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Article *in* Annals of Botany · June 2017

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Phylogeography of herbarium specimens of asexually propagated paper mulberry [*Broussonetia papyrifera* (L.) L'Hér. ex Vent. (Moraceae)] reveals genetic diversity across the Pacific

Claudia Payacan[1], Ximena Moncada[2], Gloria Rojas[3], Andrew Clarke[4], Kuo-Fang Chung[5], Robin Allaby[4], Daniela Seelenfreund[1] and Andrea Seelenfreund[6,*]

[1]Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile, [2]Centro de Estudios Avanzados en Zonas Áridas (CEAZA), La Serena, Chile, [3]Museo Nacional de Historia Natural, Santiago, Chile, [4]School of Life Sciences, University of Warwick, Coventry, UK, [5]Biodiversity Research Center, Academia Sinica, Taipei, Taiwan and [6]Escuela de Antropología, Universidad Academia de Humanismo Cristiano, Santiago, Chile

**For correspondence. E-mail aseelenfreund@gmail.com*

Received: 23 January 2017 Returned for revision: 16 February 2017 Editorial decision: 11 April 2017 Accepted: 28 May 2017

• **Background and Aims** Paper mulberry or *Broussonetia papyrifera* (L.) L'Hér. ex Vent. (Moraceae) is a dioecious species native to continental South-east Asia and East Asia, including Taiwan, that was introduced to the Pacific by pre-historic voyagers and transported intentionally and propagated asexually across the full range of Austronesian expansion from Taiwan to East Polynesia. The aim of this study was to gain insight into the dispersal of paper mulberry into Oceania through the genetic analysis of herbaria samples which represent a more complete coverage of the historical geographical range of the species in the Pacific before later introductions and local extinctions occurred.
• **Methods** DNA from 47 herbarium specimens of *B. papyrifera* collected from 1882 to 2006 from different islands of the Pacific was obtained under ancient DNA protocols. Genetic characterization was based on the ribosomal internal transcribed spacer ITS-1 sequence, a sex marker, the chloroplast *ndhF–rpl32* intergenic spacer and a set of ten microsatellites developed for *B. papyrifera*.
• **Key Results** Microsatellites allowed detection of 15 genotypes in Near and Remote Oceanian samples, in spite of the vegetative propagation of *B. papyrifera* in the Pacific. These genotypes are structured in two groups separating West and East Polynesia, and place Pitcairn in a pivotal position. We also detected the presence of male plants that carry the Polynesian chloroplast DNA (cpDNA) haplotype, in contrast to findings in contemporary *B. papyrifera* populations where only female plants bear the Polynesian cpDNA haplotype.
• **Conclusions** For the first time, genetic diversity was detected among paper mulberry accessions from Remote Oceania. A clear separation between West and East Polynesia was found that may be indicative of pulses during its dispersal history. The pattern linking the genotypes within Remote Oceania reflects the importance of central Polynesia as a dispersal hub, in agreement with archaeological evidence.

Key words: *Broussonetia papyrifera*, paper mulberry, Moraceae, Pacific, Remote Oceania, herbaria, vegetative propagation, genetic diversity, sex marker, ITS-1, chloroplast DNA, microsatellite markers.

INTRODUCTION

Museum collections, whether plant or animal, are an important source of information, as they often include extinct specimens, accessions that have been locally lost or samples collected in remote locations. In recent years, in the wake of technical innovation, a number of studies using DNA from museum collections have been published. Such studies allow the opening of windows to the past to reveal new and hidden histories (Wandeler *et al*., 2007). In particular, herbarium collections are extremely useful as they are true 'dry gardens' where worldwide plant diversity is retained, including endemic and undescribed species (Särkinen *et al*., 2012). They constitute remarkable sources of information about plants and the world they inhabited in the past, and provide the comparative material essential for taxonomic studies, population ecology, conservation biology and molecular evolution (Hartnup *et al*., 2011) of rare, extirpated or extinct species that can no longer be found in nature (Weising *et al*., 2005; Funk, 2007). There are an estimated 3400 active herbaria in the world which are true 'treasure chests', holding around 361 million specimens (Särkinen *et al*., 2012) that document the Earth's vegetation up to 400 years ago. Although much younger than archaeological samples, some herbaria pre-date the industrial revolution, large-scale modern breeding efforts or plant dispersals and introductions by colonial economies in the recent past. They thus provide meaningful information on the status *quo ante* and emerge as a rich source of information on past economies, ecology and migration (Schlumbaum *et al*., 2008). Herbarium specimens permit precise chronological control, as the date of sample collection is normally recorded, allowing comparative studies of genetic diversity between past and present

populations to determine possible continuities and pathways of process (Wandeler *et al*., 2007).

The genetic material from herbarium specimens can be used to determine the relationships between plant species, to perform species identification and to clarify taxonomic discrepancies and inconsistencies (Weising *et al*., 2005). In the case of extinct species, herbarium specimens or archaeological samples are the only source for performing genetic studies. It is also possible to estimate the magnitude of human influences on population size at different times and gene flow between populations, and also to detect species re-introductions (Wandeler *et al*., 2007). A number of these studies have focused on taxonomy and evolution of extinct or endangered plants (Korpelainen and Pietiläinen, 2008; Silva *et al*., 2017), or human-mediated plant translocations (Ames and Spooner, 2008; Malenica *et al*., 2011).

Broussonetia papyrifera *and Austronesian migrations*

Prehistoric Austronesian-speaking peoples migrated out of Asia into the vast Pacific expanse starting at about 6000 years BP. In their colonizing canoes, they carried their culturally and economically important plants and animals and introduced these species to the islands on which they settled, forming so-called 'transported landscapes' in these new and often remote localities (Kirch, 2000). Plant exploitation in Oceania relies particularly on arboriculture and vegeculture (Allaby, 2007). The main crops (taro, yams, bananas, breadfruit, sugar cane and kava), from Vanuatu to Hawaii, separated by more than 6000 km, all have the common characteristic, that they are exclusively vegetatively propagated. This feature prevents their natural distribution between islands and island groups in the Pacific unless aided/transported by humans; therefore, the introduction of such plants to islands is indicative of human agency. The study of these plants is important because it allows us to pose questions on their geographical origin and domestication process, which enabled varietal diversification (Lebot, 2002).

Multidisciplinary evidence for the histories of domestic cultivars are proxies of human processes such as their introduction, adoption and dispersal into areas beyond the natural range by people in the distant or recent past (Bird *et al*., 2004; Neumann and Hildebrand, 2009). A number of domesticated or managed plant resources were introduced over time from different source regions (Whistler, 2009; Bellwood *et al*., 2011; Storey *et al*., 2013). The study of animal and plant species transported on the colonizing canoes has been dubbed the 'commensal approach' and is based on the use of these species as a proxy for reconstructing past human migration histories (Matisoo-Smith, 2015). Each of these species was totally dependent upon humans for dispersal across major water gaps, and recent studies have shown that each of these species has a different history (Lebot, 2002; Matisoo-Smith and Robins, 2004; Storey *et al*., 2013). Studies on *Artocarpus* sp. (Zerega *et al*., 2004) and banana (Kennedy, 2008; Donohue and Denham, 2009) suggest interaction between oceanic populations and New Guinea, supporting the hypothesis that Central Polynesia was settled by humans via Melanesia. Zerega *et al.* (2004) also conclude the existence of long-distance migration from eastern Melanesia into Micronesia. Lebot (2002), employing isozyme analysis, suggests that the Pacific plantain and banana cultivars found as far distant as Hawaii originated in Papua New Guinea or Western Melanesia as a result of hybridization between members of the *Musa acuminata*/*banksii* complex and *M. balbisiana* (Lebot, 2002; Kennedy, 2008). De Langhe and collaborators (2009) posed that the first hybridizations of edible diploid *M. acuminata* (type AA) bananas with *M. balbisiana* may have occurred with the arrival in eastern Indonesia and Melanesia of Austronesian-speaking people coming from Taiwan. However, some authors have proposed models that do not invoke such a large-scale mass migration (Donohue and Denham, 2009; Perrier *et al*., 2011). Studies performed on taro (*Colocasia esculenta*) by isoenzymes indicate low genetic diversity in Oceania (Lebot *et al*., 2004); using amplified fragment length polymorphism (AFLP) analysis, the authors were able to distinguish between the populations from South-east Asia and the Pacific (Lebot *et al*., 2004; Matthews and Nguyen, 2014). Another of the Polynesian plants studied with genetic markers is kava (*Piper methysticum*). This plant is dioecious and cross-pollinated; however, it flowers rarely and is incapable of reproducing sexually. Because of its low genetic diversity, Lebot *et al.* (1999) concluded that the kava plant was probably domesticated only about 3000 years ago. The Polynesian-introduced *Cordyline fruticosa* (ti) was studied by Hinkle (2007) as a proxy for reconstructing human colonization patterns in Oceania. Because of its material, nutritional, medicinal and religious importance, green-leaved *C. fruticosa* was transferred by Polynesian settlers to virtually every habitable Pacific island before European contact. AFLP analyses on experimental greenhouse crosses showed that the Eastern Polynesian form was sterile and lacked genetic diversity, suggesting to the author (Hinkle, 2007) that the sterile forms were developed in Western Polynesia and transported to Eastern Polynesia.

Paper mulberry [*Broussonetia papyrifera* (L.) L'Hér. ex Vent., Moraceae] is a dioecious species native to continental South-east Asia and East Asia including Taiwan, that was introduced to the Pacific between 3500 and 1000 BP by Austronesian-speaking migrants (Kirch, 2000). It was transported across the full range of Austronesian expansion from Taiwan to Eastern Polynesia (Chang *et al*., 2015). In the Pacific, this species was dispersed intentionally and widely distributed throughout the islands as far as Easter Island, for the use of its inner bark for the manufacture of bark cloth textiles (Matthews, 1996; Seelenfreund *et al*., 2010). Linguistic evidence strongly suggests an ancient introduction of paper mulberry (Matthews, 1996; Whistler, 2009). Paper mulberry is one of the many economic crops in the Pacific reproduced by asexual propagation, and therefore its dispersal over the vast range of the Pacific was human mediated. Its propagation and importance across Remote Oceanic islands were well documented by the early explorers and missionaries, who also described plantations and the methods used for making bark cloth (Seelenfreund *et al*., 2010; Seelenfreund *et al*., 2016).

Today paper mulberry in the Pacific is an important crop plant in Tonga, Wallis, Fiji and to some extent in Samoa. It has seen a recent revival on islands such as Hawaii (Tanahy, 1998), Easter Island (Seelenfreund, 2013) and the Marquesas (Ivory, 1999). However, on other islands such as the Cook Islands and New Zealand, this plant has disappeared locally (Seelenfreund *et al*., 2010). On some islands, plants have been introduced

recently or re-introduced from other locations, which makes the interpretation of genetic data difficult [for example on Raiatea (Society Islands), Solomon Islands, New Zealand, New Caledonia and the Philippines]. One possibility to overcome these problems is to study and analyse herbarium specimens of old *B. papyrifera* accessions. Many of these samples were collected prior to modern re-introductions of paper mulberry. Specimens from the Pacific were collected as early as the first European expeditions into the region, about 250 years ago, allowing, therefore, an independent analysis from the recent history of modern re-introductions. Additionally, herbaria allow access to material from islands too remote to obtain fresh leaf samples (Barker, 2002; Seelenfreund *et al.*, 2010).

Many herbaria in the world house specimens of *B. papyrifera* collected in the Pacific. Among these are the Allan Herbarium (CHR, New Zealand), the New York Botanical Garden (NY, USA), B. P. Bishop Museum, Herbarium Pacificum (BISH, USA), Muséum National d'Histoire Naturelle, Herbarium (P, France), Royal Botanic Gardens Herbarium, Kew (K, UK), Auckland War Memorial Museum Herbarium (AK, New Zealand), Museo Nacional de Historia Natural Herbarium (SGO, Chile), British Museum of Natural History Herbarium (UK, England) and the Smithsonian Institution, United States National Herbarium (US, USA). In the two latter institutions we find the oldest paper mulberry herbarium specimens on record from the Pacific, collected in 1769 by J. Banks and D. Solander during Captain Cook's first voyage.

We have previously described the use of molecular markers to analyse contemporary specimens of *B. papyrifera* (Seelenfreund *et al.*, 2010, 2011; Chang *et al.*, 2015; González-Lorca *et al.*, 2015; Peñailillo *et al.*, 2016), and also 19 herbarium specimens (Chang *et al.*, 2015) in order to address the question of its dispersal in the Pacific range. In this latter work, Chang *et al.* (2015) have been able to demonstrate that the most common variant of paper mulberry found in the Pacific, and the one most likely to have been introduced by the early colonists, has a clear Taiwanese origin. Analysis of the ribosomal internal transcribed spacer ITS-1 region revealed a polymorphism specific to paper mulberry introduced into Remote Oceania (Seelenfreund *et al.*, 2011). Also, Peñailillo *et al.* (2016) have shown that contemporary paper mulberry plants in Remote Oceania are exclusively female, indicating human-mediated dispersal. The sole exception is found in Hawaii, where both sexes are present in contemporary plants. The male plants were most probably introduced in historic times to Hawaii, as suggested by González-Lorca *et al.* (2015). In addition, these authors also described a lack of genetic diversity of Pacific paper mulberry using inter-simple sequence repeat (ISSR) markers (González-Lorca *et al.*, 2015). Therefore, analysis of contemporary paper mulberry with ribosomal, sex and ISSR markers showed homogeneous Oceanian patterns, revealing no significant genetic diversity to shed light on specific dispersal patterns of this plant in the vast Pacific region.

The aims of this study were to (1) characterize herbaria samples that represent a wide coverage of the historical geographical range of the species in the Pacific, including islands where paper mulberry plants are no longer present or where modern introductions may obscure ancient dispersal patterns; (2) assess genetic diversity within Remote Oceania based on nuclear and plastid molecular markers used in the former studies and include a set of microsatellite markers; and (3) propose plausible scenarios of the human-mediated dispersal and distribution history of paper mulberry in Remote Oceania.

MATERIALS AND METHODS

Herbarium samples

Forty-seven herbarium specimens of *B. papyrifera* collected between 1882 and 2006 from different islands of the Pacific were provided by three different Museums: the Bishop Museum (BISH, Honolulu, Hawaii, USA), Auckland War Memorial Museum Herbarium (AK, Auckland, New Zealand) and the National Museum of Natural History (SGO, Santiago, Chile). Whenever possible, samples chosen were collected prior to the mid 20th century, to minimize the impact of increased connectivity between islands that spurred modern re-introductions and translocations, The majority of these specimens (32 samples, 68 %) were collected prior to 1941, i.e. >70 years ago, and only 12 specimens date to between 1953 and 1995. However, most of these come from locations that remained isolated until the late 1990s such as Île de Horn (Wallis and Futuna) and some of the Marquesas islands. Three samples were recently collected (2003–2006) and are known to have been taken from recently introduced plants. Sample codes, collectors, year of collection and geographic origin are summarized in Table 1. All necessary permissions for sampling of specimens were obtained from the respective curators, Barbara Kennedy (BISH), Ewen Cameron (AK) and Gloria Rojas (SGO).

Sampling protocol

Since herbarium specimens are fragile, unique and irreplaceable, a sampling protocol was designed. Each specimen was photographed before handling and after sampling and labelled for future use and museum records. Triplicate samples, smaller than 1 cm^2 were obtained, taking care not to alter the aesthetics of the mounting. Samples or areas with mould were not used. Each sample was weighed and then stored in a sterile 2 mL plastic tube for later use. Herbarium samples were manipulated with tweezers and latex gloves that were changed between each sample. Tweezers were cleaned prior to use and between samples with 70 % ethanol.

Precautions for work with DNA from herbarium samples

All extractions and PCRs were conducted in an exclusive physically isolated space which had never been used for isolation of contemporary plant DNA and separated from where contemporary samples were analysed. All reagents and work material, such as micropipettes, tips, gloves, etc. were used exclusively for working with herbarium DNA. During lab work, disposable overalls, hairnets, face masks, disposable shoe covers and double latex gloves were worn. A unidirectional workflow was established for this lab, with no movement of materials or workers back into this laboratory. All extraction

TABLE 1. *Sample codes of herbarium samples, field collection number, geographic origin, collectors and year of collection*

No.	Herbarium code	Collection number	Provenance		Collector	Year of collection
			Geographic origin	Locality		
1	SGO005091	6605	Santiago	Quinta Normal	F. Phillipi	1882
2	SGO141121	0633	China	–	Luo Lin Bo	1995
3	SGO058300	–	Easter Island	Easter Island	F. Fuentes	1911
4	SGO058271	–	Easter Island	Easter Island	F. Fuentes	1911
5	SGO129525	667	Easter Island	Easter Island	F. Sudzuki	1971
6	BISH161284	1009	Easter Island	Easter Island	J. P.Chapin	1935
7	BISH161285	670	Easter Island	Easter Island	C. J. F. Skottsberg	1917
8	BISH36684	19	Wallis and Futuna	Île de Horn/Alofi	M. Mackee	1968
9	BISH161275	10114	Niue	Niue	Not indicated	1940
10	BISH161276	10114	Niue	Niue	Not indicated	1940
11	BISH161287	965	Pitcairn	Pitcairn	J. P. Chapin	1934
12	BISH161288	15032	Pitcairn	Pitcairn	H. St. John	1934
13	BISH664608	81	Pitcairn	Pitcairn	W. H. Lintott	1957
14	BISH161280	899	South Cook Islands	Rarotonga	G. P. Wilder	1929
15	BISH161286	524	Society Island	Moorea	G. P. Wilder	1926
16	BISH418270	20	Wallis and Futuna	Lalosea, Asoa	P. Kirch	1974
17	BISH161278	15179	Tonga	Tongatapu	T. G. Yuncker	1953
18	BISH161279	15471	Tonga	Eua	T. G. Yuncker	1953
19	BISH750662	1071	Samoa	–	D. W. Garber	1925
20	BISH161272	1071	Samoa	–	D. W. Garber	1925
21	BISH161273	1071	Samoa	–	D. W. Garber	1925
22	BISH161277	9204	Samoa	Tau	T. G. Yuncker	1939
23	BISH161289	11847	Austral Islands	Tubuai	Anderson and F. R. Fosberg	1934
24	BISH161290	24	Austral Islands	Rapa	A. M. Stokes	1921
25	BISH161291	2	Austral Islands	Rimatara	A. M. Stokes	1921
26	BISH161292	136	Austral Islands	Rurutu	J. F. G. Stokes	1921
27	BISH161293	412	Austral Islands	Rapa	J. F. G. Stokes	1921
28	BISH161294	412	Austral Islands	Rapa	J. F. G. Stokes	1921
29	BISH161296	216	Austral Islands	Rapa	J. F. G. Stokes	1921
30	BISH161297	140	Austral Islands	Rapa	A. M. Stokes	1921
31	BISH161300	129	Austral Islands	Rapa	A. M. Stokes	1921
32	BISH161301	129	Austral Islands	Rapa	A. M. Stokes	1921
33	BISH751633	140	Austral Islands	Rapa	A. M. and J. F. G. Stokes	1921
34	BISH751635	216	Austral Islands	Rapa	J. F. G. Stokes	1921
35	BISH751636	140	Austral Islands	Rapa	A. M. and J. F. G. Stokes	1921
36	BISH404138	11396A	Austral Islands	Rapa	Anderson and F. R. Fosberg	1934
37	BISH493902	6305	Austral Islands	Rapa	J. Florence	1984
38	BISH161281	664	Marquesas Islands	Nuku Hiva	F. B. H. Brown and E. D. W. Brown	1921
39	BISH161283	387	Marquesas Islands	Hivaoa	F. B. H. Brown and E. D. W. Brown	1921
40	BISH588624	6198	Marquesas Islands	Fatu Hiva	D. H. Lorence	1988
41	BISH609116	2715	Marquesas Islands	Fatu Hiva	B. G. Decker	1974
42	BISH709092	389	Marquesas Islands	Ua Huka	J. Y. Meyer	2003
43	AK214298	7418	Solomon Islands	–	R. O. Gardener	1993
44	AK116673	219	New Guinea	–	R. N. H. Bulmer	1964
45	AK76866	NA	Niue	Niue	S. P. Smith	1901
46	AK295889	NA	New Zealand	North Island	D. S. McKenzie	2006
47	AK296981	6642	New Zealand	Auckland	P. J. de Lange	2006

procedures and PCRs were set up with dedicated micropipettes with filtered tips, and performed in a UV-treated PCR cabinet, which was cleaned with a 1 % Extran solution after work.

DNA extraction and amplification

For reproducibility, herbarium DNA extractions were performed in duplicate in two different laboratories. One replicate was processed in the Ancient DNA Laboratory at the University of Warwick (UK) using the DNeasy® Plant Mini Kit (Qiagen). In brief, samples were homogenized with liquid nitrogen and the extraction buffer containing 2 % cetyltrimethylammonium bromide (CTAB) and 1 % polyvinylpyrrolidone (PVP) was added. The solution was incubated for 2 d at 37 °C to lyse tissues and then extracted with 1 vol. of chloroform and isoamyl alcohol (24:1). The supernatant was mixed with the AP3/E buffer and transferred to the columns provided in the kit to continue the protocol according to the manufacturer's instructions. The second sample set was analysed at a separate laboratory at the Faculty of Chemical and Pharmaceutical Sciences, University of Chile where no DNA extractions, PCRs or any molecular biology work with contemporary DNA are performed. The second replicates were extracted following the manual CTAB extraction protocol described by Lodhi *et al.* (1994) and modified as described in

Moncada *et al.* (2013). RNase was not used, assuming degradation of RNA. In both extraction protocols, negative extraction controls (no sample) were included and one sample was extracted in duplicate (biological replicate) as an internal control.

The integrity of DNA was visualized on 0·8 % agarose gels. DNA concentrations were measured using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) according to the manufacturer's instructions. The quality of obtained DNA was evaluated by the absorbance ratio 260 nm/280 nm using a NanoDrop ND-2000 spectrophotometer.

PCR amplification of the ITS region. The 300 bp ITS-1 region from all herbarium samples was amplified with primers ITS-A and ITS-C (Blattner, 1999). Seven herbarium specimens were amplified with the ITS region primers ITS-5B (5′-TCG CGA GAA GTC CAC TGA A-3′) and ITS-4 (5′-GCT TAA ACT CAG CGG GTA GC-3′) designed specifically for paper mulberry by one of the authors (K.F.C.). In both cases, PCR mixtures consisted of 2 µL of genomic DNA, 2·5 mM $MgCl_2$, 0·625 mM of each dNTP, 0·25 µM of each primer, 1 mg mL^{-1} bovine serum albumin (BSA) and 0·2 U mL^{-1} of GoTaq® Flexi DNA Polymerase (Promega, Madison, WI, USA) in a final volume of 20 µL. For difficult templates, GoTaq® G2 Hot Start DNA Polymerase (Promega) at the same concentration was used. The amplification program for both primer pairs for the ITS and ITS-1 region consisted of an initial denaturation step at 94 °C for 5 min, followed by 40 cycles with a denaturation step at 94 °C for 1 min, an annealing stage at 60 °C for 1 min, an extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplicons were separated by electrophoresis on 1·5 % agarose gels, dyed with GelRed™ Nucleic Acid Gel Stain (Biotium, Inc.) and visualized under UV light. All PCRs included a negative PCR control without DNA template.

Sex marker region amplification. All herbarium samples were amplified with a paper mulberry-specific sex marker initially developed by Wang *et al.* (2012) and enhanced as a duplex PCR assay in our laboratory (Peñailillo *et al.*, 2016). Briefly, the PCR mixture consisted of 3 µL of genomic DNA, 2·5 mM $MgCl_2$, 0·625 mM of each dNTP, 0·5 µM MMFw forward primer, 0·25 µM MMRL reverse (large) primer, 0·25 µM MMRS18 reverse (short) primer, 1 mg mL^{-1} BSA and 0·125 U mL^{-1} GoTaq® Flexi DNA Polymerase in a final volume of 20 µL. Difficult templates were amplified using GoTaq® G2 Hot Start DNA Polymerase (Promega) at the same concentration. The amplification program consisted of an initial denaturation step at 94 °C for 5 min, followed by 40 cycles with a denaturation step at 94 °C for 1 min, an annealing step at 55 °C for 1 min, an extension at 72 °C for 1 min and a final extension at 72 °C for 7 min. Amplicons were analysed by electrophoresis on 1·5 % agarose gels, as described above. All PCRs included a negative PCR control without DNA template. As described in Peñailillo *et al.* (2016), gels were analysed by visual inspection. Female samples displayed a single 420 bp band, while male samples exhibited two bands at 273 and 420 bp on 1·5 % agarose gel.

PCR amplification using internal primers of the ndhF–rpl32 *chloroplast region.* The herbarium samples were amplified with primers ndhF (5′-GAA AGG TAT KAT CCA YGM ATA TT-3′) and ndhF-rpl32-5R (5′-ATA TCA GTT GAC CCA TTT TAA CC-3′), generating fragments appropriate for degraded DNA of approx. 300 bp as described in Chang *et al.* (2015). The PCR mixtures consisted of 2 µL of genomic DNA, 3 mM $MgCl_2$, 0·2 mM of each dNTP, 0·1 µM of each primer, 1 mg mL^{-1} BSA and 0·2 U μL^{-1} GoTaq® G2 Flexi DNA Polymerase in a final volume of 25 µL. The amplification program consisted of an initial denaturation at 80 °C for 5 min followed by 30 cycles with a denaturation step at 95 °C for 1 min, primer annealing at 50 °C for 1 min, followed by a ramp of 0·3 °C s^{-1} to 65 °C, primer extension at 65 °C for 4 min and a final extension of 5 min at 65 °C. Amplicons were analysed by electrophoresis on 1·5 % agarose gels, as described above. All PCRs included a negative PCR control without DNA template.

PCR amplification using microsatellite markers. All herbarium samples were amplified using four microsatellite markers Bro 07, Bro 08, Bro 13 and Bro 15 developed by one of us (K.F.C.) and six microsatellite markers Bropap 02214, Bropap 02801, mBropap 20558, Bropap 25444, Bropap 26985 and Bropap 30248, selected from an enriched library constructed by Ecogenics GmbH (Zurich, Switzerland) (J. Peñailillo *et al.*, Chile, unpubl. res.). The fluorescent labelling method used for later detection by capillary electrophoresis was as described by Schuelke (2000).

The PCR mixtures consisted of 2 µL of genomic DNA, 2·5 mM $MgCl_2$, 0·2 mM of each dNTP, 0·125 µM of forward primer with the attached M13 tail, 0·5 µM of fluorophore-labelled universal M13 forward primer, 0·5 µM of reverse primer, 1 mg mL^{-1} BSA and 0·125 U μL^{-1} GoTaq® Flexi DNA Polymerase (Promega) in a final volume of 20 µL. For difficult templates, GoTaq® G2 Hot Start DNA Polymerase was used at the same concentration. The amplification program consisted of two steps: first an initial denaturation at 95 °C for 15 min, followed by 30 cycles with a denaturation step at 95 °C for 30 s, an annealing stage at 55 or 56 °C for 45 s and an extension stage at 72 °C for 45 s. The second step consisted of eight cycles with a denaturation step at 95 °C for 30 s, an annealing stage at 53 °C for 45 s, an extension step at 72 °C for 45 s and a final extension at 72 °C for 30 min. Amplicons were separated by electrophoresis as described. All PCRs included a negative PCR control without DNA template.

Data analysis

ITS-1 sequences. The amplified ITS-1 samples were purified using the DNA Clean and Concentrator Kit™ (Zymo Research, Irvine, CA, USA) and FavorPrep Gel/PCR purification Mini Kit™ (Favorgen, Biotech Corp., Ping-Tung, Taiwan), both according to the manufacturer's instructions, and sequenced at Macrogen Inc. (Seoul, South Korea). Polymorphisms from all sequences were visualized and checked on electropherograms using Bio Edit 7.1.3.0 software (Hall, 1999). ITS-1 sequences were edited manually and aligned using the Clustal W method (Thompson *et al.*, 1994) with the same program. A tree were constructed using the Maximum Likelihood method based on the Tamura–Nei model and a bootstrapping of 10 000 resamplings computed with the MEGA6 program (Tamura *et al.*, 2013).

Chloroplast marker. The amplified samples were sequenced at Macrogen Inc. (Seoul, South Korea). The electropherograms were checked using Bio Edit 7.1.3.0 software (Hall, 1999). DNA sequences suitable for analysis were aligned with the Clustal W algorithm (Thompson *et al.*, 1994) and dendrograms were constructed using the Maximum Likelihood method using MEGA6 as described above (Tamura *et al.*, 2013). Previous sequences of each haplotype described by Chang *et al.* (2015) were included in the analysis for comparative purposes.

SSR markers. The amplified samples were analysed by capillary electrophoresis at the sequencing services from the Pontificia Universidad Católica de Chile (Santiago, Chile) and electropherograms were visualized with Peak Scanner™ v1.0 software (Applied Biosystems). Due to the M13 tail attached to each forward primer, the appropriate numbers of base pairs were subtracted from the experimentally determined amplicon size, to obtain the length of the actual alleles. These were registered in an Excel spreadsheet. For SSR cluster analysis, a minimum spanning tree (MST) was generated using BioNumerics v.7.6 (Applied Maths NV) using the categorical coefficient for the calculation of the similarity matrix. The priority rules 1 and 2 used were maximum number of N-locus variants (N = 1), weight: 10 000 and maximum number of N-locus variants (N = 2), weight: 10, respectively.

RESULTS

Sampling and DNA extraction

Samples were taken from 47 herbarium specimens provided by the three institutions. Sample weights varied between 1.0 mg (BISH161283) to 13·4 mg (SGO058300). DNA was successfully extracted by both methods for 44 of the 47 herbarium specimens. In three cases, only one of the replicates yielded DNA using the DNeasy Plant Mini Kit® (see Table 2). DNA was successfully extracted at least once from all the 47 samples (100 %). When comparing the DNA concentrations obtained by both protocols (absorbance and fluorescence), we observed that in most samples, the manual extraction method yielded more DNA than the DNeasy Plant Mini Kit®.

In Table 2, performance values from both DNA extraction and quantitation methods employed are shown. The normalized yield of each sample is shown. Overall, no correlation between the year of collection of the herbarium specimen and the amount of DNA obtained is observed, therefore sample age does not determine the amount of DNA extracted. DNA extracted from herbarium samples has a low molecular weight (200–500 bp), while contemporary DNA samples present a band of high molecular weight on 0·8 % agarose gels (data not shown).

The calculated 260/280 nm ratio for DNA purity from DNA extracted with the DNeasy Plant Mini Kit® ranged between 1·8 and 2·0 for 56·2 % of the samples, while these values were obtained in 39·1 % of the samples using the Lodhi extraction method. The use of the commercial set-up results in a larger number of samples with acceptable levels of purity.

Analysis of the ITS-1 region

Due to the high levels of DNA degradation, several authors (Pääbo *et al.*, 2004; Gugerli *et al.*, 2005) have suggested that successful amplification of ancient samples requires the use of small regions (<500 bp). In order to verify this, some of the samples were selected to amplify the complete ITS region, of approx. 700 bp for *B. papyrifera*. These were accessions collected in 2006, 2003, 1995, 1957, 1939, 1921 and 1882. As expected, it was not possible to amplify the complete ITS region. However, ITS-1 amplicons of 300 bp were obtained from all herbarium samples tested and readable sequences were obtained from 43 samples (91·5 %). Each of these sequences was compared with the database using NCBI BLAST, confirming that the samples corresponded to *B. papyrifera* in 39 of the 43 analysed sequences, while four samples were identified as other plant species, as shown in Table 3.

Maximum Likelihood analysis showed that 33 paper mulberry (31 herbarium and two contemporary) samples from Remote Oceania derive from a branch that contains all Asian (eight herbarium and two contemporary) samples (Fig. 1). The first branch (bootstrapping 100 %) included eight herbarium samples from China, New Guinea, Solomon Islands, Île de Horn (Alofi), Santiago (Chile), one sample from Niue, both samples collected in New Zealand and two contemporary samples from Taiwan and China. All these present the G variant, which in previous studies was found to be associated with Asian samples (Seelenfreund *et al.*, 2011). The derived branch includes 31 samples from Polynesia (Easter Island, Austral Islands, Marquesas Islands, South Cook Islands, Futuna, Tonga, Samoa, Pitcairn and two samples from Niue) and two contemporary samples from Tonga and Easter Island. All these samples show the T variant, identified previously as the 'Polynesian' genotype.

Sex determination

The 47 herbarium samples studied were amplified with the sex marker described in Peñailillo *et al.* (2016). Molecular amplification using this sex marker was successful at least once for 35 samples (74·5 %), and indicated that 31 samples of the herbaria samples collected in Polynesia were female and four were male. These male specimens corresponded to samples BISH161281 from the Marquesas Islands, BISH161297 from Rapa and AK295889 and AK296981 from New Zealand. Figure 2 shows the sex distribution of the herbarium specimens according to their geographical origin.

Analysis of the ndhF–rpl32 *chloroplast region.*

Amplification of a 300 bp region of the *ndhF–rpl32* chloroplast marker with primer sequences designed for amplifying herbarium collections (Chang *et al.*, 2015) was successful for 33 of the 39 *B. papyrifera* herbarium samples (84·7 %). Figure 3 shows the relationship between the herbarium samples. The 28 samples from Oceania comprising specimens from New Guinea (AK 116673) to Easter Island grouped together in one branch (bootstrapping 63 %). The remaining five samples were separated into two branches. One branch grouped the sample from China (SGO141121) and one of the samples from New

TABLE 2. *DNA extraction methods, yields and purity from herbarium samples*

No.	Herbarium code	Year	Extraction method	Weight, mg	[DNA], ng μL^{-1} PG	Yield by PG (μg DNA g^{-1} leaf)	[DNA], ng μL^{-1} ABS	Yield by ABS (μg DNA g^{-1} leaf)	$A_{260/280}$
1	SG0005091	1882	Modified Lodhi's method	4·4	2·07	47·03	53·90	1225·00	1·76
			DNeasy Plant Mini Kit	5·30	0·33	12·46	2·70	101·89	2·78
			DNeasy Plant Mini Kit	14·4	1·18	16·44	11·90	165·28	2·01
2	SGO141121	1995	DNeasy Plant Mini Kit	12·5	8·95	143·26	104·40	1670·40	1·82
3	SG0058300	1911	Modified Lodhi's method	13·4	2·15	16·04	333·90	2491·79	1·61
			DNeasy Plant Mini Kit	12·3	1·03	16·69	22·90	372·36	1·79
4	SGO058271	1911	Modified Lodhi's method	25·5	1·83	7·19	141·90	556·47	1·73
			DNeasy Plant Mini Kit	9·4	0·72	15·30	19·50	414·89	1·79
5	SG0129525	1971	Modified Lodhi's method	4·8	6·09	126·78	74·50	1552·08	1·92
			Modified Lodhi's method	10·5	3·61	34·36	133·00	1266·67	1·95
			DNeasy Plant Mini Kit	6·9	1·04	30·05	16·20	469·57	1·88
6	BISH161284	1935	Modified Lodhi's method	5·5	22·76	413·85	482·40	8770·91	1·63
			DNeasy Plant Mini Kit	9·5	1·47	30·89	35·10	738·95	1·80
7	BISH161285	1917	DNeasy Plant Mini Kit	5·8	0·12	4·25	9·70	334·48	1·89
8	BISH36684	1968	Modified Lodhi's Method	4·3	0·07	1·55	9·80	227·91	1·42
			DNeasy Plant Mini Kit	2·7	0·07	5·03	5·30	392·59	1·58
9	BISH161275	1940	Modified Lodhi's method	5·2	5·47	105·15	129·70	2494·23	2·00
			DNeasy Plant Mini Kit	3·0	0·25	16·64	16·60	1106·67	1·94
10	BISH161276	1940	Modified Lodhi's method	5·7	6·11	107·17	110·40	1936·84	2·00
			DNeasy Plant Mini Kit	6·7	0·28	8·44	19·80	591·04	1·90
11	BISH161287	1934	Modified Lodhi's method	1·5	0·57	37·81	36·00	2400·00	1·87
			DNeasy Plant Mini Kit	2·3	0·10	8·22	5·00	434·78	2·44
12	BISH161288	1934	Modified Lodhi's method	3·2	1·28	40·04	16·70	521·88	1·71
			DNeasy Plant Mini Kit	4·1	0·56	27·05	4·90	239·02	2·10
13	BISH664608	1957	Modified Lodhi's method	4·7	2·06	43·87	75·90	1614·89	1·64
			DNeasy Plant Mini Kit	7·9	2·35	59·49	17·70	448·10	1·94
14	BISH161280	1929	Modified Lodhi's method	5·4	5·53	102·49	161·50	2990·74	1·52
			DNeasy Plant Mini Kit	3·4	3·64	213·95	10·60	623·53	1·81
15	BISH161286	1926	Modified Lodhi's method	17·3	0·39	2·24	540·40	3123·70	1·87
			DNeasy Plant Mini Kit	8·9	0·14	3·04	19·50	438·20	1·81
16	BISH418270	1974	Modified Lodhi's method	3·8	2·76	72·76	155·80	4100·00	1·99
			DNeasy Plant Mini Kit	3·1	1·65	106·68	31·90	2058·06	1·91
17	BISH161278	1953	Modified Lodhi's method	3·4	2·57	75·72	107·20	3152·94	1·99
			DNeasy Plant Mini Kit	5·7	0·19	6·56	13·40	470·18	2·00
18	BISH161279	1953	Modified Lodhi's method	1·2	1·52	126·59	47·90	3991·67	2·03
			DNeasy Plant Mini Kit	2·2	0·21	19·13	20·30	1845·45	1·88
19	BISH750662	1925	Modified Lodhi's method	5·3	4·40	83·07	293·30	5533·96	1·68
			DNeasy Plant Mini Kit	2·3	0·69	59·59	3·70	321·74	1·69
20	BISH161272	1925	Modified Lodhi's method	4·1	4·58	111·62	113·90	2778·10	1·77
			DNeasy Plant Mini Kit	2·7	OR	OR	5·30	392·59	2·00
21	BISH161273	1925	Modified Lodhi's method	5·0	0·47	9·35	96·50	1930·00	1·59
			DNeasy Plant Mini Kit	3·3	0·25	15·17	12·00	727·27	1·84
22	BISH161277	1939	Modified Lodhi's method	7·8	6·38	81·77	388·70	4983·33	1·95
			DNeasy Plant Mini Kit	5·9	0·54	18·17	38·20	1294·92	1·95
23	BISH161289	1934	Modified Lodhi's method	5·9	8·49	143·93	136·60	2315·30	1·87
			DNeasy Plant Mini Kit	6·3	0·07	2·11	16·10	511·11	1·69
24	BISH161290	1921	Modified Lodhi's method	3·9	4·74	121·61	145·50	3730·80	1·78
			DNeasy Plant Mini Kit	2·7	0·18	13·51	5·70	422·22	1·80
25	BISH161291	1921	Modified Lodhi's method	4·7	4·74	100·83	135·20	2876·60	1·71
			DNeasy Plant Mini Kit	6·2	0·40	12·75	9·90	319·35	1·59
26	BISH161292	1921	Modified Lodhi's method	6·6	3·21	48·64	91·50	1386·36	1·73
			DNeasy Plant Mini Kit	4·9	0·29	12·00	13·00	530·61	1·60
27	BISH161293	1921	Modified Lodhi's Mmethod	9·1	0·58	6·34	47·70	524·18	1·66
			DNeasy Plant Mini Kit	6·2	0·23	7·50	6·70	216·13	1·63
28	BISH161294	1921	Modified Lodhi's method	8·8	2·30	26·15	112·80	1281·82	1·59
			DNeasy Plant Mini Kit	5·5	0·58	21·11	7·20	261·82	1·57
29	BISH161296	1921	Modified Lodhi's method	4·5	0·89	19·71	101·30	2251·11	1·71
			DNeasy Plant Mini Kit	3·2	0·43	26·59	15·40	962·50	1·56
30	BISH161297	1921	Modified Lodhi's method	12·1	7·35	60·73	573·60	4740·50	1·95
			DNeasy Plant Mini Kit	7·9	1·24	31·33	54·60	1382·28	1·94
31	BISH161300	1921	Modified Lodhi's method	5·2	15·48	297·60	304·10	5848·08	1·93
			DNeasy Plant Mini Kit	5·0	0·78	31·25	24·00	960·00	1·86
32	BISH161301	1921	Modified Lodhi's method	5·4	12·41	229·73	393·80	7292·59	1·76
			DNeasy Plant Mini Kit	11·0	1·46	26·60	54·40	989·09	1·85
33	BISH751633	1921	Modified Lodhi's method	5·2	2·71	52·06	69·90	1344·23	1·69

(Continued)

TABLE 2. *Continued*

No.	Herbarium code	Year	Extraction method	Weight, mg	[DNA], ng μL^{-1} PG	Yield by PG (μg DNA g^{-1} leaf)	[DNA], ng μL^{-1} ABS	Yield by ABS (μg DNA g^{-1} leaf)	$A_{260/280}$
			DNeasy Plant Mini Kit	5·6	0·46	16·48	6·20	221·43	1·87
34	BISH751635	1921	Modified Lodhi's method	8·4	13·18	156·94	263·70	3139·29	1·82
			DNeasy Plant Mini Kit	5·9	0·41	13·74	22·00	745·76	1·80
35	BISH751636	1921	Modified Lodhi's method	6·7	2·63	39·28	92·60	1382·09	1·77
			DNeasy Plant Mini Kit	4·9	0·34	14·05	24·70	1008·16	1·90
36	BISH404138	1934	Modified Lodhi's method	3·5	0·65	18·61	58·70	1677·14	1·80
			DNeasy Plant Mini Kit	7·1	0·13	3·77	10·70	301·41	1·80
37	BISH493902	1984	Modified Lodhi's method	13·0	0·24	1·88	92·30	710·00	1·47
			DNeasy Plant Mini Kit	8·1	OR	OR	5·50	135·80	1·52
38	BISH161281	1921	Modified Lodhi's method	3·5	2·21	63·11	42·70	1220·00	1·80
			DNeasy Plant Mini Kit	3·7	0·21	11·34	6·10	329·73	1·97
39	BISH161283	1921	DNeasy Plant Mini Kit	1·0	OR	OR	2·40	480·00	2·27
40	BISH588624	1988	Modified Lodhi's method	1·9	1·51	79·64	1·80	94·70	5·54
			DNeasy Plant Mini Kit	2·6	0·17	12·95	4·30	330·77	1·70
41	BISH609116	1974	Modified Lodhi's method	3·3	0·13	4·03	13·80	418·18	1·66
			DNeasy Plant Mini Kit	4·9	0·07	2·89	5·20	212·24	1·42
42	BISH709092	2003	Modified Lodhi's method	2·5	1·60	64·07	89·60	3584·00	2·05
			DNeasy Plant Mini Kit	2·9	0·91	63·05	20·50	1413·79	2·05
43	AK214298	1993	Modified Lodhi's method	8·0	0·07	0·87	67·90	848·75	2·12
			DNeasy Plant Mini Kit	11·1	2·37	42·67	18·60	335·14	1·90
44	AK116673	1964	Modified Lodhi's method	7·5	0·88	11·78	461·50	6153·33	1·83
			DNeasy Plant Mini Kit	8·0	0·84	20·92	19·50	487·50	1·79
45	AK76866	1901	Modified Lodhi's method	2·5	0·71	28·27	87·80	3512·00	1·95
			DNeasy Plant Mini Kit	4·4	1·06	48·27	22·70	1031·82	2·02
46	AK295889	2006	Modified Lodhi's method	3·0	20·72	690·72	45·60	570·00	1·95
			DNeasy Plant Mini Kit	12·0	3·33	55·57	17·50	291·67	1·97
47	AK296981	2006	Modified Lodhi's method	5·9	1·16	19·74	328·90	5574·58	1·90
			DNeasy Plant Mini Kit	7·8	9·40	240·96	31·50	807·69	1·92

ABS, absorbance; OR, out of range; PG, PicoGreen.

Zealand (AK295889) with a bootstrapping of 67 %. The second branch grouped the second sample from New Zealand (AK296981), the sample from Solomon Islands (AK214298) and the sample from Chile (SGO005091).

To determine the relationship between herbarium samples and the 48 haplotypes described by Chang *et al.* (2015), a dendrogram which included all these haplotypes was constructed (Supplementary Data Fig. S1). All samples from Remote Oceania (excluding New Zealand) and the sample from New Guinea possess the defining single nucleotide polymorphism (SNP) that corresponds to the haplotype cp17 described by Chang *et al.* (2015) in samples from Sulawesi to Easter Island. On the other hand, the branch comprising the samples AK214298, AK296981 and SGO005091 grouped with numerous haplotypes found in Asia, including Taiwan and the recent introductions found in the Philippines, Solomon Islands and New Guinea. The second branch, comprising the samples AK295889 and SGO141121 grouped with haplotypes found in China, Japan and in male plants from Hawaii. These results, like those obtained analysing the ITS-1 sequence, were consistent with their recent introductions from Asia.

Genetic characterization of herbarium samples using SSR

Samples were amplified with ten SSR markers designed specifically for *B. papyrifera* as indicated in the Materials and Methods section. The marker Bro 07 was excluded from further analysis, because it yielded inconsistent results. Of the 47 samples tested, 31 (66 %) samples were amplified using the nine SSR markers. A total of 61 alleles, 61 genotypes and 20 combinations of genotypes were identified (Table 4). The Bropap 25444 marker was the most informative, detecting 11 alleles, followed by the Bropap 02214 marker, which detected ten alleles. This last marker was the most informative at the genotype level, as it detected ten different genotypes. In turn, the Bropap 20558 and Bro 13 markers were the least informative, identifying five alleles and five genotypes. A genotype network was constructed based on the 31 *B. papyrifera* herbarium samples that amplified with the nine microsatellite markers. Using SSR, the analysed herbarium samples clustered into three distinct groups, as shown in Fig. 4. One genotype group (GG1) includes samples from China, New Zealand, Chile and the Solomon Islands. The herbarium sample from China represents the native habitat of this species. The herbarium samples from the Solomon Islands and New Zealand, being recent direct introductions from Asia, or indirect introductions via Europe (Chile), also represent lineages from the native range, as discussed below. The second genotype group (GG2) includes genotypes from Île de Horn (Futuna), Tonga and two genotypes from Samoa, representing Western Polynesia. The third group (GG3) of ten different genotypes includes samples from New Guinea, Niue, Pitcairn, Rapa, Cook Islands, Marquesas and Easter Island. All except New Guinea are part of East Polynesia.

The remaining 16 samples were not included in this analysis, as they did not amplify or presented partial amplification with the set of SSR primer pairs. Three samples did not amplify with any of the SSR markers (BISH161273, BISH161291

TABLE 3. *General overview of amplification results: species identification, ITS-1 polymorphism, sex identification, cpDNA marker amplification and amplification with nine microsatellite markers of herbarium samples*

No.	Herbarium code	ITS-1 amplification	Identified species	ITS-1 genotype	Sex marker	cpDNA	Nine SSRs
1	SG0005091*	Yes	*Broussonetia papyrifera*	G	F	Yes	Yes
2	SGO141121*	Yes	*Broussonetia papyrifera*	G	F	Yes	Yes
3	SG0058300*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
4	SGO058271*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
5	SG0129525*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
6	BISH161284	Yes	*Broussonetia papyrifera*	T	F	No	Yes
7	BISH161285*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
8	BISH36684	Yes	*Broussonetia papyrifera*	G	–	No	No
9	BISH161275*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
10	BISH161276*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
11	BISH161287*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
12	BISH161288*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
13	BISH664608*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
14	BISH161280*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
15	BISH161286	Yes	*Juglans regia*, *Prunus spinosa*		–	No	No
16	BISH418270*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
17	BISH161278	Yes	*Broussonetia papyrifera*	T	–	No	No
18	BISH161279	Yes	*Broussonetia papyrifera*	T	–	Yes	Yes
19	BISH750662	Yes	*Broussonetia papyrifera*	T	F	No	Yes
20	BISH161272	Yes	*Origanum* spp.		F	No	No
21	BISH161273	Yes	*Dendrocnide* spp.		–	No	No
22	BISH161277*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
23	BISH161289	Yes	*Broussonetia papyrifera*	T	F	Yes	No
24	BISH161290*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
25	BISH161291	Yes	*Pipturus ruber*		–	No	No
26	BISH161292*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
27	BISH161293	Yes	–	NRS	F	Yes	Yes
28	BISH161294*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
29	BISH161296	Yes	*Broussonetia papyrifera*	T	F	No	Yes
30	BISH161297	Yes	*Broussonetia papyrifera*	T	M	No	No
31	BISH161300	Yes	–	NRS	F	Yes	No
32	BISH161301	Yes	*Broussonetia papyrifera*	T	–	Yes	No
33	BISH751633*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
34	BISH751635	Yes	*Broussonetia papyrifera*	T	F	Yes	No
35	BISH751636*	Yes	*Broussonetia papyrifera*	T	F	Yes	Yes
36	BISH404138	Yes	*Broussonetia papyrifera*	T	–	Yes	No
37	BISH493902	Yes	–	NRS	–	No	No
38	BISH161281*	Yes	*Broussonetia papyrifera*	T	M	Yes	Yes
39	BISH161283	Yes	–	NRS	F	Yes	Yes
40	BISH588624	Yes	*Broussonetia papyrifera*	T	–	No	No
41	BISH609116	Yes	*Broussonetia papyrifera*	T	–	No	No
42	BISH709092	Yes	*Broussonetia papyrifera*	T	F	No	Yes
43	AK214298*	Yes	*Broussonetia papyrifera*	G	F	Yes	Yes
44	AK116673*	Yes	*Broussonetia papyrifera*	G	F	Yes	Yes
45	AK76866	Yes	*Broussonetia papyrifera*	G	–	No	No
46	AK295889*	Yes	*Broussonetia papyrifera*	G	M	Yes	Yes
47	AK296981*	Yes	*Broussonetia papyrifera*	G	M	Yes	Yes

NRS, non-readable sequence. *Amplification with ITS-1, sex, chloroplast and nine SSR markers.

and BISH493902). In another three samples (BISH36684, BISH161286, BISH588624), only one or two alleles were detected with markers Bropap 05258, Bro 08 and Bropap 3024, respectively. Sample BISH161300 amplified four alleles with four markers, and sample BISH161272 amplified five alleles also with four markers. Finally, eight samples (BISH161278, BISH161289, BISH161297, BISH161301, BISH751635, BISH404138, BISH609116 and AK76866) amplified between seven and 14 alleles with seven markers.

DISCUSSION

A critical issue when working with herbarium material is the varying quality of DNA preservation in samples. Herbaria specimens are usually prepared in order to preserve plant anatomy and morphology. Much of this material is brittle, and its genetic material partially degraded, chemically modified, contaminated by DNA from bacteria or fungi and/or handling by humans, and may contain compounds that inhibit the PCR (Weising *et al*., 2005). All these factors contribute to the challenge of obtaining amplifiable DNA. Our work shows the feasibility of fingerprinting herbarium collections using several molecular markers. As most of the herbarium accessions were $\geq$50 years old, samples were treated as ancient DNA. Among other precautions, extractions were performed in dedicated ancient DNA facilities and in two different laboratories using two different extraction procedures, as recommended by the ancient DNA protocols. Several arguments support the authenticity of

FIG. 1. Maximum Likelihood tree of ITS-1 sequence analysis.

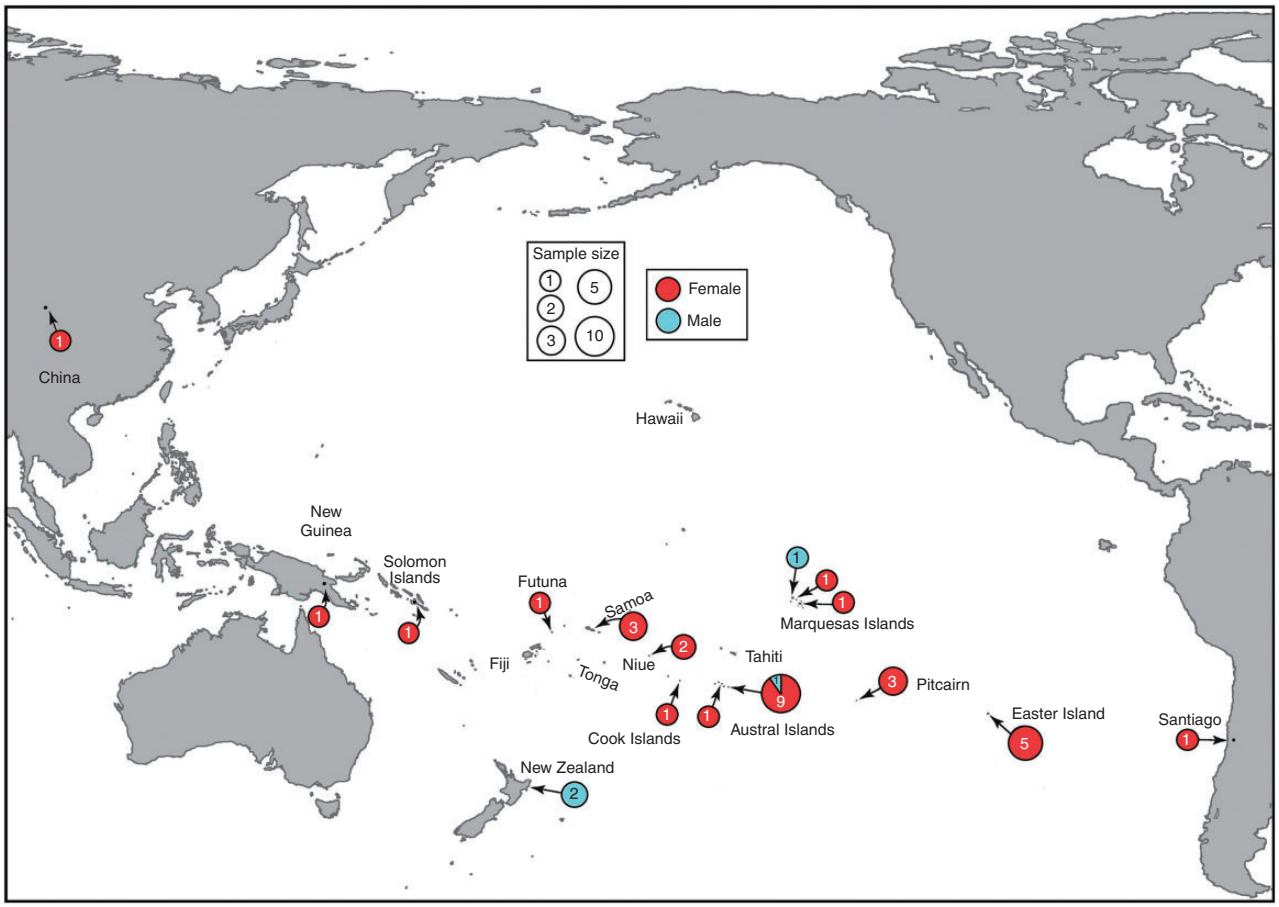

FIG. 2. Map with sampling location and sex distribution of *B. papyrifera* in the Pacific.

the obtained paper mulberry herbarium molecular profiles. Our handling of the herbarium samples always involved the use of gloves and tweezers, and in areas where no extractions or amplifications of contemporary DNA had been performed; however, evidently there is no possibility to control the prior handling of the samples. During the extraction procedure, negative controls were always included. These controls did not amplify the different markers, even when testing several dilutions.

The aim of our study was to characterize paper mulberry using a combination of molecular markers that would enable us to detect genetic diversity within a region where the plant was introduced in prehistoric times. We genotyped Pacific paper mulberry herbarium samples predominantly from the early 20th century that include islands where the plant has disappeared locally during the last century, such as the Cook Islands, or from localities that are extremely difficult to reach such as Pitcairn Island, Futuna and Rapa. The analysis combined several molecular markers but, importantly, a set of microsatellites designed for paper mulberry. Previous studies on contemporary leaf material did not detect genetic diversity among the prehistorically introduced plants within this vast region, analysing the ribosomal ITS-1 region, ISSR markers, a chloroplast and a sex marker (Seelenfreund *et al.*, 2011; Chang *et al.*, 2015; González-Lorca *et al.*, 2015; Peñailillo *et al.*, 2016). The lack of genetic diversity might be explained by the fact that these plants have been reproduced clonally (vegetative propagation) for hundreds of years and the short time span since their introduction to Remote Oceania. Mutations occur spontaneously even in the absence of recombination (Loxdale and Lushai, 2003). Some of these somatic mutations can produce phenotypic differences and, if culturally valued, these may be selected to produce clonal crop varieties. Therefore, the analysis of genetic diversity can be used to study the spread of clonally reproduced crops. For example, Moncada *et al.* (2006), analysing the widely cultivated grapevine variety Cabernet Sauvignon using nuclear microsatellites, could infer its dispersal from its centre of origin in France to different parts of the world, where new genotypes appeared within a time lapse of a few centuries.

We successfully extracted and amplified DNA with one or more markers from all 47 herbarium samples (Table 3). Out of the 47 DNA extractions, finally a total of 31 paper mulberry samples could be amplified with nine microsatellites and 24 samples with the complete set of markers (ITS-1, sex, chloroplast region and nine microsatellites) as shown in Table 3. Analysis with the ITS-1 marker allowed the successful species determination in 43 specimens. Only four samples yielded unreadable sequences, 39 were identified as paper mulberry and four extractions amplified DNA sequences

FIG. 3. Maximum Likelihood tree of cpDNA haplotypes found in herbarium samples.

from other plant species (Table 3). In those 39 samples identified as paper mulberry, the 'Polynesian' polymorphism (T) was detected in the Oceanian samples, in contrast to the samples of Asian or Near Oceanian origin, that presented the G variant at the same position (Seelenfreund *et al.*, 2011). Future studies should consider including additional herbarium samples from collections from the first European expeditions into the Pacific, particularly from New Guinea and the Society Islands, if available, to clarify the issues raised in the discussion below.

Amplification of the ITS-1 region allowed correct species identification or if the mounted specimen had been contaminated during collection, storage or general handling. From a total of 47 herbarium specimens analysed that were labelled as *B. papyrifera* and presented the expected morphological traits of this species, 39 accessions from three different museums could be positively identified as *B. papyrifera*. The four samples that amplified DNA from other species suggested that contamination occurred at different stages in these specimens, although these accessions present phenotypic characteristics of paper mulberry,

TABLE 4.

Sample	Locality	Genotype	Bro 08		Bro 13		Bro 15		Bropap 02214		Bropap 02801		Bropap 20558		Bropap 25444		Bropap 26985		Bropap 30248	
			A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
BISH161290	Rapa	G1	203	221	227	227	211	222	242	250	150	171	218	222	185	187	176	183	94	94
BISH161296	Rapa	G1	203	221	227	227	211	222	242	250	150	171	218	222	185	187	176	183	94	94
BISH751633	Rapa	G1	203	221	227	227	211	222	242	250	150	171	218	222	185	187	176	183	94	94
BISH751636	Rapa	G1	203	221	227	227	211	222	242	250	150	171	218	222	185	187	176	183	94	94
BISH161293	Rapa	G2	203	207	227	227	211	222	242	250	150	171	218	222	185	187	176	183	94	94
BISH161294	Rapa	G2	203	207	227	227	211	222	242	250	150	171	218	222	185	187	176	183	94	94
BISH161281	Marquesas	G3	193	203	227	227	211	222	242	252	150	171	218	222	185	187	178	183	94	94
BISH161283	Marquesas	G3	193	203	227	227	211	222	242	252	150	171	218	222	185	187	178	183	94	94
BISH161292	Rapa	G4	203	207	227	227	211	222	242	250	150	171	218	222	185	187	178	183	94	94
BISH709092	Marquesas	G4	203	207	227	227	211	222	242	250	150	171	218	222	185	187	178	183	94	94
BISH161287	Pitcairn	G5	203	207	227	227	211	215	242	250	150	171	218	222	185	187	178	183	94	94
BISH161288	Pitcairn	G5	203	207	227	227	211	215	242	250	150	171	218	222	185	187	178	183	94	94
BISH664608	Pitcairn	G5	203	207	227	227	211	215	242	250	150	171	218	222	185	187	178	183	94	94
BISH161280	Cook Islands	G6	203	207	227	227	207	211	242	250	150	171	218	222	185	187	178	183	94	94
SGO058300	Easter Island	G7	203	207	227	227	211	215	244	250	150	171	218	222	185	187	178	183	94	94
SGO058271	Easter Island	G7	203	207	227	227	211	215	244	250	150	171	218	222	185	187	178	183	94	94
BISH161285	Easter Island	G7	203	207	227	227	211	215	244	250	150	171	218	222	185	187	178	183	94	94
SGO129525	Easter Island	G8	203	207	227	228	211	215	244	250	150	171	218	222	185	189	178	183	94	94
BISH161284	Easter Island	G9	203	207	227	227	211	215	248	252	150	171	218	222	185	187	178	183	94	94
AK116673	New Guinea	G10	203	207	227	227	211	215	242	252	150	171	218	222	185	187	178	183	94	94
BISH161275	Niue	G11	203	207	227	227	211	222	242	250	150	173	218	222	185	187	178	183	94	94
BISH161276	Niue	G11	203	207	227	227	211	222	242	250	150	173	218	222	185	187	178	183	94	94
BISH418270	Futuna	G12	203	207	227	227	211	215	242	250	150	173	218	222	185	187	178	183	94	94
BISH161279	Tonga	G13	205	207	227	227	211	215	242	250	150	173	218	222	185	187	178	183	94	94
BISH750662	Samoa	G14	203	207	227	227	211	215	244	252	150	173	218	225	185	187	178	181	94	94
BISH161277	Samoa	G15	203	207	227	227	211	215	244	252	150	161	218	225	185	187	178	181	94	94
AK296981	New Zealand	G16	209	209	227	227	203	203	240	242	150	150	222	222	176	217	178	191	94	98
SGO141121	China	G17	205	205	227	227	211	211	224	240	148	150	218	222	178	191	183	183	100	108
AK214298	Solomon Islands	G18	195	207	222	223	211	211	232	240	150	150	218	224	178	180	183	183	124	124
AK295889	New Zealand	G19	207	209	221	228	206	211	232	244	150	155	218	221	184	207	185	185	102	124
SGO005091	Santiago	G20	209	217	228	228	206	206	248	248	155	155	218	221	205	207	183	183	124	124
BQUCH0012	Easter Island	-	203	207	227	227	211	215	244	250	150	171	218	222	185	187	178	183	94	94
BQUCH0077	Samoa	-	203	207	227	227	211	215	242	250	150	173	218	222	185	187	178	181	94	94

such as leaf size, morphology and hairiness. Repeated amplification with the universal ITS-1 primers revealed contamination with DNA from different species, in most cases with high e-values (data not shown). The same DNA preparations were also assayed with the species-specific sex and microsatellite markers. In particular, sample BISH161272 amplified twice an ITS-1 sequence corresponding to *Origanum* spp., although the leaves morphologically correspond to *B. papyrifera*. On the other hand, the species-specific sex marker yielded an amplicon characteristic of female paper mulberry with this sample. Also, amplification with four microsatellite markers was obtained from accession BISH161272. Our interpretation is that the primers for amplification of the ITS sequences are universal and preferentially amplified the modern contaminating DNA, while the species-specific markers amplified sequences from the herbarium specimen. Samples BISH161286 amplified twice as *Juglans regia* and once as *Prunus spinosa*, and accession BISH161272 was identified twice as *Origanum* spp. These results suggest modern environmental contamination. On the other hand, samples BISH161273 and BISH161291 identified as Urticaceae species *Dendrocnide* spp. and *Pipturus ruber*, respectively, suggest misidentification or contamination with samples from the Pacific, either in the field or during the handling/storing of the collection in the museum.

In the Pacific, paper mulberry plants are periodically cut and harvested, and stems are used to obtain the inner bark for the manufacture of bark cloth textiles, and therefore flowering is seldom observed (see Seelenfreund *et al.*, 2010; Peñailillo *et al.*, 2016). In addition, since this species is propagated asexually like many crops in this region, a molecular marker is needed to determine the sex of individuals of this dioecious species. Analysis with the sex marker was successful in 35 of the 39 paper mulberry accessions. Results on the analyses of these 35 paper mulberry samples indicated that 31 of these accessions are female plants. Unlike reported by Peñailillo *et al.* (2016), where all contemporary *B. papyrifera* samples analysed from Polynesia were female, we found four male samples. Of these, two samples were collected in New Zealand (AK295889 and AK296981) and correspond to recent introductions to this country. However, accession BISH161281 from the Marquesas archipelago collected on the island of Nuku Hiva in 1921 and accession BISH161297, collected in 1921 on Rapa by the Stokes expedition to the Pacific, were male plants, which was an unexpected finding. It is noteworthy that these two accessions present the 'Polynesian' ITS-1 polymorphism, attesting to their Oceanic origin. The fact that these accessions were sampled in very small and isolated locations prior to modern plant translocations suggests that they represent the ancient

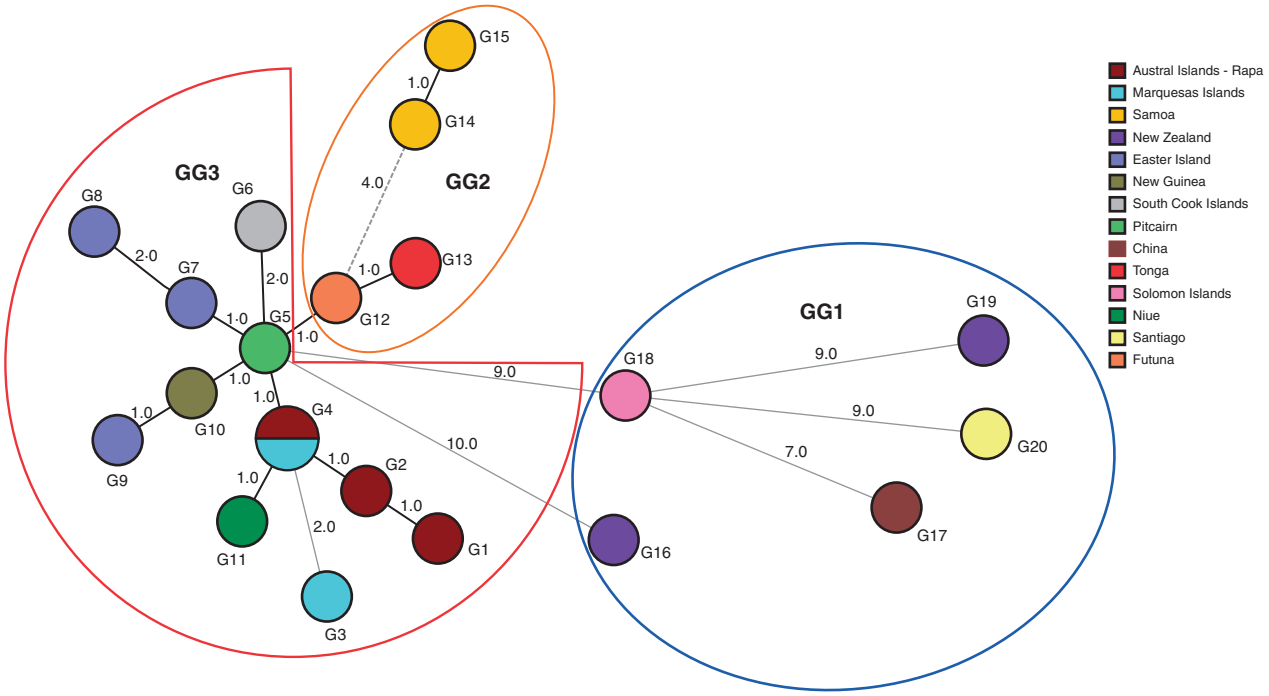

FIG. 4. Relationship between the detected genotypes. Minimum spanning tree (Bionumerics v.7.6) showing the differences between the genotypes based on a categorical analysis. Each circle represents a unique genotype (shown as pies). Numbers correspond to the number of differences between the genotypes. Thick, short lines connect genotypes differing by one mutation; thin, longer lines connect genotypes differing by two mutations, and dotted lines connect genotypes differing by three or more mutations.

genetic diversity that is no longer found in the few contemporary plants still remaining on Nuku Hiva. In the case of Rapa, it is possible that male plants still survive; however, extensive sampling of the current extant plants has not been performed. It is important to point out that the presence of both sexes on an island does not necessarily imply sexual reproduction. As long as plants of either or both sexes are periodically harvested for bark cloth production, the bearing of fruits and therefore sexual reproduction is precluded. Therefore, provided that the cultural use of this plant is continued, the clonal propagation of this species on Pacific islands will be retained. The discovery of male plants on some islands indicates that both sexes were probably present in the past and were involved in the 'out of Taiwan' dispersal of this species (Chang *et al.*, 2015). However, an additional complication is that there is also the possibility of sex reversion in plants, so that previously female plants may for some reason produce male flowers, or vice versa. This is also a little understood phenomenon, but is known to happen in *Broussonetia*, where male plants have been described to change to females (Sykes, 1969).

Analysis of the chloroplast DNA region was successful in 33 of the 39 paper mulberry accessions. The specific primer pair for the chloroplast *ndhF–rpl32* region was chosen because it contains the distinct polymorphism that identifies the cp17 haplotype (Chang *et al.*, 2015). The haplotypes identified in these herbarium specimens are also consistent with the haplotype distribution found in contemporary samples. The hypervariable chloroplast *ndhF–rpl32* region distinguishes 48 haplotypes in this species, of which 43 are exclusively found in the native range in Asia. The most widely distributed haplotype in the Pacific, and identified in contemporary and some other herbaria samples from Remote Oceania is cp17, as previously described (Chang *et al.*, 2015). This haplotype has a clear south/central Taiwanese origin and is the only lineage from the native range found in Sulawesi, Fiji and in all the Polynesian islands with the exception of Hawaii (Chang *et al.*, 2015). These results indicate that the most common variant of paper mulberry most probably introduced by the prehistoric Austronesian-speaking colonists is of Taiwanese origin, providing a direct genetic link between Taiwan and one of the Pacific commensal species (Matisoo-Smith, 2015).

The two accessions from New Zealand and the accessions from Solomon Islands, Chile and China showed haplotypes consistent with their original Asian provenance (Fig. S1) and are also consistent with the information provided by the nuclear ribosomal marker (G variant). These characteristics indicate a more recent introduction to New Zealand, Solomon Islands and also to continental Chile. The Solomon Island samples were collected outside Honiara in 1993, and derive from modern introductions (Matthews, 1996). The samples from New Zealand were collected in 2006 and also represent modern introductions, as reported by the collectors. Paper mulberry plants introduced by Polynesians to New Zealand disappeared after European colonization and were rare even at the time of contact (Neich, 1996) and became extinct in New Zealand after 1846 (Colenso, 1880). The sample from Chile derives from a tree introduced from Europe and planted in the nascent Santiago Botanical Garden, and sampled in 1882 by the eminent German botanist R. A. Philippi. All these samples therefore correspond to five

genotypes derived from Asian stock, representing the native range of this species.

In contrast to our previous results, the use of microsatellites allowed for the first time the detection of genetic diversity in paper mulberry within Remote Oceania, a region outside its native range. A sub-group of 31 out of the 39 paper mulberry accessions could be analysed with nine SSR markers. In these specimens, we identified 20 different genotype combinations, as shown in Fig. 4. The constructed network shows an interesting broad geographical distribution of these genotype combinations. The most distant genotype combinations (GG1) are found in the five specimens from China, New Zealand, Solomon Islands and Chile. Except for the sample from China (the native range), the other specimens represent introductions to these countries at different times in the recent past derived from Asian stock. The microsatellite profiles from these samples are very different from all other Pacific samples, which is consistent with their non-Oceanian genotype, as demonstrated by their chloroplast haplotype and ribosomal ITS-1 sequence.

The genotype groups GG2 and GG3 represent a single lineage in the Pacific, linked to unknown genotypes in the native habitat. For the first time, we have found 15 different genotype combinations in Near and Remote Oceanian paper mulberry samples. All the genotypes included in GG2 and GG3 are very distant from the Asian genotypes (GG1). Interestingly all Oceanian samples cluster around a network centred on the specimen from the remote island of Pitcairn. One branch (GG2) includes all the samples from West Polynesia (Samoa, Tonga and Futuna) and the second branch (GG3) comprises samples from East Polynesia (Rapa, Marquesas, Niue, Pitcairn, Southern Cook Islands and Easter Island) and New Guinea.

The Asian genotypes (GG1) found in the five herbarium samples analysed in this work probably represent a very small fraction of the diversity of the native range for this species. An extensive sampling in the native range should reveal the presence of high genetic diversity and several lineages. We hypothesize that one of these lineages gave rise to the accessions found in the Pacific, where new genotypes appear. The herbarium specimens selected for this work were strongly skewed in favour of Oceanic accessions. Therefore, the higher genetic diversity found in the GG2 and GG3 groups representing accessions from Remote Oceania is related to a higher number of samples from this region and does not reflect an ancestral group or the diversity at the centre of origin. The bias in the sampling of the herbaria reflects our search of genetic diversity in Remote Oceania and derives from accessions sampled on different islands at different times, by different collectors, following diverse criteria, and therefore do not conform to a systematic sampling procedure and do not represent populations.

The network analysis showed a central genotype within the Pacific lineage and that all branches of this lineage are connected to this central genotype. Surprisingly, this connecting genotype corresponds to specimens collected on Pitcairn Island. This genotype articulates all the Pacific genotypes from West and East Polynesia and also the sample from New Guinea. The central position of the genotype found on Pitcairn either suggests a relatively ancient lineage that survived on this remote island and/or reveals a central position of this island as part of an extensive interaction sphere that connected East and West Polynesia. This scenario is supported by the fact that Pitcairn had excellent stone-tool resources that were exported to the Gambier Islands, and to the Society Islands. Archaeological basalt adzes have been sourced to the Pitcairn basalt quarry. As stated by Weisler (2002), Mangareva (Gambier Islands) was central to an interaction sphere that included the Pitcairn group to the east, the eastern Tuamotus to the west and the Marquesas to the north-east. This scenario can be further sustained by recent archaeological findings of basalt adzes found on the Cook Islands that are indicative of an extensive network that connected the Austral Islands with the Cook Islands and these with the Marquesas, and Samoa, up to 2400 km distant (Weisler *et al*., 2016). In turn, adzes from basalt sources in the Marquesas have been found on Pitcairn and other islands of the Austral Group (Collerson and Weisler, 2007). In addition, basalt tools from the Kaho'olawe quarry in Hawaii have been reported in the Tuamotu Archipelago (Collerson and Weisler, 2007). These authors suggest that Pitcairn at some point in time was part of an extensive network that connected a number of these islands until long-distance voyaging ceased during the 15th century. The Pitcairn Island paper mulberry genotype found in these herbarium specimens collected at the beginning of the 20th century possibly corresponds to remnant plants transported by the original Polynesian colonizers. However, we cannot rule out that these plants were introduced by the Tahitian women that accompanied the Bounty mutineers in the late 18th century. The name of '*Aute Walley*' on Pitcairn Island is suggestive of the existence of a large number of paper mulberry plants found by the Tahitian women on their arrival (Reynolds, 2008). Prior to the Bounty settlement, there were a number of settlements on the island at different times. A Tahitian legend details voyages between Tahiti and Pitcairn, prehistorically known as Hitiaurevareva to the Tahitians (Reynolds, 2008). Alternatively we cannot rule out that the Bounty settlers chose this location for the planting of their own cuttings. Morrison (2010: 70), a midshipman on the Bounty, wrote that when departing from Tahiti the second time, she was filled with livestock 'together with plants of all kinds that are common in these Islands'. Teehuteatuaonoa or Jenny, one of the Tahitian women that accompanied the Bounty mutineers to Pitcairn, reported that on their arrival on Pitcairn the settlers set to work at planting the yams, taro, bananas and *aute* they had brought with them (Maude, 1968: 26). Therefore we cannot rule out that the plants present today on Pitcairn are a mix of very ancient stock and those brought by the women of the mutineers. At present, since the DNA from the single specimen from the Society Island (BISH161286) was contaminated apparently with contemporary DNA, we are unable to solve this question.

The Pitcairn genotype (G5) articulates genotypes between West and East Polynesia through the genotype found in the single specimen from the island of Futuna. The genotype from Futuna (G12) is connected by one mutation with the genotype found in Tonga and more distantly connected with the two genotypes found on Samoa. The close connection between Pitcairn and Futuna is also unexpected, since both islands are >2000 km apart from each other and Futuna is also relatively isolated today. The isolation of these two islands possibly reflects the survival of ancient paper mulberry stock. To our knowledge, there is no evidence of modern introductions of paper mulberry to either island. The inclusion of the genotypes from Futuna, Tonga and Samoa in the same group is to be expected, since they are part of the same broad geographic area of West Polynesia. However,

Samoa is closer both geographically and linguistically to Futuna than Tonga (Green, 1966). The position of the Samoan genotypes reflect a more distant relationship with Futuna, while the position of the Tongan samples reflects a closer relationship with Futuna (one mutation). These results are somewhat unexpected, considering the linguistic relationship and geographic proximity between Samoa and Futuna. The central position of the Pitcairn samples in the network possibly accounts for the genotypes originally found in central Polynesia (Society Islands) that are absent or rare today. Again, the remoteness of Pitcairn permitted the survival of paper mulberry plants of central Polynesian stock. In this regard, the central position of Pitcairn in the network acts as a reflection of central Polynesia as a dispersal hub, in agreement with archaeological evidence.

The third group of genotypes (GG3) presents the highest diversity found in this study. Within this group, we find the central genotype from Pitcairn (G5) connecting with the genotypes from the Cook Islands, Marquesas Islands, Rapa, Easter Island from East Polynesia and also from Niue. The genetically most diverse branch is represented by a genotype shared by specimens from Rapa and Marquesas (G4) suggesting a common ancestry. This genotype gives rise to four additional genotypes: one found on Niue (G11), one on the Marquesas (G3) and two on Rapa (Austral Islands) (G1 and G2). A second branch that closely connects to the Pitcairn genotype is represented by the sample from the Cook Islands (G6). The genotypes from the Cook Islands represent a genetic diversity that is no longer present, as there are no extant paper mulberry plants today. A third branch represented by two genotypes from Easter Island (G7 and G8) is also closely related to Pitcairn. Finally, a fourth branch includes one sample from New Guinea in Near Oceania (G10) and one from Easter Island (G9). Surprisingly, the single specimen from New Guinea is located in the East Polynesian group, and presents a genotype derived by mutation from the Pitcairn genotype. This specimen is female, presents a Polynesian chloroplast DNA (cp17) haplotype and an East Polynesian microsatellite pattern. However, its ITS-1 polymorphism is 'G', which suggests for the first time that the G to T transversion occurred somewhere between New Guinea and West Polynesia. The genetic closeness of the microsatellite profile of this specimen and the genotype from the Pitcairn samples across a distance of > 5000 km suggests the survival of an ancient genotype on Pitcairn Island. There are three genotypes found on Easter Island that are found on two branches. Genotypes G7 and G8 form one branch, where G7 is closely related to Pitcairn (by one mutation) and G8 derives from G7 by two mutations. Genotype G9 is found on a different branch that is closely connected to G10 from New Guinea (one mutation), and G10 in turn connects with Pitcairn by one mutation. The close relationships between the specimens from extremely distant locations, such as inland New Guinea and Easter Island, may again reflect the survival of ancient paper mulberry genotypes until the early 20th century in isolated locations in East Polynesia. Finally, the Marquesas sample BISH161281 is a male specimen that presents an East Polynesian genotype profile (G3). This genotype combination also reflects the survival of ancient stock as there are no male plants in the Marquesas today. Another male plant was found on Rapa (BISH161297), but unfortunately its genotype could not be assessed, as it did not amplify with all microsatellites. However, the 14 detected alleles (from seven SSRs) are identical to those found in another female sample from Rapa (BISH161296). The observed genetic diversity could have been created by both sexual reproduction and somatic mutations, as long as plants were allowed to flower and reproduce. In clonally propagated crops that are periodically harvested, new genetic diversity can occur only through somatic mutations. If these mutations produce distinct phenotypes, and these are culturally valued, human selection will lead to a cluster of distinct varieties that are genetically similar (Scarcelli *et al.*, 2011). Contemporary ethnographic data do not support the existence of sexual reproduction of paper mulberry in Oceania (Florence, 2004). Our results suggest that the observed genetic diversity may be the result of one or more of the following non-excluding processes: somatic mutation, a single introduction of several genotypes from the native range, multiple introductions of plants of both sexes bearing a reduced number of genotypes from a specific region within the native range and/or sexual reproduction on those islands where plants of both sexes were present and allowed to flower. In consequence, the observed diversity in Remote Oceania is probably the product of some sexual reproduction in the past and somatic mutations that occurred after prehistoric colonization of the islands. Today, in the absence of male plants on most islands, further diversity can only occur through somatic mutations.

Despite the relatively small sample size of herbaria specimens used, significant genetic diversity has been uncovered in this study. A clear separation between West and East Polynesia was found that may be indicative of pulses during its dispersal history. The pattern linking the genotypes within Remote Oceania reflects the importance of central Polynesia as a dispersal hub, in agreement with archaeological evidence. The genetic diversity of Pacific paper mulberry herbarium specimens detected in this study also needs to be compared with the genetic diversity present in contemporary plants from this broad geographic region. Several scenarios may be envisaged for extant paper mulberry plants in Oceania: (1) the most 'conservative' possibility would be to find the same genotypes on the same islands today; (2) the same genotypes may be also found in different localities; (3) due to the relatively reduced number of herbarium specimens analysed, more extensive sampling may detect new genotypes in contemporary plants that were not found in this work; and (4) that extant plants present less genetic diversity due to genetic erosion or clonal propagation. The analysis of somatic mutations in herbarium and contemporary specimens could allow an estimation of age of these genotypes within Oceania. An estimation of relative clone age has been performed on African yams, an important clonally propagated crop (Scarcelli *et al.*, 2013). This analysis in turn may infer whether the genetic diversity detected by microsatellites reflects the genetic make-up of the plants dispersed by the Austronesian voyagers or later somatic mutations on the different islands. A further and different approach in the study of the interaction between this plant and humans can be provided by the genetic characterization of historic bark cloth textiles from museum collections, housed in many museums around the world. The application of ancient DNA methods to identify genotypes in artefacts made of bark cloth will further our understanding of the intertwined dispersal history of humans and this culturally important plant.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of Figure S1: Maximum Likelihood tree of cpDNA haplotypes found in herbarium samples and contemporary paper mulberry haplotypes.

ACKNOWLEDGEMENTS

This work was supported by the Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) from the Government of Chile [Fondecyt 1120175 to A.S. and a Master of Science scholarship Number 221320693 to C.P.] and by the University of Chile [Scholarship for research visit to C.P.]. We wish to thank the B. P. Bishop Museum, Hawaii, USA and the Auckland War Memorial Museum, New Zealand for issuing sampling permits. Results were obtained by a temporary BioNumerics evaluation licence from Applied Math NV, with permission to publish. We thank B. Peña-Ahumada for valuable assistance.

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