moraceae) are keystone species in many tropical and subtropical ecosystems, providing food for more frugivorous animal species than other plants (Shanahan et al., 2001). Fig tree species rely on highly species-specific pollinating fig wasps (Agaonidae) for pollen dispersal and seed production, and in turn provide food for fig wasp larvae. With more than 750 species, a diverse range of life forms (trees, shrubs, stranglers, and vines), breeding systems (monoecy and dioecy), and pollination modes (active and passive) among *Ficus* species (Herre et al., 2008), the interaction between *Ficus* species and their pollinators continues to stimulate and inform evolutionary and conservation questions (Cook and Rasplus, 2003; Herre et al., 2008).

Ficus tikoua Bureau was previously attributed to subgenus *Ficus* (Corner, 1965), but was transferred into subgenus *Sycomorus* (Gasp.) Miq. in a recent phylogenetic study (Cruaud et al., 2012). It is a functionally dioecious shrub with an unusual prostrate life form, so that the figs (syconia) in which the seeds and pollinating fig wasps develop are close to, or partially buried in, the soil. The position of the figs of *F. tikoua* makes them unusually hidden from its pollinator, potentially restricting the gene flow of both host and pollinator populations. Significant genetic differentiation was detected between *F. tikoua* populations separated by only 31 km using microsatellite primers developed for other *Ficus* species (Chen et al., 2011). However, these transferred primers showed low resolution in *F. tikoua*, with no more than four alleles per locus (see table 1 in Chen et al., 2011). High-resolution microsatellite markers for this species are therefore needed to assess its fine-scale genetic structure and degree of inbreeding, given its highly restricted gene flow.

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METHODS AND RESULTS

Leaves of 76 individuals were collected from three natural populations of *F. tikoua* in southwestern China, with two in Sichuan Province (Mianyang: 31°33′N, 104°26′E; Yanyuan: 27°14′N, 101°51′E) and one in Yunnan Province (Mengzi: 23°20′N, 103°25′E) (voucher specimen information is shown in Appendix 1). Genomic DNA of sampled individuals was extracted from silica gel–dried leaves using the Tiangen Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China).

Microsatellite primers were developed using the biotin-streptavidin capture method following the procedure of Kijas et al. (1994) and Tong et al. (2012). Approximately 250 ng of genomic DNA from each individual was digested with the restriction enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA). The digested fragments were linked to an MseI-adapter pair (F: 5′-TACTCAGGACTCAT-3′, R: 5′-GACGATGAGTCCTGAG-3′) and then amplified with an MseI-N primer (5′-GATGAGTCCTGAGTAAN-3′) using a protocol of 95°C for 3 min; followed by 20 cycles of 94°C for 30 s, 54°C for 1 min, and 72°C for 1 min; with a final extension at 72°C for 5 min. PCR products were hybridized with a 5′-biotinylated probe $(AG)_{15}$ at 48°C for 2 h, and microsatellite motifs were then captured with streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). The enriched motifs were again amplified with an MseI-N primer and purified with a multifunctional DNA Extraction Kit (Sangon Biotech, Shanghai, China). The purified products were ligated into a pMD 19-T vector (TaKaRa Biotechnology Co., Dalian, China) and transformed into *Escherichia coli* strain JM109. Positive clones were detected by PCR using $(AG)_{10}$ and $M13^+/M13^-$ primers.

A total of 106 positive clones were selected and sequenced with $M13^+/M13^-$ primers on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA) at Sangon Biotech. Ninety-five clones were found to contain simple sequence repeats, of which 51 primer pairs were designed using Primer Premier version 5.0 (PREMIER Biosoft International, Palo Alto, California, USA).

The amplification stability and polymorphism of each primer pair were tested using randomly selected individuals. The 10-μL PCR reaction volumes included 50 ng of genomic DNA, 0.2 mM of each dNTP, 0.1 μM of each primer, 1× PCR buffer (Mg^{2+} free), 2.5 mM Mg^{2+}, and 1 unit *Taq* DNA polymerase

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TABLE 1. Characteristics of 14 polymorphic microsatellite loci developed for *Ficus tikoua.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	*A*	T_a (°C)	GenBank accession no.
FT01	F: TACAAGCGGTAAACTG	$(AG)_8$	216–238	4	54	KT591855
	R: TTGGCAACCACTGACT					
FT02	F: CAAAATCGGAATAATGACAGC	$(CT)_{13}$	143–157	5	56	KT591856
	R: AAAGACAACGAAACTAAGAGG					
FT03	F: GCCACAAAAGCCATATC	$(TC)_7...(TC)_{24}$	156–188	15	51	KT591857
	R: TAATCTACGCACGCACT					
FT04	F: TCTCAACTCCCAAACACTCG	$(CT)_{12}$	107–109	3	62	KT591858
	R: ACCCCATTCTCAGCCATACC					
FT05	F: TGACGATGGACCAGGTTAGT	$(TC)_{13}$	337–361	5	62	KT591859
	R: AACGGATCTTCTTCAAAGCAAT					
FT06	F: CGAGAATAACGAGGCAATG	$(GA)_9$	209–217	4	62	KT591860
	R: ACGGAAGCCCTAAACCTA					
FT07	F: CCACAACTACCCAACCAAG	$(TG)_7A(GA)_8$	248–254	5	60	KT591861
	R: GCTGGAGCCAAATCATCTA					
FT08	F: TTACAAGCTCGGAAACAGT	$(TC)_9$	191–215	7	62	KT591862
	R: TTAGCACGTTGGTATCCTT					
FT09	F: TGAAGCGTTGGAGGATAG	$(TC)_{14}$	206–220	6	56	KT591863
	R: TGCCGTGAACATCAAGAG					
FT10	F: TCCTCCCTCTGTCCCTTCT	$(TC)_9$	126–130	13	58	KT591864
	R: ACCTTGGGTTCTGCCTCC					
FT11	F: GCGGAATCTTTGAGGGAA	$(GA)_{13}$	190–218	8	58	KT591865
	R: AAGGCTGGAGCAATGAAC					
FT12	F: TTTCCTTCTCCAACACTG	$(GA)_6$	286–308	4	58	KT591866
	R: ACAGCACAAACAGCACCA					
FT13	F: GTGGGTGACATTGGTGAAG	$(CT)_{18}$	195–199	13	54	KT591867
	R: GCCATAAATACAAGAGGGA					
FT14	F: GAAGAGGCCCTGAGATAA	$(GA)_{12}$	195–200	11	56	KT591868
	R: GATCAAGCGATGACAACC					

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

(Sangon Biotech), which was performed under the following conditions: 94°C for 5 min; 30 cycles with each cycle lasting 30 s at 94°C, 30 s at a primer-specific annealing temperature (Table 1), and 30 s at 72°C; and a final extension of 72°C for 8 min. PCR products were first checked on 1.2% agarose gels, resolved on 8% polyacrylamide denaturing gel, and then visualized by silver staining, with pUC19 DNA/MapI (HpaII) (Fermentas International, Burlington, Ontario, Canada) as the ladder.

To rate the polymorphism of each locus, genomic DNA templates of all 76 *F. tikoua* individuals from the three natural populations were used (Table 2). The forward primers of each polymorphic locus were labeled with fluorescent dyes (5′TAMRA, 5′ROX, 5′FAM; Sangon Biotech) for scoring fragment length on an ABI 3130 automated sequencer (Applied Biosystems), using GeneScan 500 LIZ (Applied Biosystems) as an internal lane standard. Fragment lengths were calculated by GeneMapper version 4.0 software (Applied Biosystems).

The linkage disequilibrium (LD) and deviation from Hardy–Weinberg equilibrium (HWE) for each locus in the three populations were evaluated using GENEPOP version 4.2.2 (Rousset, 2008). The genetic diversities of each population were assessed using FSTAT 2.9.3.2 (Goudet, 2001) for the following indexes: the number of alleles per locus (*A*), observed heterozygosity (H_o), and unbiased expected heterozygosity (H_e).

In total, 14 loci proved to be polymorphic, with allele numbers ranging from three to 16 in total (Table 1) and from one to 11 within populations. No significant LD was found between any pair of loci, and no loci were found to deviate

TABLE 2. Genetic diversity measures for 14 polymorphic microsatellite loci in three *Ficus tikoua* populations.[a]

	Mianyang (*N* = 30)			Yanyuan (*N* = 29)			Mengzi (*N* = 17)		
Locus	*A*	H_o[b]	H_e	*A*	H_o[b]	H_e	*A*	H_o[b]	H_e
FT01	4	0.700	0.697	3	0.103	0.101	2	0.250	0.484
FT02	4	0.600	0.581	1	0.000	0.000	2	0.231	0.471
FT03	8	0.625	0.804	11	0.696	0.824	9	0.588*	0.870
FT04	2	1.000*	0.509	3	1.000*	0.527	2	0.294	0.259
FT05	1	0.000	0.000	1	0.000	0.000	5	0.600	0.602
FT06	2	0.133	0.127	3	0.035*	0.068	4	0.235	0.223
FT07	1	0.000	0.000	3	0.464	0.413	4	0.214	0.323
FT08	5	0.267*	0.446	5	0.586	0.677	4	0.294*	0.570
FT09	4	0.500	0.596	5	0.846	0.784	2	0.133	0.129
FT10	4	0.433	0.584	5	0.380	0.555	9	0.688*	0.845
FT11	4	0.333	0.427	5	0.714	0.714	5	0.333	0.614
FT12	2	0.033	0.033	4	0.192	0.360	2	0.294	0.508
FT13	8	0.400	0.738	7	0.414*	0.795	4	0.563	0.569
FT14	4	0.667	0.696	7	0.379	0.420	9	0.706	0.786
Average	3.786	0.369	0.445	4.500	0.397	0.446	4.500	0.350	0.518

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = sampled individuals from each population.

[a] See Appendix 1 for locality and voucher information.

[b] Significant deviation from Hardy–Weinberg equilibrium after sequential Bonferroni correction (*$P < 0.05$).

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TABLE 3. Amplification of 14 microsatellite primers developed for *Ficus tikoua* in 12 other *Ficus* species.

Ficus species	Subgenus	FT01	FT02	FT03	FT04	FT05	FT06	FT07	FT08	FT09	FT10	FT11	FT12	FT13	FT14
F. deltoidea Jack	*Ficus*	—	+	+	+	+	+	+	+	—	—	—	—	—	+
F. stenophylla Hemsl.	*Ficus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. montana Burm. f.	*Sycidium*	—	+	—	+	+	+	+	+	+	+	+	+	+	+
F. hispida L. f.	*Sycomorus*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. rumphii Blume	*Synoecia*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. altissima Blume	*Urostigma*	+	+	+	+	+	+	+	+	—	+	+	—	—	+
F. benjamina L.	*Urostigma*	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. concinna (Miq.) Miq.	*Urostigma*	+	+	+	+	+	+	+	—	+	—	+	—	+	+
F. geniculata Kurz	*Urostigma*	+	+	—	+	+	+	+	+	+	+	+	+	+	+
F. martini H. Lév. & Vaniot	*Urostigma*	—	+	+	+	+	+	+	—	+	+	+	+	+	+
F. microcarpa L. f.	*Urostigma*	+	+	+	—	+	+	—	—	+	+	+	+	+	+
F. virens Dryand.	*Urostigma*	+	+	+	+	+	+	+	+	+	—	+	—	+	+

Note: + = primers could be successfully amplified; — = primers could not be amplified.

from HWE in all three populations (Table 2). H_o and H_e ranged from 0 to 1 and from 0 to 0.87, respectively, and substantial between-population differences were found at loci FT02, FT05, and FT07 (Table 2). The genetic diversity values of developed loci were comparable with those of microsatellite loci developed for other *Ficus* species, such as for *F. hirta* Vahl (Zheng et al., 2015). In addition, relatively low genetic variation was found in Mianyang, the northernmost population, despite its large sample size, which further verified the validity of these loci.

Cross-species amplification of the 14 developed primers was tested in 12 other *Ficus* species, using the same procedures described above. The involved species covered most *Ficus* subgenera distributed in the Asian-Australasian region (four out of five subgenera). All primers successfully amplified in at least eight additional species (Table 3), indicating good transferability of these primers.

CONCLUSIONS

The 14 microsatellite loci developed for *F. tikoua* showed high genetic diversity and substantial differences among populations. They will be useful for further studies of fine-scale genetic structure and gene flow in *F. tikoua*.

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APPENDIX 1. Voucher information for *Ficus tikoua* specimens used in this study.[a]

Accession no.[b]	Collection locality	Geographic coordinates
SZ-MYNU-00000002-LsZ,YC	Mianyang, Sichuan	31°33′N, 104°26′E
SZ-MYNU-00000001-LsZ	Yanyuan, Sichuan	27°14′N, 101°51′E
SZ-MYNU-00000003-LsZ,RhF,JyD	Mengzi, Yunnan	23°20′N, 103°25′E

[a] Vouchers are deposited at the Plant Specimen Museum of Sichuan University (SZ), Chengdu, Sichuan, China.
[b] Collectors: LsZ = Lushui Zhang; YC = Yan Chen; RhF = Ronghua Fu; JyD = Junyin Deng.

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