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Development and polymorphism of microsatellite primers in *Ficus pumila* L. (Moraceae)[1]

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- *Premise of the study*: Microsatellite primers were developed in the functionally dioecious *Ficus pumila* L. to provide polymorphic markers for further population genetic studies and parentage analysis.
- *Methods and Results*: Eleven polymorphic microsatellite loci were developed in *F. pumila*. These loci were successfully amplified in four *F. pumila* populations from eastern China (Fodu, Xiangshan, Xianju, and Hexi). These loci had 3–11 alleles across all 80 *F. pumila* individuals. At the population level, the number of alleles per locus varied from 1 to 8, and the observed (H_O) and expected (H_E) heterozygosities ranged from 0.000 to 0.900 and from 0.000 to 0.830, respectively. Linkage disequilibrium between loci *FP213* and *FP435* was found in three of the four tested populations.
- *Conclusions*: These loci showed high levels of polymorphism, indicating the utility of these primers in population genetic studies as well as parentage analysis of *F. pumila*.

Key words: *Ficus pumila*; genetic variation; microsatellite primers; polymorphism.

Ficus, with roughly 800 species, is one of the largest genera of angiosperms, and is also one of the most important plant genera in lowland rainforests (Harrison, 2005). A distinct feature of this genus is the specific mutualism with its pollinating wasps, which has long fascinated ecologists and evolutionists. Each fig species has its own pollinating wasp species from the family Agaonidae; and in turn, the wasp species depends upon the fig for development and reproduction (Cook and Rasplus, 2003; Chen et al., 2010). The functionally dioecious *Ficus pumila* L. (Moraceae), one of the most northward-distributed figs, is widely distributed in subtropical areas of eastern Asia. High genetic variation was generally found in long-lived, widespread, outcrossing species (Hamrick and Godt, 1996). However, using microsatellites developed in other figs, low genetic variation was found in *F. pumila* populations (Chen et al., 2008; Wang et al., 2009). A possible cause is that the probability of occurrence of a null allele may be higher than in the species from which the microsatellites were isolated and developed (Smulders et al., 1997). In this study, we isolated microsatellite loci and evaluated their polymorphism in *F. pumila*. These loci will be useful for analyzing genetic structure, parentage, and phylogeographic pattern in *F. pumila* and its close relatives.

METHODS AND RESULTS

Total genomic DNA of *F. pumila* was extracted from silica-gel-dried leaves using the Plant Genomic DNA Kit (Tiangen, Beijing, China). Microsatellites were developed according to Liu et al.'s (2009) protocol. Approximately 250 ng of genomic DNA was digested with *Mse* I restriction enzyme (NEB) and fragments of 200–800 bp in length were fractionated. An *Mse* I-adapter pair was ligated to DNA fragments of the appropriate length. Five μL of a 1:10 dilution of the adapter-ligated fragments were used as templates for PCR in 20 μL reactions using *Mse* I-N primer (5′-GATGAGTCCTGAGTAAN-3′) with the following conditions: 3 min denaturation at 95°C; followed by 30 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C; with a final extension of 72°C for 5 min. The PCR products were denatured at 95°C for 5 min, and then hybridized with 5′-biotinylated probe $(AG)_{15}$ in 250 μL hybridization solution at 48°C for 2 h. Hybridization products were selectively captured with streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA). After stringent washing, the captured DNA fragments were eluted in 50 μL of 1 × TE. The enriched product was amplified with *Mse* I-N as primers for 30 cycles as described above. After purification with a multifunctional DNA Extraction Kit (Bioteke, Beijing, China), PCR products were ligated into pMD 19-T vector (Takara, Dalian, China), and these plasmids were used to transform *E. coli* strain JM109. A total of 690 positive clones were chosen and tested by PCR using $(AG)_{10}$ and $M13^+$ /$M13^-$ as primers, respectively.

Of the 690 positive clones, 192 were sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA), and 58 were found to contain simple sequence repeats. Nine of the 58 sequences were discarded because the repeat was too short to design primers, and 49 primer pairs were designed using the program Primer Premier 5.0 (http://www.premierbiosoft.com). These primers were tested for polymorphism in 21 *F. pumila* individuals drawn randomly from different populations from eastern China. PCR was performed in a 20-μL final volume, which included approximately 50 ng of genomic DNA, 0.2 mM each dNTPs, 0.1 μM of each primer, 1× PCR buffer, 1.5 mM Mg^{2+}, 0.4 U of DNA Taq polymerase (Sangon, Shanghai, China). Microsatellite loci were amplified under the following conditions: 5 min denaturation at 94°C; 35 cycles of 30 s at 94°C, 45 s at 52–62.5°C (Table 1), 45 s at 72°C; and a final extension of 72°C for 8 min. Amplified PCR products were resolved using 8% polyacrylamide denaturing gels and visualized by silver staining using pUC19DNA/Msp I (Hpa II) (Fermentas, Vilnius, Lithuania) as the ladder.

Finally, we obtained 11 loci that had clear polymorphic products (Table 1). The applicability of these 11 loci was further tested in 80 individuals from four *F. pumila* populations, which are located in Fodu (122°01′39″E, 29°44′53″N), Xiangshan (121°46′29″E, 29°32′19″N) and Xianju (120°36′23″E, 28°47′01″N) of Zhejiang Province, and Hexi (117°13′40″E, 24°52′44″N) of Fujian Province,

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TABLE 1. Eleven polymorphic microsatellite loci developed in *Ficus pumila*. Forward and reverse primers, repeat motif, allele size range, number of alleles (A), annealing temperature (*Ta*), and GenBank accession numbers are given. The fluorescent dye labels for these loci are also shown.

Locus	Sequences of forward (F) and reverse (R) primers	Motif	Size range (bp)	A	*Ta* (°C)	GenBank Accession No.
FP9	F: <6-FAM> TACTCTGACCAACAATAACG R: CATGTAATCTGTTCTGAGGA	$(AG)_{10}$	166–180	6	54.5	HQ148556
FP38	F: <6-FAM> ATAATGTCTTTTACGCGTGT R: ATTCCAAGGTCCTCCTACTA	$(AG)_{12}$	123–151	11	54.3	HQ148557
FP102	F: <TAMRA> GACAGTACCATTTCCGATTC R: ATGTAAGTTCCGCATGAGTT	$(CT)_{14}$	205–243	10	62.5	HQ148558
FP134	F: <ROX> ATTTGATGGGTTTTCTGTTT R: TCTTCTTCATCGTTTCATTC	$(AG)_{13}$	116–142	11	58	HQ148559
FP213	F: <TAMRA> CATGCAACAAACTTCCTTAC R: GTACTAGGATTGTTCGTGCT	$(CT)_7 \ldots (CT)_9$	183–201	6	61	HQ148560
FP327	F: <6-FAM> GCCAAAGAAAGAAGGGAGAA R: TGGTTGCCGAGAAAACATA	$(AG)_{11}$	119–137	7	62	HQ148561
FP328	F: <6-FAM> TCAATTCTTTTCGCTATCTC R: GAAGCTACGTTGTGCTTTAT	$(CT)_{11} \ldots (CT)_{14}$	129–143	8	59	HQ148562
FP435	F: <ROX> GTTATTACAAGGTTTGGTCG R: GCAACAAACTTCCTTACATT	$(AG)_{10} \ldots (AG)_{16}$	153–171	6	53.5	HQ148563
FP540	F: <ROX> TCAATTCTTTTCGCTATCTC R: GAAGCTACGTTGTGCTTTAT	$(CT)_{14} \ldots (CT)_{14}$	133–149	7	52	HQ148564
FP556	F: <6-FAM> ACCCTAACTCAGCAACTATT R: AAAATGGTATGAAATGGTAT	$(CT)_{12} \ldots (CT)_4$	96–114	4	61	HQ148565
FP601	F: <HEX> TGGGTTATTCCGAATGGTCT R: CGAAATGCCTTTCCTTTGAG	$(CT)_8GC(CT)_3$	182–190	3	53.5	HQ148566

TABLE 2. Characteristics of 11 polymorphic microsatellite loci tested in four *F. pumila* populations. Number of samples genotyped (*N*), number of alleles (A), observed (H_O) and expected (H_E) heterozygosities.

	Fodu				Xiangshan				Xianju				Hexi			
Locus	*N*	A	H_O	H_E	*N*	A	H_O	H_E	*N*	A	H_O	H_E	*N*	A	H_O	H_E
FP9	20	5	0.600	0.749	20	5	0.750	0.708	20	3	0.700	0.573	20	4	0.700	0.585
FP38	20	8	0.800	0.786	19	4	0.790	0.627	20	1	0.000	0.000	20	6	0.750	0.754
FP102	19	8	0.737	0.794	20	6	0.500	0.689	20	3	0.650	0.676	20	4	0.850	0.735
FP134	20	5	0.400	0.430	20	7	0.500	0.676	20	7	0.900	0.830	20	4	0.400	0.458
FP213	20	5	0.750	0.750	20	4	0.550	0.514	20	3	0.550	0.465	20	4	0.650	0.727
FP327	20	4	0.100	0.677	20	5	0.150	0.735	20	5	0.100	0.769	20	5	0.550	0.719
FP328	20	6	0.700	0.621	20	5	0.700	0.609	20	5	0.600	0.523	20	5	0.850	0.753
FP435	20	5	0.750	0.750	20	4	0.600	0.572	20	3	0.550	0.465	20	4	0.550	0.668
FP540	20	6	0.550	0.649	20	5	0.650	0.609	20	5	0.550	0.523	20	5	0.750	0.740
FP556	20	3	0.550	0.595	20	3	0.600	0.528	20	2	0.200	0.185	20	4	0.500	0.573
FP601	20	3	0.650	0.524	20	3	0.150	0.145	20	1	0.000	0.000	20	3	0.750	0.580
Mean	19.9	5.3	0.599	0.666	19.9	4.6	0.540	0.583	20.0	3.5	0.436	0.455	20.0	4.4	0.664	0.663

respectively (Appendix 1). Polymorphic primers were labeled with fluorescent dye 5′ HEX, 5′ TAMRA, 5′ ROX, or 5′6-FAM (Sangon, Shanghai, China) (Table 1), which was carried out in a 15-μL reaction volume. The amplification products were combined into three pools (pool 1: FP328, FP134, and FP556; pool 2: FP9, FP213, FP327, and FP435; pool 3: FP38, FP102, FP540, and FP601), and each pool was scanned on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, California, USA) using an internal lane standard (GS500 (–250)Liz). Allele binning and calling were performed using then software GENEMAPPER 4.0 (Applied Biosystems).

All 11 microsatellite primers yielded polymorphic products. The number of alleles (A) present at each locus was 3–11, with an average of 7.2 (Table 1). At the population level, number of alleles per locus varied from 1 to 8, and the observed (H_O) and expected (H_E) heterozygosities ranged from 0.000 to 0.900 and from 0.000 to 0.830 (Table 2), respectively, as calculated using the software TFPGA v1.3 (Miller, 1997). Linkage disequilibrium between loci *FP213* and *FP435* was found in populations Foudu, Xianju, and Hexi using software the GENEPOP v4.0(Rousset, 2008) followed by sequential Bonferroni correction (Rice, 1989).

Using polymorphic loci isolated from *F. pumila*, we revealed more alleles per polymorphic locus in *F. pumila* than reported in previous studies based on loci isolated from other *Ficus* species (Chen et al., 2008; Wang et al., 2009), even though we genotyped fewer samples than in previous studies.

CONCLUSIONS

The development of polymorphic microsatellite primers is an important step to study the genetic variation of *F. pumila* populations. The high levels of polymorphism of these microsatellite loci render them ideal for population genetic studies. We will use them to analyze genetic differentiation among populations as well as to identify parentage within populations of *F. pumila*, and thus, to understand the potential role of pollinating wasps in this species.

LITERATURE CITED

CHEN, Y., H.-Q. LI, M. LIU, AND X.-Y. CHEN. 2010. Species-specificity and coevolution of figs and their pollinating wasps. *Biodiversity Science* 18: 1–10.

CHEN, Y., M. M. SHI, B. AI, J. M. GU, AND X. Y. CHEN. 2008. Genetic variation in island and mainland populations of *Ficus pumila* (Moraceae) in eastern Zhejiang of China. *Symbiosis* 45: 37–44.

COOK, J. M., AND J.-Y. RASPLUS. 2003. Mutualists with attitude: Coevolving fig wasps and figs. *Trends in Ecology & Evolution* 18: 241–248.

HAMRICK, J. L., AND M. J. W. GODT. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London* 351: 1291–1298.

HARRISON, R. D. 2005. Figs and the diversity of tropical rainforests. *Bioscience* 55: 1053–1064.

LIU, M., M.-M. SHI, M.-H. LIU, AND X.-Y. CHEN. 2009. Isolation and characterization of microsatellite loci in *Fagus longipetiolata* Seem. (Fagaceae). *Conservation Genetics* 10: 1981–1983.

MILLER, M. P. 1997. Tools for population genetic analyses (TFPGA) v1.3: A windows program for the analysis of allozyme and molecular genetic data. Department of Biological Sciences, Northern Arizona University, Flagstaff.

RICE, W. R. 1989. Analyzing tables of statistical tests. *Evolution; International Journal of Organic Evolution* 43: 223–225.

ROUSSET, F. 2008. Genepop'007: A complete re-implementation of the genepop software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

SMULDERS, M. J. M., G. BREDEMEIJER, W. RUS-KORTEKAAS, P. ARENS, AND B. VOSMAN. 1997. Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. *Theoretical and Applied Genetics* 94: 264–272.

WANG, R., B. AI, B. Q. GAO, S. YU, Y. Y. LI, AND X. Y. CHEN. 2009. Spatial genetic structure and restricted gene flow in a functionally dioecious fig, *Ficus pumila* L. *pumila* (Moraceae). *Population Ecology* 51: 307–315.

APPENDIX 1. Information on voucher specimens deposited at East China Normal University.

Taxon—- *Voucher specimens*, Locality in East China.	
Ficus pumila – *FD-01*and *FD-02*, Fodu, Zhejiang, China.	*XJ-01* and *XJ-02*, Xianju, Zhejiang, China.
XS-01 and *XS-02*, Xiangshan, Zhejiang, China.	*HE-01* and *HE-02*, Hexi, Fujian, China.