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Primer Note

Characterization of microsatellite markers for *Broussonetia papyrifera* (Moraceae)[1]

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- *Premise of the study: Broussonetia papyrifera* (Moraceae) is native to Asia and is used as a medicinal plant and as a source of fiber for making paper. It was dispersed into the Pacific region as a fiber source for making nonwoven textiles (barkcloth). Microsatellites were developed to trace the human-mediated dispersal of this species into the Pacific region.
- *Methods and Results:* A set of 36 microsatellites was isolated and initially assayed on 10 accessions to assess polymorphism. We found that 20 markers were polymorphic, with the number of alleles per marker ranging from four to 35 in 70 accessions genotyped from three Asian populations. Observed and expected heterozygosities ranged from 0.04 to 0.85 and from 0.19 to 0.94, respectively. These markers were tested in four Moraceae species and one Rosaceae species.
- *Conclusions:* These markers will be useful for the assessment of genetic diversity in *B. papyrifera*. They show low transferability to other species tested.

Key words: *Broussonetia papyrifera*; genetic diversity; microsatellite; Moraceae.

Paper mulberry (*Broussonetia papyrifera* (L.) L'Hér. ex Vent.), belonging to the family Moraceae, is a multifunctional tree of cultural importance in Asia that has been used for centuries in the manufacture of high-quality paper. *Broussonetia papyrifera* is native to southern and central China, Vietnam, Thailand, and Taiwan (Matthews, 1996; Chang et al., 2015), where it is common in secondary forests growing at moderate elevations. *Broussonetia papyrifera* was intentionally transported into the Pacific region by prehistoric voyagers for making barkcloth, a nonwoven textile, and several centuries ago to Japan as a high-quality fiber source (Whistler, 2009).

The genetic diversity of *B. papyrifera* has been studied using intersimple sequence repeat (ISSR) markers (Ho and Chang, 2006; Liao et al., 2014; González-Lorca et al., 2015), sequence-related amplified polymorphism (SRAP) markers (Liu et al., 2009), hypervariable chloroplast DNA (cpDNA) sequences, and internal transcribed spacer (ITS) sequences of ribosomal DNA (Chang et al., 2015). These molecular markers have been useful for characterizing the genetic diversity and population structure of this species within its native range. However, studies using ITS sequences and ISSR data (Seelenfreund et al., 2011; Gonzalez-Lorca et al., 2015) and a sex marker (Peñailillo et al., 2016) in *B. papyrifera* in the introduced range in the Pacific region have not provided the resolution to understand its dispersal across this geographic area where it has been propagated asexually (Peñailillo et al., 2016). Therefore, the development of microsatellite markers is crucial to genotype the species' genetic diversity in Japan and the Pacific region. Here, we present the isolation and characterization of 20 microsatellite markers that will provide information on the fine structure of *B. papyrifera* populations both in its native and introduced range.

METHODS AND RESULTS

To isolate microsatellites, genomic DNA from 10 samples of *B. papyrifera* collected in Taiwan, Japan, Chile, and several islands in Oceania was used (Appendix 1). These samples are deposited at the University of Chile, and one sample (BQUCH0152) has a voucher (SGO162505) at the herbarium of the National Museum of Natural History, Chile. Total DNA was extracted from young silica gel–dried leaves following the cetyltrimethylammonium bromide (CTAB) extraction protocol (Lodhi et al., 1994) and modified as described in Moncada et al. (2013). Approximately 1 cm^2 of tissue was homogenized, mixed with extraction buffer (50 mM EDTA, 100 mM Tris-HCl, 0.3 M NaCl, 2.0% [w/v] CTAB, 0.5% [v/v] 2-mercaptoethanol [pH 8.0]), and incubated at 65°C for

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25 min, followed by organic extraction. Total DNA was precipitated and stored at −20°C until analysis. Purified DNA was quantified by spectrophotometric absorbance (Nanodrop, ThermoFisher Scientific, Wilmington, Delaware, USA) and Picogreen (Synergy H1, Winooski, Vermont, USA) and its integrity verified by 0.8% agarose gel electrophoresis.

Ecogenics GmbH (Zurich, Switzerland) constructed an enriched library using magnetic streptavidin beads and biotin-labeled CT and GT repeat oligonucleotides to develop size-selected fragments of genomic DNA for enrichment of microsatellite sequences. The microsatellite-enriched library was analyzed with GS FLX Titanium chemistry on a Roche 454 platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). A total of 32,947 reads was found with an average length of 321 bp. Microsatellite simple sequence repeats (SSRs) with tri- or tetranucleotide motifs, repeated at least six times, and dinucleotides of at least 10 repeat units, were found in 10,024 reads. Primers were designed for 190 of these reads, and 36 were tested for the presence of polymorphisms on 10 samples from different geographic areas.

Polymorphisms in these 36 loci were assessed using the procedure described by Schuelke (2000). A universal 18-bp fluorophore-labeled M13 tail (5′-TGTAAAACGACGGCCAGT-3′) was incorporated into the PCR products during the first PCR cycles. In subsequent cycles, these products function as templates for the fluorophore-labeled universal M13 primer to produce fluorescent PCR products. PCR reactions were performed in a reaction volume of 10 µL containing 2–10 ng of genomic DNA, 1× buffer with 15 mM $MgCl_2$, 200 µM dNTP mix, 0.04 µM forward primer, 0.16 µM of the reverse and the M13 primer, and 0.05 µL of HotStarTaq DNA polymerase (QIAGEN, Hilden, Germany). PCR amplifications were conducted under the following conditions: an initial denaturation of 15 min at 95°C; 30 cycles at 95°C for 30 s, at an annealing temperature specific for each primer for 45 s, and at 72°C for 45 s; followed by eight cycles at 95°C for 30 s, at 53–55°C for 45 s, and at 72°C for 45 s; and a final extension step at 72°C for 30 min.

The amplified products were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) with GeneScan 500 ROX Size Standard (Applied Biosystems). Genotypes were determined using GeneMapper version 3.2 (Applied Biosystems) with default settings. Due to the M13 tail attached to each forward primer, 18 bp were subtracted from the experimentally determined amplicons to obtain the length of actual alleles.

Twenty primer pairs were successfully amplified, with the expected sizes and banding patterns displaying clear polymorphisms in *B. papyrifera* (Table 1). Polymorphisms were evaluated in samples of *B. papyrifera* from its native range (three Asian populations, $n = 70$). Table 2 shows the number of alleles per marker, observed and expected heterozygosity (H_o and H_e), polymorphism information content (PIC), coefficient of inbreeding (F_{IS}), null allele frequency (r), and Hardy–Weinberg equilibrium (HWE) of the analyzed samples from three populations. H_o, H_e, and F_{IS} were estimated using Arlequin 3.5.2.2 (Excoffier and Lischer, 2010). CERVUS 3.0.7 was used to calculate PIC (Kalinowski et al., 2007). Null allele frequency was calculated using MICRO-CHECKER (van Oosterhout et al., 2004) and HWE with GenAlEx 6.502 (Peakall and Smouse, 2006, 2012). The total number of alleles ranged from four to 35 with a mean of 19.2 (Table 1). H_o and H_e ranged from 0.038 to 0.846 and from 0.191 to

Table 1. Characteristics of 20 polymorphic microsatellite loci developed in *Broussonetia papyrifera.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	A	T_a (°C)	GenBank accession no.
Bropap_01931	F: GGCATCGTTTTGACTGTTGG	$(AC)_{16}$	170–219	20	54	KU598930
	R: GCAGTGAGGTAAGGCCCAG					
Bropap_02075	F: GGTGGTGATGCAGAACATGC	$(TAG)_8$	224–256	12	55	KU598931
	R: TTTCCCTCGCGTCAAGATCG					
Bropap_02214	F: CGGAAGAGAGAGATTACAAACTAGC	$(GA)_{19}$	220–262	24	55	KU598932
	R: ACTTGACCATTCTAAGCAAGACC					
Bropap_02359	F: ATTTGTTGCTGCCCAAGGTC	$(AGA)_8$	168–183	4	56	KU598933
	R: TTCGCTCCCTTCCCTATTGC					
Bropap_02560	F: CATGTGGGACCCTGAAAACC	$(AC)_{15}$	179–254	21	55	KU598934
	R: AGAAGCCGTCTTACTGGGAG					
Bropap_02801	F: GACATCTCATAAAATGTTTAAATCCAG	$(AC)_{11}$	102–179	20	56	KU598935
	R: AATTGCCCTGTAGCATTCCG					
Bropap_03147	F: CAGACTAGGTTGACCGCAAG	$(GT)_{11}$	192–201	8	55	KU598936
	R: CACGTTTTGAACCTGGGAGC					
Bropap_07697	F: GGTGCATCGCTGATTTTTGC	$(AC)_{13}$	185–256	20	55	KU598937
	R: CCTGCCAAATACACCAACCG					
Bropap_15123	F: TTACGTTTCCTCCCACGTCC	$(GA)_{15}$	147–184	19	55	KU598938
	R: CGCCATTACAAAGTAAACTGCC					
Bropap_16591	F: CCTCCTCTACATAACAGGCAG	$(AG)_{17}$	62–89	23	56	KU598939
	R: TCGCGATCTTGTAGCCTTCG					
Bropap_20558	F: TCCACCATCCAACGATGAAG	$(TAT)_7$	214–248	13	55	KU598940
	R: GGGCTAAACTACCTTGTCCG					
Bropap_20939	F: GACACATGCACTCACAAATGC	$(AG)_{13}$	119–178	31	56	KU598941
	R: GGGGCGATGAAAGACTTGAG					
Bropap_21091	F: TGCTAGTTTGGTTTAAGACAGG	$(AC)_{22}$	146–191	30	55	KU598942
	R: CAAGGGACAACAGCAATGAAAG					
Bropap_22348	F: CCTCCAAGCACCTTTTAGGC	$(TC)_{12}$	110–116	5	54	KU598943
	R: CGTACGTTGAACAAAATGGACAC					
Bropap_23758	F: CGTCATTTTGCCTTGCCTTC	$(AGA)_7$	199–225	14	55	KU598944
	R: AACAAACCACCCAAGATGCC					
Bropap_23810	F: AAAATGCCTCGCCATTGACC	$(GA)_{12}$	156–197	20	55	KU598945
	R: CCCACCAAACCTCGAAAACC					
Bropap_25444	F: TCACACTTACACACGGAGGG	$(GA)_{14}$	146–212	28	55	KU598946
	R: GGTACGTAATTCCCACCACC					
Bropap_26773	F: CCTGCATCTGACACCAAGTC	$(TC)_{13}$	169–264	29	56	KU598947
	R: ATTGCTCTCTTGAGGGGTGG					
Bropap_26985	F: AGAATCACCACTCTCCCTTGG	$(TG)_{12}$	178–191	8	56	KU598948
	R: TGGTTTGCTTCATTCAAAAAGTG					
Bropap_30248	F: AGAGCAGGGCAAGCAATATC	$(GA)_{14}$	83–195	35	56	KU598949
	R: GGTCATCCATTTGTCTGAACCTC					

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

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TABLE 2. Genetic properties of the 20 newly developed polymorphic microsatellites of *Broussonetia papyrifera*.[a]

Locus	Taiwan (n = 24)						China-Guangdong (n = 26)						China-Yunnan (n = 20)					
	A	H_o	H_e	PIC	F_{IS}	r	HWE[b]	A	H_o	H_e	PIC	F_{IS}	r	HWE[b]	A	H_o	H_e	PIC	F_{IS}	r	HWE[b]
Bropap_01931	5	0.083	0.684	0.618	0.880	0.396	82.206***	13	0.731	0.778	0.736	0.061	0.016	95.908^{ns}	9	0.579	0.811	0.762	0.292	0.137	44.281^{ns}
Bropap_02075	2	0.083	0.284	0.239	0.711	0.243	11.760***	9	0.231	0.587	0.541	0.612	0.288	147.262***	5	0.158	0.602	0.532	0.743	0.327	39.474***
Bropap_02214	17	0.792	0.931	0.905	0.152	0.064	152.385^{ns}	10	0.769	0.830	0.795	0.075	0.031	41.515^{ns}	12	0.684	0.834	0.792	0.183	0.083	54.562^{ns}
Bropap_02359	2	0.125	0.191	0.169	0.349	0.117	2.617^{ns}	3	0.192	0.242	0.217	0.209	0.075	1.669^{ns}	3	0.263	0.317	0.275	0.174	0.061	0.971^{ns}
Bropap_02560	5	0.208	0.660	0.608	0.689	0.317	46.039***	13	0.692	0.823	0.792	0.161	0.088	113.967**	10	0.526	0.839	0.796	0.379	0.182	61.204^{ns}
Bropap_02801	8	0.542	0.677	0.636	0.204	0.101	42.756*	9	0.692	0.680	0.610	–0.018	–0.037	37.294^{ns}	11	0.684	0.853	0.811	0.203	0.083	99.592***
Bropap_03147	4	0.458	0.646	0.577	0.295	0.144	35.458***	5	0.423	0.613	0.545	0.314	0.140	14.972^{ns}	6	0.474	0.728	0.676	0.356	0.164	21.984^{ns}
Bropap_07697	10	0.417	0.760	0.708	0.457	0.226	101.345***	14	0.346	0.911	0.885	0.625	0.296	219.180***	11	0.474	0.856	0.817	0.454	0.215	105.556***
Bropap_15123	7	0.500	0.791	0.742	0.373	0.168	32.367^{ns}	12	0.538	0.766	0.735	0.301	0.134	107.792***	8	0.263	0.720	0.675	0.641	0.295	65.363***
Bropap_16591	11	0.667	0.855	0.817	0.224	0.106	54.653^{ns}	16	0.462	0.928	0.903	0.507	0.243	201.769***	12	0.316	0.890	0.853	0.652	0.315	148.766***
Bropap_20558	5	0.458	0.682	0.607	0.332	0.150	20.954*	8	0.577	0.676	0.634	0.149	0.076	58.285***	9	0.684	0.752	0.698	0.093	0.041	30.828^{ns}
Bropap_20939	9	0.333	0.560	0.520	0.410	0.199	41.092^{ns}	23	0.769	0.920	0.895	0.167	0.074	328.229**	12	0.842	0.859	0.819	0.020	–0.004	73.210^{ns}
Bropap_21091	17	0.833	0.922	0.895	0.098	0.038	155.884^{ns}	19	0.692	0.891	0.863	0.226	0.111	215.801*	14	0.789	0.913	0.880	0.139	0.052	89.625^{ns}
Bropap_22348	4	0.125	0.487	0.428	0.747	0.304	28.744***	4	0.038	0.360	0.321	0.895	0.320	52.015***	2	0.211	0.273	0.231	0.234	0.088	0.825^{ns}
Bropap_23758	5	0.667	0.652	0.571	–0.024	–0.027	18.217^{ns}	9	0.654	0.732	0.693	0.109	0.050	36.555^{ns}	8	0.421	0.458	0.434	0.083	0.056	37.515^{ns}
Bropap_23810	7	0.625	0.647	0.601	0.035	0.011	32.872 *	13	0.731	0.798	0.763	0.086	0.023	140.218***	10	0.579	0.829	0.784	0.308	0.149	52.140^{ns}
Bropap_25444	14	0.542	0.691	0.658	0.219	0.126	124.820*	15	0.423	0.900	0.873	0.535	0.260	176.389***	12	0.579	0.879	0.842	0.348	0.167	100.278**
Bropap_26773	9	0.708	0.843	0.805	0.163	0.069	49.938^{ns}	19	0.846	0.934	0.911	0.096	0.040	180.859^{ns}	19	0.737	0.935	0.904	0.216	0.099	179.181^{ns}
Bropap_26985	5	0.667	0.715	0.651	0.068	0.016	19.153*	6	0.269	0.609	0.539	0.563	0.255	43.514***	4	0.263	0.587	0.496	0.559	0.242	27.144***
Bropap_30248	14	0.458	0.881	0.852	0.485	0.237	165.102***	16	0.308	0.902	0.876	0.663	0.319	281.041***	15	0.526	0.927	0.895	0.439	0.206	219.606***

Note: A = number of alleles; F_{IS} = coefficient of inbreeding; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; PIC = polymorphism information content; r = null allele frequency.

[a]Voucher and locality information are provided in Appendix 2.

[b]Deviations from HWE using χ^2 tests: *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$; ns = not significant.

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TABLE 3. Transferability of the 20 microsatellite markers developed in *Broussonetia papyrifera* across three Moraceae and one Rosaceae species.

Locus	*B. papyrifera*	*Morus* sp.[a]	*Ficus elastica*[a]	*Ficus carica*[a]	*Prunus dulcis*[a]
Bropap01931	+	—	—	+	*
Bropap02075	+	+	*	+	*
Bropap02214	+	—	*	+	+
Bropap02359	+	—	*	*	*
Bropap02560	+	—	*	+	*
Bropap02801	+	—	—	—	—
Bropap03147	+	—	*	—	*
Bropap07697	+	—	—	—	—
Bropap15123	+	—	*	+	—
Bropap16591	+	—	*	*	+
Bropap20558	+	—	—	*	—
Bropap20939	+	+	*	*	+
Bropap21091	+	—	—	*	—
Bropap22348	+	—	—	—	—
Bropap23758	+	—	—	—	—
Bropap23810	+	—	+	+	+
Bropap25444	+	—	—	—	—
Bropap26773	+	—	—	—	—
Bropap26985	+	—	—	—	—
Bropap30248	+	—	—	—	—

Note: + = successful amplification; — = failed amplification; * = amplification in fewer than four of five samples.

[a]Transferability was tested on five independent specimens from Santiago, Chile.

0.935 with averages of 0.495 and 0.722, respectively. PIC values ranged from 0.169 to 0.911 with averages of 0.678 and F_{IS} values ranged from −0.024 to 0.895 with an average of 0.329. Null allele frequency values ranged from −0.037 to 0.396 (Table 2). No significant deviation of HWE in terms of heterozygosity deficiency was detected for four markers (Bropap02214, Bropap02359, Bropap23758, Bropap26773) in the three populations (Table 2). Across the three populations (in Guangdong and Yunnan in southern China and Taiwan), 384 alleles were scored. Samples were from nonadjunct individuals because *B. papyrifera* is a widely distributed and common species in East Asia.

The reported genetic diversity for *B. papyrifera* represents only part of the diversity found in Asia. The transferability of these markers was tested in three additional Moraceae and one Rosaceae species (Table 3, Appendix 2). Some of the developed markers exhibited limited interspecific transferability. Six markers showed transferability to *Ficus carica* L. Eight markers exhibited no transferability to any of the tested species (Table 3).

CONCLUSIONS

We identified and characterized 20 highly polymorphic and informative microsatellite markers for *B. papyrifera*, presenting an average of 19.2 alleles per marker. Some of the markers show limited transferability to other Moraceae and one Rosaceae species. The described genetic diversity represents a subset of the genetic diversity in the native range. These microsatellite markers may be able to serve as useful tools to analyze genetic diversity, population genetic structure, and gene flow of *B. papyrifera* in its native range, to trace its worldwide dispersal history, and to help in germplasm conservation.

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Appendix 1. Locality and voucher information for the 10 samples of *Broussonetia papyrifera* used for microsatellite development.

Species	Location	Range	Voucher no.[a]	Collector[b]	Latitude[c]	Longitude[c]
B. papyrifera	Fatu Hiva, Marquesas Island, French Polynesia	Introduced	BQUCH0045	AS	−10.512452	−138.683993
B. papyrifera	Waimea, Big Island, Hawaii, USA	Introduced	BQUCH0064	AS	20.028473	−155.660508
B. papyrifera	Kanokupolu, Tongatapu, Tonga	Introduced	BQUCH0100	AS	−21.075553	−175.334735
B. papyrifera	Votua, Viti Levu, Fiji	Introduced	BQUCH0115	AS	−18.208704	177.709575
B. papyrifera	Santiago, Chile	Introduced	BQUCH0134	AS	−33.469052	−170.519744
B. papyrifera	Taichung, Taiwan	Native	BQUCH0138	KFC	24.077374	120.664456
B. papyrifera	Wulai District, Taiwan	Native	BQUCH0140	KFC	24.853237	121.548910
B. papyrifera	Kyoto, Honshu, Japan	Introduced	BQUCH0141	PM	35.025817	135.781519
B. papyrifera	Kyoto, Botanical Garden, Honshu, Japan	Introduced	BQUCH0144	PM	35.050188	135.763272
B. papyrifera	Roiho, Easter Island, Chile	Introduced	BQUCH0152	AS	−27.113048	−109.404041

[a] Specimens are deposited at the University of Chile, Santiago, Chile; specimen BQUCH0152 is also deposited (voucher SGO162505) at the herbarium of the National Museum of Natural History, Santiago, Chile.

[b] AS = Andrea Seelenfreund; KFC = Kuo-Fang Chung; PM = Peter Matthews.

[c] Latitude and longitude are provided in decimal degrees.

Appendix 2. Locality and voucher information for samples used in this study.

Species	Location[a]	Voucher no.[b]	*N*	Collectors[c]	Latitude[d]	Longitude[d]
Broussonetia papyrifera (L.) L'Hér. ex Vent.	Taiwan (T1–T24)	HAST Chung 7-1	24	KFC, HLL, KYH	24.8506600	121.5698400
B. papyrifera	Guangdong, China (G1–G26)	HAST Chung 1742	26	KFC	24.4823900	113.7839700
B. papyrifera	Yunnan, China (Y1–Y20)	HAST Chung 016	20	KFC, CSA	22.6588300	99.603500
Ficus carica L.	Santiago, Chile	BQUCHZ0010	5	DS, BPA	−33.4229130	−70.6529900
F. elastica Roxb. ex Hornem.	Santiago, Chile	BQUCHZ0009	5	DS, BPA	−33.4225410	−70.6539970
Morus L. sp.	Santiago, Chile	BQUCHZ0011	5	DS	−33.4132500	−70.5568700
Prunus dulcis (Mill.) D. A. Webb	Santiago, Chile	BQUCHZ0012	5	DS	−33.4132700	−70.5571000

Note: *N* = number of individuals sampled.

[a] Number of accessions from Taiwan, Guandong, and Yunnan are indicated in parentheses.

[b] Voucher specimens identified as HAST are deposited at the Herbarium at the National Taiwan University, Taipei, Taiwan; voucher specimens identified as BQUCHZ are deposited at the University of Chile, Santiago, Chile.

[c] KFC = Kuo-Fang Chung; HLL = Hsiao-Lei Liu; KYH = Kuen-Yi Ho; CSA = Chi-Shan Chang; AS = Andrea Seelenfreund; PM = Peter Matthews; DS = Daniela Seelenfreund; BPA = Barbara Peña-Ahumada.

[d] Latitude and longitude are provided in decimal degrees. Coordinates for *B. papyrifera* samples are representative of the approximate range of the sampled populations.

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