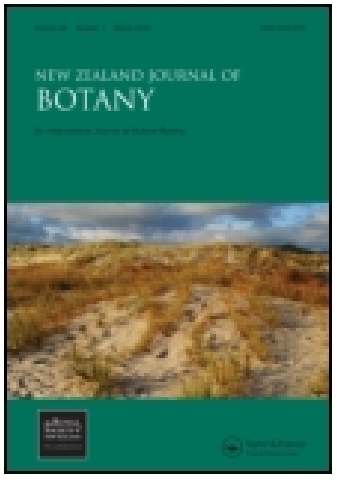

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Ancient and modern introduction of Broussonetia papyrifera ([L.] Vent.; Moraceae) into the Pacific: genetic, geographical and historical evidence

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RESEARCH ARTICLE

Ancient and modern introduction of *Broussonetia papyrifera* ([L.] Vent.; Moraceae) into the Pacific: genetic, geographical and historical evidence

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Broussonetia papyrifera (L.) Vent. (Moraceae), or paper mulberry, is a species of cultural importance in South East Asia, East Asia and the Pacific. Originally from mainland South East Asia or East Asia, this plant was introduced into the Pacific range by prehistoric Austronesian voyagers. We used non-coding internal transcribed spacer (ITS) sequences of nuclear ribosomal DNA and inter-simple sequence repeat (ISSR) on 79 samples of *B. papyrifera* from different islands of Remote Oceania, and South East Asia and East Asia. Our results show an absence of genetic diversity in the introduced range of Remote Oceania, with the sole exception of Hawaii. By contrast, Asian samples show genetic diversity. The data obtained suggest a prehistoric human-mediated introduction of this species from East Asia to Remote Oceania and a second, possibly historic, human-mediated introduction to Hawaii.

Keywords: Asia; human-mediated dispersal; ISSR; ITS; paper mulberry; Polynesia

Introduction

Paper mulberry, *Broussonetia papyrifera* (L.) Vent., belongs to the Moraceae family, a diverse and widespread taxonomic group that includes a suite of useful plants that are important food and fibre sources (Whistler 2009). Using nuclear and chloroplast DNA markers, the family has been partitioned into several clades and tribes (Clement & Weiblen 2009). The clade that gave rise to the *Broussonetia* genus likely originated in the upper Eocene. Fossil fruits have been recorded from the upper Eocene in southern England and Germany (Zerega et al. 2005). The species *B. papyrifera* (L.) Vent. is of great cultural importance in Asia because its branches supply the bark that is used as a source of raw material for the manufacture of high-quality paper, from which its common name 'paper mulberry' is derived.

Taxonomically, the nearest wild relatives of *B. papyrifera* are *B. kazinoki* and *B. kaempferi*. Matthews (1996) argues that the most common recent ancestor of this species was probably located in South East Asia, where all three species are known to occur. Therefore, the nominal native range of *B.*

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AS and DS developed the project and did field collections. JG performed the experiments. JG, AR, XM and SL analysed the data. AS, DS and XM wrote the article. All authors read, edited and approved the final manuscript.
Supplementary file 1: Table S1. Optimisation of PCR reagents for ISSR using a Taguchi orthogonal design; **Supplementary file 2:** Figure S1. Genetic diversity of *Broussonetia papyrifera* samples from Asia and Island Oceania using UPGMA analysis.

papyrifera appears to be mainland South East and East Asia, as far east as Taiwan. It is a common and widespread tree in its native range and grows at moderate elevations in secondary forests, in subtropical to temperate climates. It is naturalised in parts of India, Ghana, Southern Europe and the USA (Matthews 1996; Morgan & Overholt 2013). In many places, it is considered a pioneer plant, requiring a moist forest environment on flat or sloping land (Saito et al. 2009). It grows spontaneously in degraded forests and in some places, such as Laos, it is often used as a plantation tree in forests and fields (Aubertin 2004).

Paper mulberry reached Polynesia through human-mediated introduction from Asia sometime between 3500 and 1000 yr BP (Kirch 1997). It has been argued that its introduction into Remote Oceania, and cultivation as far east as Rapa Nui (Easter Island), was intentional due to its cultural importance, because its bark is the main fibre used to make textiles in this region (barkcloth) (Matthews 1996; Seelenfreund et al. 2010). Paper mulberry is thought to have been dispersed through its native habitat by birds. However, in its introduced range in the Pacific, and even between islands within Near and Remote Oceania, its dispersal should be considered essentially human-dependent, just like other plants used and introduced by Polynesian people (Lebot 2002; Whistler 2009), given that seeds of Moraceae, including *B. papyrifera*, although large, have short survivorship (Berg 2001). As suggested previously (Seelenfreund et al. 2010, 2011), these attributes allow *B. papyrifera* to be considered as an excellent candidate for studying human mobility patterns across the broad geographic area of the Pacific, with a specific emphasis on Remote Oceania.

Molecular markers are useful tools to provide information about genetic diversity. To date, only one molecular analysis of *B. papyrifera* populations in Oceania has been performed using internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA region (rDNA ITS) (Seelenfreund et al. 2011). In that study, our results showed limited genetic variation within the small area of Taiwan and no genetic variability within the broad geographic region of island Oceania. Because rDNA ITS represents a single region in the nuclear genome (Nieto Feliner & Rosselló 2007), with moderate diversity at the intra-species level (Weising et al. 2005), we might expect to detect more genetic diversity by using a multilocus marker that encompasses several genomic regions. For this reason, we analysed a larger group of samples using the ITS region and complemented this study using multilocus inter-simple sequence repeat (ISSR) markers. The ISSR technique requires PCR amplification of genomic DNA using a single primer that targets a known simple sequence repeat (SSR) or microsatellite motif (Liu & Wendel 2001), thus generating a pattern of multiple bands representing regions between SSR loci. ISSR markers avoid the need to develop species-specific primers and can be applied to a species whose genome has not been sequenced, providing a useful, affordable and fast analytical tool. This method has been used to determine genetic diversity (Taheri et al. 2012; Ribeiro et al. 2013; Giustina et al. 2014), detect gene-pool origin (Galván et al. 2003), the genetic structure of crops (Carrasco et al. 2009) and for the construction of genetic maps (Feng et al. 2013). The genetic diversity of paper mulberry has also been assessed using ISSR within Taiwan (Ho & Chang 2006) and recently in southern China (Liao et al. 2014). In this study, we assess the genetic diversity of paper mulberry introduced into the Pacific using the information provided by the ITS-1 region and ISSR markers, in order to test the hypothesis that *B. papyrifera* in the Pacific is a homogenous population that represents a single human-mediated introduction.

Materials and methods

Sampling and study area

Remote Oceania

Fresh young leaves were collected in the field between 2008 and 2012, from Western Polynesia (Fiji, Samoa and Tonga) and Eastern Polynesia (Marquesas Islands, Hawaii and Rapa Nui). Leaf samples collected in 2008 in Fiji, Samoa, Tonga, Marquesas and Rapa Nui were preserved at −20 °C in the laboratory (Seelenfreund et al. 2010). Leaf samples collected in 2012 in Hawaii were dried

immediately with silica gel and stored at room temperature.

Asia

Leaf samples dried in silica gel from Taiwan were kindly collected and provided by Dr Kuo-Fang Chung (School of Forestry and Resource Conservation, National Taiwan University, Taipei, Taiwan). Samples from Vietnam and Japan and a sample of *B. kazinoki* were sent by Dr Peter Matthews (National Museum of Ethnology, Osaka, Japan) and Mr Hiroyuki Nikuto (Kyoto Botanic Gardens, Japan).

The origin and number of samples are shown in Fig. 1 and Table 1, respectively. The collection consisted of 68 samples from Remote Oceania and 11 from Asia. One additional sample of *B. kazinoki* was used as an outgroup. Sampling in Oceania was not uniform, because the number and density of the plants on each island or island group are highly variable, as described in Seelenfreund et al. (2010). To obtain samples from Asia, we depended on donations from colleagues working in the areas concerned and therefore the number of samples received was limited.

DNA extractions and PCR amplification

Genomic DNA was extracted following a CTAB extraction protocol described by Lodhi et al. (1994) and modified as described in Moncada et al. (2013). Extracted DNA was stored in double-distilled sterile water at −20 °C until analysis. High-quality DNA was obtained mainly from silica-gel-dried samples stored at room temperature. Samples from Oceania comprised 80 accessions, of which only 68 provided high-quality DNA (a single band of high molecular DNA with an absorbance ratio $A_{260/280}$ between 1.8 and 2.0). Asian specimens yielding high-quality DNA were restricted to three samples from Taiwan, five from Vietnam and three from Japan. In total, high-quality DNA was obtained from 79 samples of *B. papyrifera* and one sample of *B. kazinoki* as outgroup. These were used for sequencing the ITS-1 region and ISSR analysis.

ITS-1 amplification and data analysis

The ITS-1 region was amplified using ITS-A (5′-GGAAGGAGAAGTCGTAACAAGG-3′) and ITS–C (5′–GCAATTCACACCAAGTATCGC-3′)

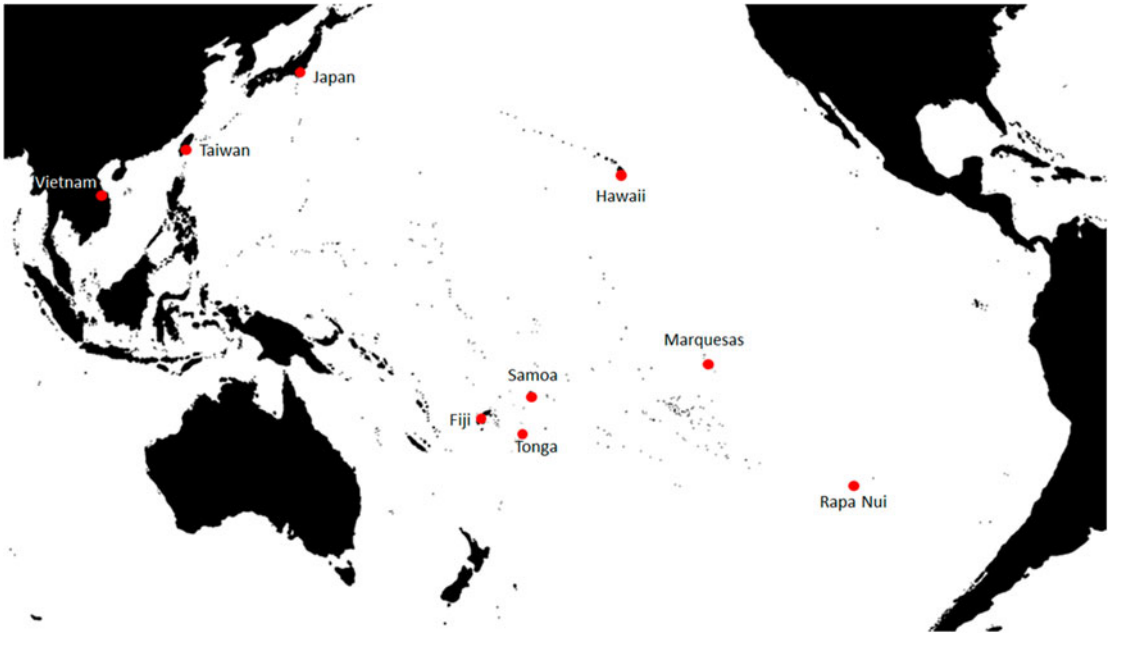

Figure 1 Map of the Pacific showing *B. papyrifera* sampling locations.

Table 1 Sampling locations, sample accession numbers and geographical coordinates.

Location			UTM coordinates				
Country	Archipelago/ Island/Region	Island/Locality	Sample No.	Zone	East	North/ South	Hemisphere
Chile (RN)	Rapa Nui	Roiho	BQUCH0002	12 J	658282	6999883	S
	Rapa Nui	Roiho	BQUCH0003	12 J	658282	6999883	S
	Rapa Nui	Roiho	BQUCH0005	12 J	658088	6999883	S
	Rapa Nui	Maunga Toatoa	BQUCH0014	12 J	668684	6997597	S
	Rapa Nui	Rano Raraku	BQUCH0023	12 J	670319	6998489	S
	Rapa Nui	Rano Kao	BQUCH0027	12 J	654421	6991509	S
	Rapa Nui	Rano Kao	BQUCH0028	12 J	654449	6991595	S
	Rapa Nui	Rano Kao	BQUCH0030	12 J	654588	6991542	S
	Rapa Nui	Rano Kao	BQUCH0031	12 J	654620	6990505	S
	Rapa Nui	Ara Tataki Rereo	BQUCH0036	12 J	656512	6998933	S
	Rapa Nui	Hanga Oteo	BQUCH0037	12 J	662483	7005684	S
	Rapa Nui	Hanga Oteo	BQUCH0038	12 J	662483	7005685	S
	Rapa Nui	PuToki Toki	BQUCH0135	12 J	667502	7002011	S
	Rapa Nui	Te Karava/Poike	BQUCH0147	12 J	671059	6999051	S
	Rapa Nui	Roiho	BQUCH0149	12 J	658282	6999883	S
	Rapa Nui	Ana Te Pahu	BQUCH0152	12 J	658088	7001652	S
	Rapa Nui	Ana Te Pahu	BQUCH0153	12 J	658089	7001652	S
	Rapa Nui	Oroi	BQUCH0155	12 J	668684	6997597	S
	Rapa Nui	Oroi	BQUCH0156	12 J	668684	6997597	S
	Rapa Nui	Oroi	BQUCH0157	12 J	668684	6997597	S
	Rapa Nui	Oroi	BQUCH0158	12 J	668684	6997597	S
	Rapa Nui	PuToki Toki	BQUCH0208	12 J	667517	7002017	S
	Rapa Nui	Ahu Kihi Kihi Rau Mea	BQUCH0211	12 J	656512	6998933	S
	Rapa Nui	MaungaToaToa	BQUCH0212	12 J	668684	6997597	S
	Rapa Nui	MaungaToaToa	BQUCH0213	12 J	668684	6997597	S
	Rapa Nui	Te Karava/Poike	BQUCH0215	12 J	671059	6999051	S
	Rapa Nui	Vai Tara Kai Ua	BQUCH0218	12 J	663657	7005502	S
	Rapa Nui	National Park Service Nursery	BQUCH0220	12 J	654649	6994386	S
	Rapa Nui	Rano Kao	BQUCH0221	12 J	654908	6992526	S
	Rapa Nui	Rano Kao	BQUCH0224	12 J	654910	6992532	S
	Rapa Nui	Hanga Oteo	BQUCH0225	12 J	661086	7006106	S
	Rapa Nui	Hanga Oteo	BQUCH0226	12 J	662498	7005729	S
Samoa (SA)	Savaii	Safu'a	BQUCH0075	2 L	369464	8483379	S
	Savaii	Faga	BQUCH0077	2 L	371327	8490104	S
	Savaii	Palauli	BQUCH0086	2 L	357751	8479413	S
	Upolu	Maangiangi	BQUCH0093	2 L	415731	8450887	S
	Upolu	Apia	BQUCH0094	3 L	420121	8468643	S
Fiji (FJ)	Viti Levu	Votua	BQUCH0117	60 K	575029	7986675	S
French	Marquesas	Tahuata/Hapatoni	BQUCH0051	7 L	705731	8897319	S
Polynesia (MA)	Marquesas	Nukuhiva/Hatiheu	BQUCH0233	7 L	600999	9024025	S
	Marquesas	Fatuhiva/Omoa	BQUCH0234	7 L	753479	8836997	S
Tonga (TG)	Tongatapu	Fatai	BQUCH0095	1 K	678952	7661967	S
	Tongatapu	Fatai	BQUCH0096	1 K	678952	7661967	S
	Tongatapu	Teekiu	BQUCH0097	1 K	674537	7662944	S

Table 1 (*Continued*)

Location			UTM coordinates				
Country	Archipelago/ Island/Region	Island/Locality	Sample No.	Zone	East	North/ South	Hemisphere
	Tongatapu	Teekiu	BQUCH0098	1 K	674537	7662944	S
	Tongatapu	Foui	BQUCH0099	1 K	672658	7664354	S
	Tongatapu	Kanokupolu	BQUCH0100	1 K	673088	7668514	S
	Tongatapu	Kanokupolu	BQUCH0101	1 K	673088	7668514	S
	Tongatapu	Liahona	BQUCH0103	1 K	679102	7659056	S
	Tongatapu	Niutoua	BQUCH0105	1 K	703140	7660724	S
	Tongatapu	Navutoka	BQUCH0107	1 K	698178	7663183	S
	Tongatapu	Malapo	BQUCH0109	1 K	691752	7654064	S
	Tongatapu	Pelehake	BQUCH0110	1 K	694042	7647088	S
	Tongatapu	Pelehake	BQUCH0111	1 K	694043	7647089	S
	Tongatapu	Fatumu	BQUCH0113	1 K	695999	7653459	S
USA (HW)	Hawaii	Big Island/Waimea	BQUCH0059	5 Q	222236	2217080	N
	Hawaii	Big Island/Waimea	BQUCH0066	5 Q	222236	2217080	N
	Hawaii	Big Island/Waimea	BQUCH0177	5 Q	222236	2217080	N
	Hawaii	Big Island/Waimea	BQUCH0181	5 Q	222236	2217080	N
	Hawaii	Big Island/Kona	BQUCH0184	5 Q	94368	2157724	N
	Hawaii	Big Island/Kona	BQUCH0187	5 Q	194368	2157724	N
	Hawaii	Big Island/Kona	BQUCH0189	5 Q	194368	2157724	N
	Hawaii	Oahu/Waimea	BQUCH0190	4 Q	597940	2392647	N
	Hawaii	Big Island/Kona	BQUCH0191	5 Q	194368	2157724	N
	Hawaii	Big Island/Kona	BQUCH0192	5 Q	194368	2157724	N
	Hawaii	Big Island	BQUCH0193	5 Q	194368	2157724	N
	Hawaii	Oahu	BQUCH0194	4 Q	581493	2374443	N
	Hawaii	Oahu	BQUCH0195	4 Q	581493	2374443	N
Taiwan (TW)	Taiwan	Taichung	BQUCH0138	51 R	262551	2664757	N
	Taiwan	Wulai District	BQUCH0139	51 R	355499	2749171	N
	Taiwan	Wulai District	BQUCH0140	51 R	353385	2749479	N
Vietnam (VT)	North Vietnam	Sapa Town	BQUCH0201	48 Q	380542	2470619	N
	North Vietnam	Hanoi	BQUCH0202	48 Q	580968	2325060	N
	North Vietnam	Ba Vi Mountain	BQUCH0203	48 Q	537948	2329519	N
	North Vietnam	Ba Vi Mountain	BQUCH0204	48 Q	537948	2329519	N
	North Vietnam	Ba Vi Mountain	BQUCH0205	48 Q	537948	2329519	N
Japan (JA)	Honshu	Kyoto	BQUCH0141	53 S	571342	3876355	N
	Honshu	Kyoto	BQUCH0142	53 S	572337	3877424	N
	Honshu	Kyoto	BQUCH0143	53 S	572337	3877424	N
Japan[a]	Honshu	Kyoto	BQUCHZ0002	53 S	569618	3878760	N

Samples in Fig. 2 and Fig. S1 are identified by their country code and sample number (e.g. sample BQUCH0002 is indicated as RN002).

[a]BQUCHZ0002 corresponds to a *B. kazinoki* sample used as outgroup.

Zone refers to the longitudinal band in the UTM (Universal Transversal Mercator) coordinates system.

primers as described by Blattner (1999). Amplification was as described in Seelenfreund et al. (2011). All polymerase chain reactions (PCR) were set up in a UV-treated PCR cabinet.

Sequences were aligned using the MUSCLE algorithm and CLC Sequence Viewer 7.5 software (Robert 2004). A dendrogram was constructed using neighbour-joining (NJ) (Saitou & Nei 1987) and unweighted pair-group method with arithmetic mean (UPGMA) clustering (Sneath & Sokal 1973), and evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004). Bootstrapping analysis (9999 resampling) was conducted with the MEGA 6 program (Tamura et al. 2013).

ISSR amplification and data analysis

The ISSR analysis was based on a set of 13 ISSR primers reported by Ho & Chang (2006). An optimisation strategy for the ISSR–PCR protocol was performed using the Taguchi method (Taguchi 1986; Cobb & Clarkson 1994). The range for each parameter was obtained using the Minitab (version 15) statistical software (Minitab, State College, PA, USA), with an orthogonal arrangement of five PCR reagents and four concentrations. This approach reduced 1024 combinations to only 16 amplification reactions (Table S1). Reagent concentrations defined by this procedure were 1.5 units of GoTaq DNA polymerase (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM of each primer and 40 ng of template DNA in a final reaction volume of 25 µL (reaction 9 in Table S1).

PCR amplifications were performed as reported by Ho & Chang (2006), with an initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 50 s at the optimal annealing temperature for each primer and an extension of 2 min at 72 °C, with a final extension at 72 °C for 10 min. Annealing temperatures for each primer are indicated in Table 2. All PCR were also set up in a UV-treated PCR cabinet.

Amplified samples were separated by electrophoresis at constant 75 V in 0.5× TBE buffer for 2 h 30 min on 2.5% agarose gels (lane width = 5 mm, gel length = 10 cm). A 1 kb plus DNA ladder (Thermo Scientific GeneRuler 1kb Plus DNA ladder # SM1331) was included at each side of every gel. After electrophoresis, gels were stained in 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide for 10 min. In order to remove excess staining, gels were subjected to a wash cycle in 1 mM $MgSO_4$ for 10 min followed by electrophoresis at 75 V for 15 min in 0.5× TBE buffer. Bands were photographed under a UV transilluminator and images were analysed using

Table 2 Primer names, annealing temperatures, sequences, number of bands and genetic diversity for nine primers.

Primer[a]	Annealing T (°C)	5′→3′ Sequence	Number of assigned bands	Number of polymorphic bands	Percentage of polymorphic bands	Genetic diversity (Nei)
UBC823	56	TCTCTCTCTCTCTCTCC	11	6	54.6	0.1447
UBC825	53	ACACACACACACACACT	15	10	66.8	0.0625
UBC827	53	ACACACACACACACACG	17	15	88.2	0.1184
UBC855	50	ACACACACACACACACYT	8	2	25.0	0.0413
UBC856	50	ACACACACACACACACYA	10	3	30.0	0.0194
UBC866	53	CTCCTCCTCCTCCTCCTC	14	6	42.9	0.0379
UBC888	56	BDBCACACACACACACA	18	8	44.4	0.0616
UBC889	56	DBDACACACACACACAC	19	12	63.2	0.0673
UBC891	58	HVHTGTGTGTGTGTGTG	24	19	79.2	0.0985
Mean (Total)			15.11 (136)	9.00 (81)	59.6	0.0761

A, adenine; T, thymine; G, guanine; C, cytosine; Y = C or T; D = A, G or T; B = C, G or T; H = A, C or T; V = A, C or G.
[a]Forward and reverse correspond to exactly the same sequence.

Corel PaintShop Pro X6 Ultimate software, version 16.1.0.48.

Band images were scored by visual inspection by two observers and weak or smeared bands were excluded. Equally sized fragments present in all samples were designated as common or monomorphic bands, whereas those bands present in only a few samples were designated as polymorphic bands. Each distinct and reproducible band was considered as a locus. For each sample, bands were designated as present (1) or absent (0) and entered into a binary matrix.

Data were analysed using the program POPGENE version 1.32, determining the number of polymorphic bands, the percentage of polymorphic bands and Nei's genetic diversity (Yeh et al. 1999). Relationships among populations were determined by NJ (Saitou & Nei 1987) and UPGMA clustering (Sneath & Sokal 1973) using a distance matrix based on the Sørensen–Dice index (Dice 1945). Cluster analyses were performed from a similarity matrix using SplitsTree4, version 4.13.1 (Huson & Bryant 2006) and dendrograms were built on the basis of 10,000 permutations.

Analysis of molecular variance (AMOVA) was performed using GenAlEx 6.501 (Peakall & Smouse 2012). This analysis was performed using a distance matrix constructed on presence/absence data and based on the Sørensen–Dice index with 1000 permutations (Excoffier et al. 1992). The representation of genetic distance of native and introduced populations of *B. papyrifera* was obtained using a principal coordinate analysis (PCoA) implemented in GenAlEx 6.501 (Peakall & Smouse 2012).

Results

Analysis of the ITS-1 region

Seventy-seven of 79 samples were used for the alignments of the ITS-1 sequence, including the ITS sequence previously deposited in GenBank under accession number HM623778.1 (Seelenfreund et al. 2011). Two samples (BQUCH0028, BQUCH0234) were eliminated from the ITS analysis because of an illegible sequence.

The multiple alignment of a 260 bp sequence corresponding to the 3′-end of the 18S gene and the ITS-1 region of the 77 samples of paper mulberry showed two variable positions defining three genotypes. Genotypes 1 and 3 are defined by a G/T transversion found at position '203', as described in our former study (Seelenfreund et al. 2011). Genotypes displaying the G variant in this position are found in Asian samples from Japan, Taiwan and Vietnam and also three samples from Hawaii (BQUCH187, BQUCH189 and BQUCH191), as shown in Table 3. Genotype 3, presenting the T variant, is found in 60 samples from Polynesia, which include 7 samples from Hawaii of a total of 13 from this island group. Genotype 2 corresponds to a C/T transition at position 99. This genotype (not described in Seelenfreund et al. 2011) is found in two samples from Vietnam (BQUCH202 and BQUCH205) and three samples from Hawaii (BQUCH177, BQUCH192 and BQUCH194).

Genetic analysis of ISSR markers

From a set of 13 ISSR primers reported by Ho & Chang (2006), nine giving reproducible banding profiles were chosen (Table 2). The reproducibility of banding profiles was verified by amplifying genomic DNA from two biological replicates for

Table 3 Genotypes of *B. papyrifera* found by ITS–1 analysis.

Genotype	Origin	Number of samples	Position in ITS-1 region	
			99	203
1	Taiwan, Japan, Vietnam	9	C	G
	Hawaii	3	C	G
2	Hawaii	2	T	G
	Vietnam	3	T	G
3	Rapa Nui, Samoa, Fiji, Marquesas, Tonga, Hawaii	60	C	T

Relative polymorphic positions in the ITS-1 region are indicated and referred to the GenBank sequence HM623778.1.

each of the nine primers. In all cases, the same amplification pattern was obtained for the same individuals. In addition, two technical replicates for each experiment, such as PCRs (repeated on different days), also showed identical genomic profiles for the same individual (data not shown).

The analysis of 79 samples yielded a total of 136 registered bands for the nine ISSR primers. Of these, 81 were polymorphic and represent 59.6% of the total number of assigned bands (Table 2). Furthermore, the average Nei genetic diversity (Nei 1973) for all loci was of 0.0761, with primer UBC823 generating the highest value (0.1447).

Using the NJ clustering method, a dendrogram was constructed which split the samples in two main clusters with high bootstrap values and five smaller clusters with variable bootstrap values. One main cluster included three groups: Group I comprises most Polynesian samples (bootstrap value 100), including nine samples from Hawaii that cluster with all samples from Rapa Nui, Samoa, Fiji, Marquesas and Tonga, as shown in Fig. 2. Samples from Japan (Group II) and Taiwan (Group III) are clustered next to one another and separated from Group I by a bootstrap value of 32.8. The split between Group I and Groups II and III is consistent with the suggested dispersal history of this species in the Pacific. The other main cluster comprises all samples from Vietnam (Group IV), and Group V includes additional samples from Hawaii (subgroup 2), which separate from each other with a bootstrap value of 44.1.

Individuals from Hawaii were split into two different clusters, and were the only group of samples from one geographic locality to present a dual origin. Most of the Polynesian plants have a closer genetic association with Japan and Taiwan than with mainland Asia (represented here by Vietnam). However, five of the Hawaiian samples (BQUCH177, BQUCH189, BQUCH191, BQUCH 192 and BQUCH194) have a closer genetic relationship with Vietnam (Fig. 2). These correspond to the same samples that grouped with Asian specimens in the ITS-1 analysis. The only exception is sample BQUCH187, which groups with the Asian samples in the ITS analysis, although it groups with Polynesian samples using ISSR.

The UPGMA distance tree showed a similar structure for the ITS-1 sequence analysis (Fig. S1), compared with NJ analysis. ITS-1 results present three genotypes in two clusters, which group most Oceania samples in one branch and a second branch that groups all Asian samples and six Hawaiian samples. ISSR results show two main clusters, one including most Polynesian samples (Group I), and the Asian samples from Japan (Group II), Taiwan (Group III) and Vietnam (Group IV) on separate branches, wherein the Japan and Taiwan clusters are connected. Group I is defined again by the samples from the islands of Tonga, Samoa, Marquesas, Rapa Nui, Fiji and part of the Hawaiian samples. The other cluster is composed of the same five samples from Hawaii that were described in the NJ analysis (Group V).

Analysis of molecular variance

For the AMOVA, a distance matrix was generated for the samples from the Polynesian islands or island groups corresponding to Rapa Nui, Marquesas, Hawaii, Samoa and Tonga. The results of this analysis showed greater genetic diversity within populations (71%), compared with the genetic variation observed among populations (29%). Both results are statistically significant ($P = 0.006$), as shown in Table 4. Although most of the genetic variability occurs within populations, there is sufficient interpopulation variability to detect at least two distinct populations (corresponding to Groups I and V in Fig. 2). When the Hawaiian samples were excluded from the AMOVA of the Polynesian plants, all remaining individuals presented similar genetic diversity between islands ($P = 0.901$) and can be considered a single genetic population (Table 5). Because the significance level is greater than 0.05 we can infer that all samples from the Polynesian islands (except for Hawaii, which was excluded from this analysis) can be considered a single genetic group.

Principal coordinate analysis

PCoA was performed for the 79 *B. papyrifera* samples from Remote Oceania and Asia. PCoA

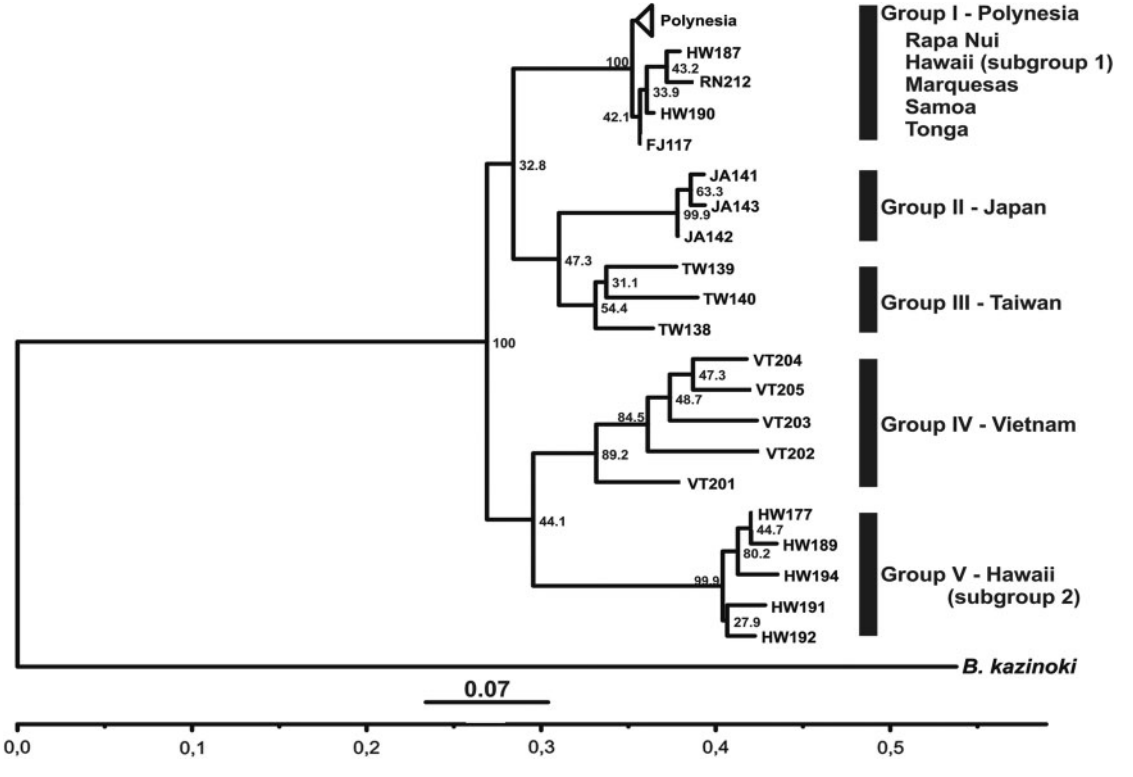

Figure 2 Dendrogram representing the genetic diversity of ISSR analyses among *B. papyrifera* samples from Asia and Island Oceania using neighbour-joining analysis. Numbers on branches correspond to the bootstrap values after performing 10,000 permutations. Outgroup: *B. kazinoki* from Japan.

Table 4 Intra- and interpopulation AMOVA of ISSR data applied to genetic distances obtained from five populations of *Broussonetia papyrifera* in Polynesia (Tonga, Samoa, Marquesas, Hawaii and Rapa Nui).

Source of variation	df	SS	MS	FST	%	*P*
Between populations	4	0.368	0.092	6.399	29.2	0.006
Within populations	62	0.894	0.014		70.8	
Total	66	1.262			100.0	

%, percentage of variance partition; df, degrees of freedom; FST, F-statistic; MS, mean square; *P*, significance (over 1000 permutations); SS, sum of squares.

clustered individuals into four groups (see Fig. 3). All samples from Polynesia (Group 1), with the exception of a subset of five Hawaiian specimens, are closely grouped and separate from all Asian samples. Samples from Taiwan and Japan cluster in Group 2; specimens from mainland Asia (Vietnam) form Group 3. Group 4 is defined by the subset of the five Hawaiian samples not included in Group 1. Therefore, the samples from Hawaii separate into two clearly distinguishable groups: one subgroup of individuals which are indistinguishable from all other samples from Polynesia (Group 1), and a second group that is more closely related to the Asian plants (Group 4). These five samples correspond to the same specimens that cluster separately in both the NJ and UPGMA analyses. The first two coordinates explain 60% of the total observed genetic variation (45.5% accounted for by the coordinate 1 and 14.5% accounted for by coordinate 2) (Fig. 3). The third coordinate accounts for 12% of the variation, and the cumulative percentage of variation for the first three coordinates is 72%.

Discussion

Broussonetia papyrifera is a plant of great cultural importance in Oceania and is believed to have been introduced into this area by Austronesian language-speaking people as part of their transported cultural landscape (Kirch 1982; Matthews 1996). Populations of *B. papyrifera* in the Pacific islands are derived from cultivated individuals via vegetative

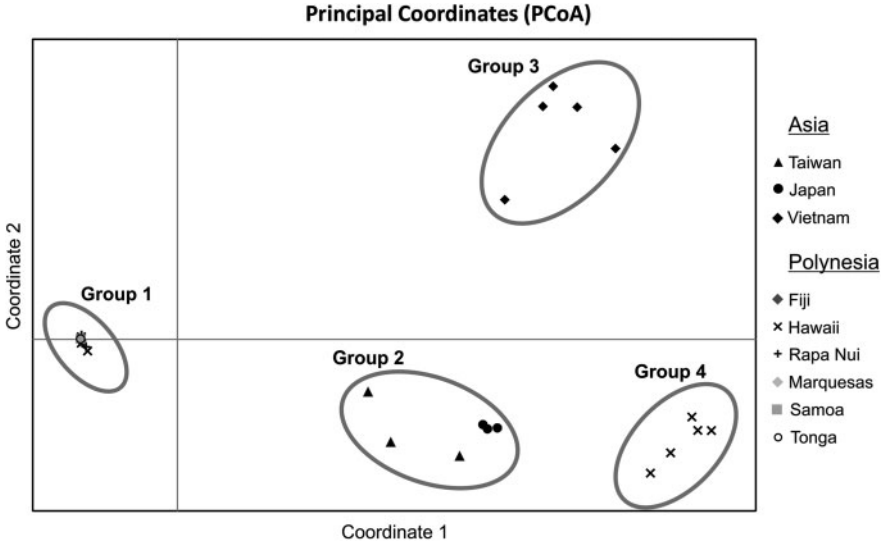

Figure 3 Principal coordinates analysis of ISSR data applied to 79 individual genotypes of *B. papyrifera* from Asia and the Pacific.

Table 5 Intra- and interpopulation AMOVA of ISSR data excluding Hawaii and applied to genetic distances obtained from four populations of *B. papyrifera* in Polynesia (Tonga, Samoa, Marquesas, and Rapa Nui).

Source of variation	df	SS	MS	FST	%	*P*
Between populations	3	0.0010	0.0003	0.2878	1.6	0.901
Within populations	50	0.0602	0.0012		98.4	
Total	53	0.0612			100.0	

%, percentage of variance partition; df, degrees of freedom; FST, F-statistic; MS, mean square; *P*, significance (over 1000 permutations); SS, sum of squares.

reproduction and have been dependent on human dispersal and cultivation (Matthews 1996; Seelenfreund et al. 2010). To date, there are very few studies that compare the genetic diversity of *B. papyrifera* in its native habitat (South East to East Asia) and in introduced regions, where lower genetic diversity is expected. However, lower genetic diversity need not limit establishment of an introduced population, as release from competitors or natural enemies or a broad environmental tolerance may contribute to success in the introduced range (Sakai et al. 2001). In the case of paper mulberry, the presence of extant populations over the vast Pacific range attests to its ability to survive since its prehistoric introduction, both with and without protection and propagation as a cultigen.

Our results show that within Remote Oceania, paper mulberry samples from all islands were genetically homogeneous with the sole exception of some Hawaiian samples, suggesting that the dispersal history of this plant in the Pacific is more complex than we expected. We discuss our results in relation to the genetic diversity of *B. papyrifera* found in Asia, within Oceania and finally within Hawaii.

The genetic diversity of the nuclear ribosomal sequence (ITS-1) and ISSR markers from *B. papyrifera* samples was analysed. Analysis of the ITS-1 sequence confirms the results of Seelenfreund et al. (2011), indicating that samples from Taiwan consistently present the 'G' polymorphism at position '203'. The additional samples from other Asian locations tested in this work also share the same 'G' variant, suggesting that this genetic variant may correspond to an ancestral genotype and seems to be widely distributed in the native region. In addition, a new polymorphism is detected in some of the Vietnamese samples. In combination with the genotype information presented in Seelenfreund et al. (2011), we have detected three genotypes within the Asian range. In comparison, in a much larger number of samples, the Polynesian specimens exhibit the described 'T' variant, with the exception of six of the Hawaiian samples that present an Asian 'G' genotypic variant. Of these, three also present the second polymorphism detected in some of the Vietnamese samples (Genotype 2), strongly suggesting a different history of some of the Hawaiian samples compared with the main group of Polynesian plants. The genetic diversity revealed by analysis of the ITS-1 sequence probably does not represent all the genetic diversity within the Asian region, whereas the genetic diversity found in Polynesia most likely represents the diversity presently found in the plant patches on the different islands of Remote Oceania for this DNA sequence.

In this work, the analysis of ITS-1 sequences allowed the detection of a limited genetic diversity, finding two genotypes from Asian samples and one genotype from Oceanian samples, whereas the analysis of ISSR loci, which detects polymorphisms of multiple genomic regions, showed five different groups, thus constituting a more informative marker system for this species.

Using ISSR, a higher genetic diversity was found within our Asian samples (the nominal native range), where both natural and human-mediated dispersal of the plant is known to occur. In China, a significant correlation between genetic variation and geographical distribution has been described (Zhiyuan et al. 2009; Liao et al. 2014). Paper mulberry in Taiwan also shows considerable genetic

variation according to geographical distribution, and natural populations cluster into two major geographic groups (Ho & Chang 2006). Our previous study of rDNA also revealed genetic diversity within Taiwan (Seelenfreund et al. 2011) and, likewise, in this study Asian samples do present genetic diversity. Interestingly, in Vietnam, Taiwan and Japan both male and female plants are observed (Matthews 1996), so wild populations can spread by both seeds and suckers, while cultivated forms are generally spread by cuttings. The high genetic diversity within this region presumably reflects the multiple modes of reproduction and dispersal.

The low genetic diversity of paper mulberry observed in island Oceania is not unexpected. Introduced populations of a species typically represent only part of the genetic diversity in the natural range. This can be explained by many factors including genetic and demographic bottlenecks and adaptation to novel environments (Bossdorf et al. 2005; Le Roux et al. 2013). As Matthews (1996, 2007) suggested, probably few cultivars were introduced into Oceania from Asia in the form of seeds or vegetative cuttings (clones). In addition, in the Pacific paper mulberry is propagated asexually by cuttings or root shoots, as are other Pacific cultivars. Paper mulberry in Oceania is grown exclusively for making barkcloth, so its branches are cut and harvested before flowering, preventing sexual reproduction even if both female and male plants are present. This agricultural practice may have helped to reduce genetic diversity in locations where breeding was a possibility. The analyses of NJ and UPGMA of ISSR data display high bootstrap values (100) separating Groups I, II and III from Groups IV and V. However, bootstrap values are low within each node, suggesting that this might be a random clustering. This is the case for Groups I, II and III and also for Groups IV and V. Nonetheless in the case of Groups I, II and III, archaeological and linguistic evidence suggest a close relationship between Taiwan and Remote Oceania (Green 1979; Ko et al. 2014; Lipson et al. 2014) that is coherent with the clustering shown in the dendrogram (Fig. 2). It is difficult to define the relationship between Taiwan and Japan, because the origin of this species in Japan is still under discussion. Natural populations of paper mulberry in Japan are not known (Matthews 1996). In the case of Groups IV and V, it needs to be stressed that Group IV corresponds to plants from the South East Asian mainland (Vietnam); there is no archaeological or linguistic evidence to link this area with Hawaii or Remote Oceania. The possible explanations for the origin of Group V (subgroup 2), from Hawaii, are discussed below.

PCoA of ISSR data also suggests that there is a close relationship between the main group of Remote Oceania samples and those from the islands of Taiwan and Japan (our East Asian set). This is consistent with the current evidence that points to Taiwan as the homeland of Austronesian-speaking colonisers of the Pacific.

In Polynesia, gene flow among populations is limited by the extreme geographic distances between islands and island groups. The AMOVA of the Polynesian samples determines that the major source of genetic diversity comes from within the populations. The level of significance below 0.05 indicates that the genetic diversity of *B. papyrifera* is similar between locations, again with the exception of Hawaii, and confirms that this island group has genetically distinct populations ($P < 0.05$). In all these analyses, a subgroup of samples from Hawaii consistently forms a separate cluster that is genetically closer to the Asian samples than to the Pacific samples. In our previous study, we did not include specimens from Hawaii, which explains the homogeneity initially found in Remote Oceania. Within Remote Oceania, Hawaii is the only island group to have greater genetic diversity of paper mulberry, and genetic affiliation with both Polynesia and Asia, suggesting that there were at least two different introduction events. This is not consistent with the hypothesis of a single introduction of paper mulberry into the Pacific.

Consequently, we propose that these results reflect an independent, second introduction of *B. papyrifera* to Hawaii. The present analysis does not allow us to ascribe a specific origin or timescale for the proposed second introduction. A number of possibilities arise in order to explain the occurrence of this population. One is that paper mulberry was carried by contracted workers from China who

started arriving at the end of the eighteenth century and during the late nineteenth century. Another option, which does not exclude the former possibility, is that it was introduced from Japan by labourers brought to the sugarcane plantations in the late nineteenth century (1876 to 1899) (Northrup 1995). We speculate that some of the workers or their families brought a few plants, seeds or cuttings of paper mulberry, for medicinal purposes or as a source of fibre. A further explanation might be the introduction of paper mulberry from the American mainland, where the plant has become invasive (Morgan & Overholt 2013). However, we have no evidence to prove this. According to the NJ analysis, the Hawaiian samples that do not present the general Pacific haplotype form a distinct group that is closer to the Vietnamese samples than to those from Taiwan, Japan and all other Pacific samples. However, the UPGMA analysis shows that these Hawaiian samples cluster independently from samples from Vietnam, Taiwan, Japan and from the Pacific. Finally, the PCoA also separates these five Hawaiian samples into a distinct group.

Our data provide new insights into the dispersal of paper mulberry in the Pacific and serves to enhance our understanding of the prehistoric Austronesian expansion and historic introductions. Further resolution will require more comprehensive sampling within Asia and East Asia, as well as more variable genetic markers to determine more precise origins and dispersal routes for Pacific paper mulberry.

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Supplementary data

Supplementary file 1: Table S1. Optimisation of PCR reagents for ISSR using a Taguchi orthogonal design.

Supplementary file 2: Figure S1. Genetic diversity of *Broussonetia papyrifera* samples from Asia and Island Oceania using UPGMA analysis. **A,** Dendrogram representing the genetic diversity of the ITS-1 region. Numbers on branches correspond to the bootstrap values after performing 10,000 permutations. **B,** Dendrogram representing the genetic diversity of ISSR analysis. Numbers on branches correspond to the bootstrap values after performing 10,000 permutations. Outgroup: *B. kazinoki* from Japan.

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