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REGULAR PAPER – TAXONOMY/PHYLOGENETICS/EVOLUTIONARY BIOLOGY

Plastome phylogenomics of *Allaeanthus*, *Broussonetia* and *Malaisia* (Dorstenieae, Moraceae) and the origin of *B.* × *kazinoki*

Wen-Hsi Kuo[1,2] · Shih-Hui Liu[3] · Chiung-Chih Chang[1] · Chia-Lun Hsieh[1] · Yi-Hsuan Li[1] · Takuro Ito[4] · Hyosig Won[5] · Goro Kokubugata[6] · Kuo-Fang Chung[1]

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Abstract

Species of *Broussonetia* have been essential in the development of papermaking technology. In Japan and Korea, a hybrid between *B. monoica* and *B. papyrifera* (= *B.* × *kazinoki*) known as *kōzo* and *daknamu* is still the major source of raw materials for making traditional paper *washi* and *hanji*, respectively. Despite their cultural and practical significance, however, the origin and taxonomy of *kōzo* and *daknamu* remain controversial. Additionally, the long-held generic concept of *Broussonetia s.l.*, which included Sect. *Allaeanthus* and Sect. *Broussonetia*, was challenged as phylogenetic analyses showed *Malaisia* is sister to the latter section. To re-examine the taxonomic proposition that recognizes *Allaeanthus*, *Broussonetia*, and *Malaisia* (i.e., *Broussonetia* alliance), plastome and nuclear ribosomal DNA (nrDNA) sequences of six species of the alliance were assembled. Characterized by the canonical quadripartite structure, genome alignments and contents of the six plastomes (160,121–162,594 bp) are highly conserved, except for the pseudogenization and/or loss of the *rpl22* gene. Relationships of the *Broussonetia* alliance are identical between plastome and nrDNA trees, supporting the maintenance of *Malaisia* and the resurrection of *Allaeanthus*. The phylogenomic relationships also indicate that the monoecy in *B. monoica* is a derived state, possibly resulting from hybridization between the dioecious *B. kaempferi* (♀) and *B. papyrifera* (♂). Based on the hypervariable *ndhF-rpl32* intergenic spacer selected by sliding window analysis, phylogeographic analysis indicates that *B. monoica* is the sole maternal parent of *B.* × *kazinoki* and that *daknamu* carries multiple haplotypes, while only one haplotype was detected in *kōzo*. Because hybridizations between *B. monoica* and *B. papyrifera* are unidirectional and have occurred rarely in nature, our data suggest that *daknamu* might have originated via deliberate hybrid breeding selected for making *hanji* in Korea. On the contrary, *kōzo* appears to have a single origin and the possibility of a Korean origin cannot be ruled out.

Keywords Asymmetrical hybridization · *daknamu* · Homoploid · *kōzo* · Phylogeography · Traditional papermaking

Introduction

Paper is one of the greatest inventions of all time (Hunter 1978). Amongst a variety of raw materials used for making paper, the moraceous tree species paper mulberry (*Broussonetia papyrifera*) was essential in the development of the technology in ancient China (Barker 2002). Although the importance of *B. papyrifera* in the modern paper industry has decreased, hybrid paper mulberry trees known as *kōzo* and *daknamu* remain essential in the production of *washi* and *hanji*, traditional paper products from Japan and Korea, respectively (Mizumura et al. 2017; Won 2019). Despite the cultural and practical importance of *washi* and *hanji*, however, relationships between *kōzo* and *daknamu* and their origin(s) have been puzzling (Ohba and Akiyama 2014; Yun and Kim 2009). To settle controversies surrounding *kōzo*

✉ Kuo-Fang Chung
bochung@gate.sinica.edu.tw

1 Research Museum and Herbarium (HAST), Biodiversity Research Center, Academia Sinica, Taipei 115201, Taiwan

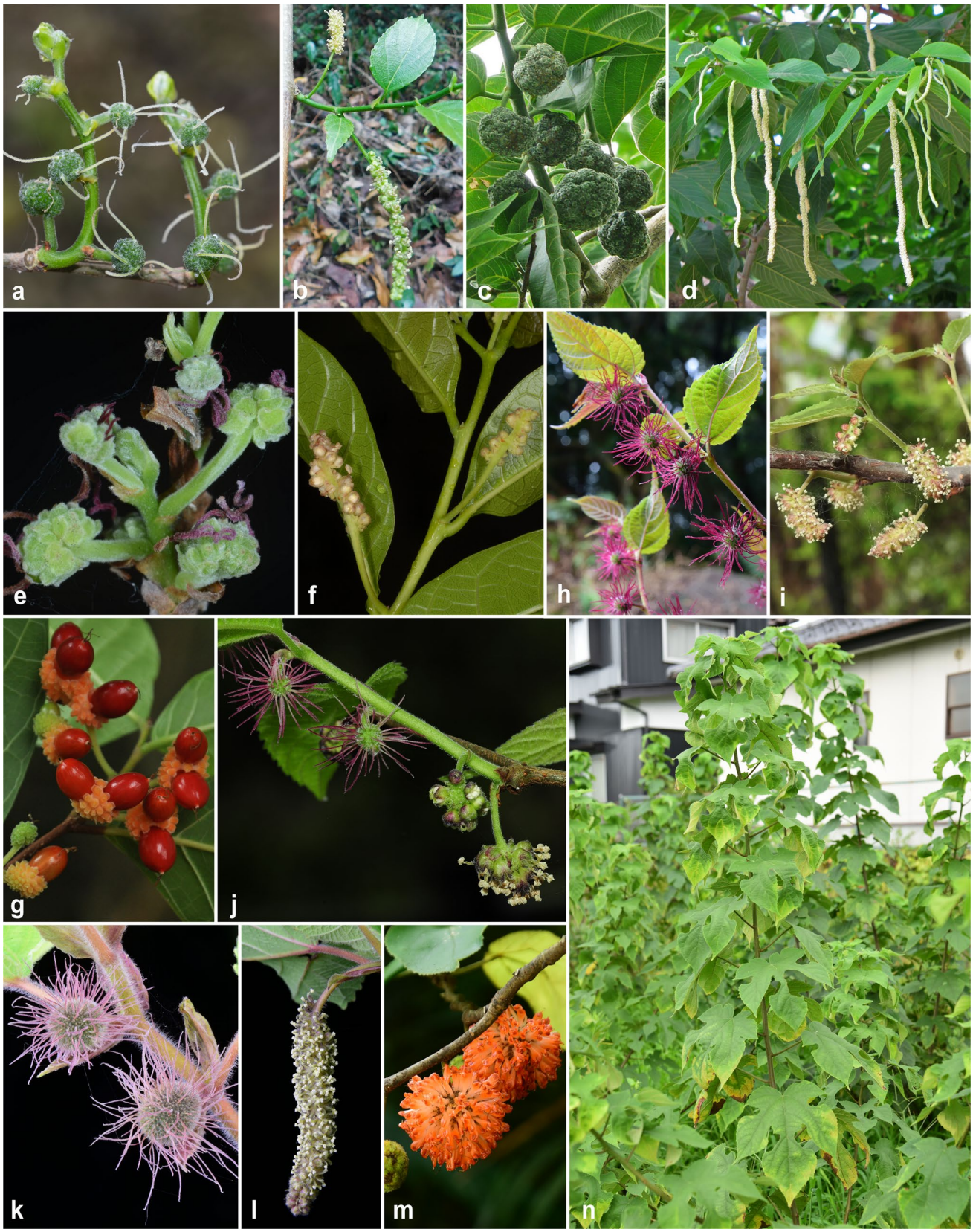
2 Department of Biology, Washington University, St. Louis, MO, USA

3 Department of Biological Sciences, National Sun Yat- sen University, Kaohsiung, Taiwan

4 The Center for Academic Resources and Archives/Botanical Gardens, Tohoku University, Sendai, Miyagi, Japan

5 Department of Biological Science and Institute of Natural Sciences, Daegu University, Gyungsan, Gyungbuk, South Korea

6 Department of Botany, National Museum of Nature and Science, Tsukuba, Ibaraki, Japan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◂**Fig. 1** Morphology of *Allaeanthus*, *Malaisia*, and *Broussonetia*: (**a**) *A. kurzii*, pistillate inflorescences, (**b**) *A. kurzii*, staminate inflorescences, (**c**) *A. luzonicus*, pistillate inflorescences, (**d**) *A. luzonicus*, staminate inflorescences, (**e**) *M. scandens*, pistillate inflorescences, (**f**) *M. scandens*, staminate inflorescences, (**g**) *M. scandens*, syncarps, (**h**) *B. kaempferi*, pistillate inflorescences, (**i**) *B. kaempferi*, staminate inflorescences, (**j**) *B. monoica*, pistillate (left two) and staminate (right two) inflorescences, (**k**) *B. papyrifera*, pistillate inflorescences, (**l**) *B. papyrifera*, staminate inflorescence, (**m**) *B. papyrifera*, syncarps, (**n**) *kōzo* (*B.* × *kazinoki*) plantation in Shiroishi, Miyagi, Japan. **a**, **b**: courtesy of Preecha Karaket; **c**, **i**: courtesy of Danilo N. Tandang; **d**: photo by Forest & Kim Starr/CC BY 3.0 US; **e**, **f**: courtesy of Pi-Fong Lu; **j**, **k**, **l**, **m**, **n**: photos by K.-F. Chung; **i**: Alan Kwok/Ada Tai/CC BY-NC 4.0

and *daknamu*, the taxonomy of *Broussonetia* first needs to be clarified. However, Corner (1962)'s long-held generic concept of *Broussonetia* (i.e., *Broussonetia s.l.*) that comprised seven species in two sections (i.e., Sect. *Allaeanthus* and Sect. *Broussonetia*) was recently challenged (Chung et al. 2017). This article addresses these issues using phylogenomic and phylogeographic approaches.

Corner (1962)'s *Broussonetia* L'Hér. ex Vent. sect. *Allaeanthus* (Thwaites) Corner included four species distributed in Madagascar [*B. greveana* (Baillon) C.C.Berg], Sri Lanka [*B. zeylanica* (Thwaites) Corner], Indochina [*B. kurzii* (Hook.f.) Corner; Fig. 1a, b], and the Philippines [*B. luzonica* (Blanco) Bureau; Fig. 1c, d]. All four species have economic and cultural importance, with the tough-fibered *B. zeylanica* historically used for papermaking in Sri Lanka (Thwaites 1854), *B. luzonica* and *B. kurzii* used as culinary ingredients (LaFrankie 2010; Tangkanakul et al. 2006), and *B. greveana* sought after for its valuable wood (Louppe 2008). *Broussonetia* sect. *Broussonetia* comprised three species, *B. kaempferi* Siebold (Fig. 1 h, i), *B. monoica* Hance (Fig. 1j), and *B. papyrifera* (L.) L'Hér. ex Vent. (Fig. 1k–m) distributed in East Asia and northern Indochina (Chung et al. 2017). All three species contain long fibers suitable for papermaking (Chûjô 1950; Mizumura et al. 2017). In addition to papermaking, *B. papyrifera* is also the prime material for making bark cloth by the Austronesian-speaking peoples in the Pacific (Chang et al. 2015; Penailillo et al. 2016) and known for its medicinal and nutritional properties and fast-growing habit (Peng and Shen 2018). Consequently, this fibrous tree has been widely translocated since prehistorical times (Chang et al. 2015) and become an invasive tree species worldwide (Chung et al. 2017).

Despite being a small genus, the extensive intraspecific morphological variation (Fig. 2), lack of type designation (Akiyama et al. 2013; Chung et al. 2017), and long history of cultivation, selection and hybrid breeding (Peng and Shen 2018; Peng et al. 2014) has led to much confusion and controversy in the taxonomy of the multi-purpose *Broussonetia s.l.*. The rationale used by Corner (1962) for reducing *Allaeanthus* Thwaites to a section of *Broussonetia* was: "*There are no major differences between these sections, which are not generically distinct*." Although Sections *Allaeanthus* and *Broussonetia* appear to be similar in their capitate, globose pistillate inflorescences and racemose to spicate staminate inflorescences (Corner 1962), the former is a tropical group, while the latter is mainly distributed in temperate to subtropical regions (Chung et al. 2017). Morphologically, reproductive organs of the two sections are also different. The slender catkins characterized by Sect. *Allaeanthus* (Fig. 1b, d) can be distinguished from the cylindric (Fig. 1 h, m) or globose (Fig. 1j) staminate inflorescences of Sect. *Broussonetia*. Styles of Sect. *Allaeanthus* are white (Fig. 1a), while those in Sect. *Broussonetia* are pinkish to purple when mature (Fig. 1h, j, k). Corner (1962) also noted that drupes of Sect. *Broussonetia* are stipitate within the sessile perianth, while drupes of Sect. *Allaeanthus* are sessile. The two sections can also be distinguished by the fruit color, with green to yellowish in Sect. *Allaeanthus* (Fig. 1c) and orange-red in Sect. *Broussonetia* (Fig. 1 m).

Although Corner (1962)'s circumscription of *Broussonetia* has been widely followed (e.g., Berg et al. 2006; Chang et al. 1998; LaFrankie 2010; Rohwer 1993; Wunderlin 1997; Yun and Kim 2009; Zhou and Gilbert 2003), whether the genus should be placed in Tribe Artocarpeae (Corner 1962), Tribe Broussonetieae (Chang et al. 1998), or Tribe Moreae (Berg et al. 2006; Rohwer 1993) has been disputed. Based on the plastid *ndhF* and nuclear ribosomal 26 S subunit sequences, Clement and Weiblen (2009) showed that molecular data did not support previous morphology-based classifications of Moraceae. In their revised tribal classification, *Broussonetia* was placed in Tribe Dorstenieae (Clement and Weiblen 2009). Within Tribe Dorstenieae, molecular data also showed that *B. papyrifera* is sister to the lianaceous *Trophis scandens* (Lour.) Hook. & Arn. [*Trophis* sect. *Malaisia* (Blanco) C.C.Berg], while other sampled species of *Trophis* form a clade sister to *Morus* L. (Clement and Weiblen 2009). To rectify the polyphyletic *Trophis*, Clement and Weiblen (2009) reinstated the generic status of *Malaisia* Blanco [i.e., *Malaisia scandens* (Lour.) Planch.; Fig. 1e–g]. Both the tribal classification of *Broussonetia* in Dorstenieae and its sister group relationship with *Malaisia* are also consistent with recent studies using Hyb-Seq data (Gardner et al. 2021; Zerega and Gardner 2019). Meanwhile, based on a nearly complete taxon sampling, Chung et al. (2017) further showed that, while both Sect. *Allaeanthus* and Sect. *Broussonetia* are monophyletic, *Broussonetia s.l.* is paraphyletic, with Sect. *Broussonetia* sister to *M. scandens* before the former joining Sect. *Allaeanthus* to form a monophyletic *Broussonetia s.l.*. To rectify the paraphyletic *Broussonetia s.l.*, Chung et al. (2017) reinstated *Allaeanthus* to maintain the generic status of *Malaisia*, avoiding nomenclatural changes that would have also generated an expanded *Broussonetia* without obvious diagnostic characters. The

Fig. 2 Leaf shape variation in *Broussonetia kaempferi* (**a**), *B. monoica* (**b**), and *B. papyrifera* (**c**). ad: adaxial view; ab: abaxial view

three genera can be identified by the following key (Berg et al. 2006; Corner 1962):

1. Syncarps globose, thickly set with slender stalked bracts of various shapes more or less covering the drupes; seeds 2–3 mm long; endocarps crustaceous to ligneous; cotyledons equal, conduplicate to plane.....2
1. Syncarps with few strongly projecting drupes each invested by the utricular perianth, the bracts short; seeds 6–7 mm long; endocarps membranous; cotyledons very unequal, the large one thickly fleshy and folded..... .....*Malaisia*
2. Styles white; exocarps green to yellowish, sessile; seeds compressed, smooth, ligneous, the keel not double .....*Allaeanthus*
2. Style pinkish to purple; exocarps orange-red, stipitate within the sessile perianth; seeds slightly compressed, papillate-asperate, crustaceous, the keel double at the base .....*Broussonetia*

Although *Malaisia* has never been synonymized under *Broussonetia*, Corner (1962) placed the two genera in the same couplet of the taxonomic key to the Asian genera of Artocarpeae, implicitly suggesting their morphological similarity. Nevertheless, the female florets of *M. scandens* form contracted capitate to short-spicate inflorescences (Fig. 1e) which has caused much difficulty in placing it within Moraceae (Corner 1962). Geographically, *M. scandens* is distributed from Taiwan and southern China to throughout SE Asia and the western Pacific islands (Berg et al. 2006), partially overlapping with the Southeast Asian *Allaeanthus* and the mainly East Asian *Broussonetia* (Chung et al. 2017). The unique morphology of *Malaisia* and its overlapping geographic distribution with *Allaeanthus* and *Broussonetia* stimulated the current study to further test Chung et al. (2017)'s recent taxonomic treatment using phylogenomic data.

By resolving phylogenetic relationships and taxonomic controversies of *Allaeanthus*, *Broussonetia*, and *Malaisia* (i.e., the *Broussonetia* alliance), this study also aims to track the origin of the hybrid paper mulberry trees *kōzo* and *daknamu* used for traditional papermaking in Japan and Korea, respectively. Papermaking technology was introduced from China to Korea during the 3rd to 6th century (Song and Munn 2004; Yun and Kim 2009), primarily using the native *Broussonetia papyrifera* (Jeong 2015). According

to *Nihon Shoki* (Chronicles of Japan), papermaking, as well as *B. papyrifera*, was introduced from Korea to Japan in 610 AD by the Korean monk Dam Jing (Kitamura and Murata 1980; Mizumura et al. 2017; Song and Munn 2004), though there is documentation that papermaking was introduced earlier to Japan by the Japanese Buddhist monk Dōkyō (Hunter 1978).

In Japan, *Broussonetia* is called *kōzo zoku* (*zoku* means 'genus' in Japanese) and the name '*kōzo*' has long been applied to all *Broussonetia* species used by craftspeople for making *washi* and growers of these trees (Chûjô 1950). Taxonomically, early Japanese botanists regarded *B. kazinoki* Siebold as the correct scientific name for *kōzo* (Chûjô 1950; Kitamura and Okamoto 1962; Ohwi 1965). However, such definition was challenged after Kitamura and Murata (1980) studied the name *hime-kōzo* ('*hime*' means small in Japanese) recorded in two Japanese classic herbal books, *Honzō Kōmoku Keimō* (Dictated Compendium of Materia Medica) by Ranzan Ono and *Sōmoku-Zusetsu* (An Iconography of Herbaceous and Woody Plants of Japan) by Yokusai Iinuma. Kitamura and Murata (1980) concluded that *B. kazinoki* should be the correct name for *hime-kōzo*, a monoecious (Fig. 1j) shrub with leaning branches (Chung et al. 2017) and variable leaf shapes (Fig. 2b) distributed in Japan (Okamoto 2006), Korea (Yun and Kim 2009), China (Zhou and Gilbert 2003), and Taiwan (Liao 1996). In ancient Japan, *hime-kōzo* was simply called *kōzo* and used for papermaking (Kitamura and Okamoto 1962). By the Edo Period (1603–1868 AD), however, a hybrid became the preferred material for making *washi* and gradually the name *kōzo* (Fig. 1n) was applied specifically to the hybrid (Kitamura and Murata 1980), inevitably resulting in subsequent and widespread confusion regarding its definition. By distinguishing *hime-kōzo* from the broadly defined *kōzo* (*sensu* Chûjô 1950, Kitamura and Okamoto 1962; Ohwi 1965), Kitamura and Murata (1980) applied *kōzo* specifically to those plants cultivated for making *washi* and regarded *kōzo* as the hybrid between *B. kazinoki* and *B. papyrifera*, though no evidence was provided.

Although Kitamura and Murata (1980)'s concept of Japanese *Broussonetia* had been immediately and widely followed (e.g., Mizumura et al. 2017; Okamoto 2006; Yamazaki 1989), the identity of *B. kazinoki* remained confusing. In Japan, '*kazinoki*' has been the common name for *B. papyrifera* since ancient times, and rightfully cited in Siebold (1830) when the name *B. kazinoki* was first proposed (though the name was not validly published until 1846; Akiyama et al. 2013). However, because the modern type concept had not yet been developed in the 19th Century (Fosberg 1992), neither Siebold (1830) nor the subsequent work (i.e., Siebold and Zuccarini 1846) specified specimens that were essential to resolve the puzzling identity of *B. kazinoki*. To stabilize the taxonomy of Japanese plant names, "Siebold collection of Japanese plants" was investigated and names described by Siebold (and Zuccarini) were lectotypified (Akiyama et al. 2013). As a result, a specimen from Siebold's collection at Botanische Staatssammlung München (i.e., M0120984) was selected as the lectotype of *Broussonetia kazinoki* Siebold, with the comment that "*This specimen is not the real Japanese "Kazinoki", i.e., Broussonetia papyrifera (L.) Vent., but Japanese kōzo*" (Akiyama et al. 2013). Based on their lectotypification, Ohba and Akiyama (2014) revised *Broussonetia* of Japan. Because *B. kazinoki* was taken by the nothospecies *kōzo*, *B. monoica*, a name long synonymized under *B. kazinoki* (e.g., Zhou and Gilbert 2003), succeeded as the earliest validly published and correct name for *hime-kōzo* (Ohba and Akiyama 2014). Subsequently, the multiplication sign was added (i.e., *B.* × *kazinoki*) by Chung et al. (2017) according to Article H.3 of the *Code* (McNeill et al. 2012).

In Korea, *daknamu*, also regarded as a hybrid species (Kim et al. 1992; Yun and Kim 2009), has been the favored material and clonally propagated for making *hanji* for centuries (Won 2019). Although widely cultivated in Korea since ancient times, *daknamu* was not botanically described until Yun and Kim (2009) published the name *Broussonetia* × *hanjiana* M.Kim. However, while *kōzo* has long been considered as a cultivated plant in Japan, Yun and Kim (2009) regarded *B.* × *hanjiana* a natural hybrid because its type specimen was collected in the pristine evergreen broadleaved forest of Gageo Island, the only place in Korea where both *B. monoica* and *B. papyrifera* co-occur naturally with *B.* × *hanjiana*. Because *kōzo* and *daknamu* are both the hybrid between *B. monoica* and *B. papyrifera*, Chung et al. (2017) synonymized *B.* × *hanjiana* under *B.* × *kazinoki*. Before the introduction of machine-made paper, both *kōzo* and *daknamu* were extensively cultivated and clonally propagated for a wide range of paper products in Japan (Mizumura et al. 2017) and Korea (Yun and Kim 2009). If *kōzo* had originated in Japan, the hybridization must have occurred after the introduction of *Broussonetia papyrifera* in the 7th Century (Won 2019). Alternatively, *kōzo* might derive from *daknamu*, given that both *B. monoica* and *B. papyrifera* are native to Korea (Yun and Kim 2009). Using plastid and nuclear markers, Won (2019) confirmed that *daknamu* is indeed a hybrid between *B. monoica* (♀) and *B. papyrifera* (♂). Additionally, a likely incidence of back-cross and introgression of *daknamu* (♂) to *B. monoica* (♀) was also detected (Won 2019). However, no studies have yet investigated these two hybrids jointly.

This study aims to settle taxonomic disputes of the *Broussonetia* alliance and track origins of *kōzo* and *daknamu*. We assembled full plastid genome (plastome) sequences of the alliance which so far are only available for *B. papyrifera* (KX828844), "*B. kazinoki*" (MH223642 and MW465960), and the synthetic hybrid paper mulberry "*B. kazinoki* × *B.*

papyrifera" (Xu et al. 2018). Additionally, nuclear ribosomal DNA sequences were assembled to test for the congruence between plastid and nuclear genomes. Based on phylogenomic relationships, Chung et al. (2017)'s taxonomic proposition is tested and character (e.g., gene loss and reproductive syndrome) state evolution is discussed. Based on the hypervariable region of plastome sequences identified in this study, we also address the following questions: Do *kōzo* and *daknamu* each have single or multiple origins? Do they share haplotypes? Have *kōzo* and *daknamu* experienced bottlenecks from the hybridization? Does *kōzo* or *daknamu* have higher haplotype diversity? If *kōzo* was introduced from Korea to Japan, as historical documents, had *kōzo* experienced a second bottleneck and showed reduced haplotype diversity?

Materials and methods

Plastome and nrDNA assembly

Based on Chung et al. (2017), we sampled six species of the *Broussonetia* alliance, including all three species of *Broussonetia*, two of the four species of *Allaeanthus*, and the monotypic *Malaisia scandens*. All voucher specimens were deposited at the Herbarium, Biodiversity Research Center, Academia Sinica, Taipei (HAST). Genomic DNA was extracted using CTAB method (Doyle 1991) and further purified by Monarch® PCR & DNA Cleanup Kit. Purified genomic DNAs were sent to the Genomic Core Lab of Institute of Molecular Biology, Academia Sinica for library preparation using TruSeq® Nano DNA Library Prep Kit (Illumina Inc., San Diego, CA, USA). The DNA was fragmented into target 350 bp by M220 Focused-ultrasonicator™ (Covaris, Woburn, MA, USA) and ligated to TruSeq Single Index adapters. Sequencing was done by MiSeq (Illumina Inc., San Diego, CA, USA) using Illumina Reagent Kit v3 (600 cycle) with pair-end mode, read length = 2×300 bp, and insert size = 350–550 bp (determined by Bioanalyzer High Sensitivity DNA Analysis). The samples were multiplexed with other *Broussonetia* samples not included in the current study. Plastomes were assembled using Geneious software version 11 (Kearse et al. 2012) with the following procedure: The adapter and barcode sequences were first removed. Then, we removed the low-quality bases at the start and the end of the reads by the modified Mott algorithm in Geneious (Error probability limit = 0.05). Then, trimmed reads were mapped to the plastome of *Morus notabilis* C.K.Schneid. (KP939360) using the "Map to Reference" function in Geneious with "Medium-Low Sensitivity" and default settings. Regions that contained indel(s) between the focal sample and the reference genome might not be correctly assembled by the reference-based method and could exhibit conflicts in the mapping results. Those regions were corrected by extending the reliably assembled regions into problematic parts and 'iteratively mapping' the reads to the contigs to obviate the effect of the reference genome. To obtain a complete plastome, the remaining gaps and uncertain regions that could not be resolved by iterative mapping were filled by Sanger sequencing using the primers designed from the reference genome and PCR condition listed in Table S1. Meanwhile, junctions between large single copy (LSC), small single copy (SSC), and inverted repeats (IRs) were verified by PCR. Nuclear ribosomal DNA (nrDNA) sequences, spanning across the partial external transcribed spacer (ETS), 18 S rRNA gene, internal transcribed spacer (ITS) 1, 5.8 S rRNA gene, ITS2, 26 S rRNA gene, and partial nontranscribed spacer (NTS) of the six species of *Broussonetia* alliance were assembled by retrieving the raw reads and mapping to the published nrDNA sequences of *Ficus tikoua* Bureau. (JF317367, JF317386, and EU091641) using the "Map to Reference" function of Geneious with "Medium-Low Sensitivity" and default settings.

Genome annotation and comparison

The newly assembled plastomes were annotated based on seven published plastomes of urticalean rosids (i.e., Sytsma et al. 2002), including Moraceae [*Broussonetia papyrifera* (KX828844), *Ficus carica* L. (NC035237; Rabah et al. 2017), and *Morus notabilis* (KP939360; Chen et al. 2016)], Urticaceae [*Debregeasia saeneb* (Forssk.) Hepper & Wood (KY419997; Zhang et al. 2017)], Cannabaceae [*Humulus lupulus* L. (KT266264; Vergara et al. 2016) and *Cannabis sativa* (KY084475; Vergara et al. 2016)], and Ulmaceae [*Ulmus pumila* L. (KY244086; Zuo et al. 2017)], using the transfer annotation feature in Geneious (similarity = 65%). To further verify the identified tRNAs, the structures and anti-codons of putative tRNAs were checked in tRNAscan-SE 2.0 (Lowe and Chan 2016). The genome maps were visualized by OrganellarGenomeDraw (Lohse et al. 2013). The numbers of variable sites were calculated using PAUP* version 4.0 (Swofford 2002). To detect genome rearrangement, multiple genome alignment of the 13 urticalean rosids plastomes was conducted using Mauve version 1.1.3 (Darling et al. 2004) launched in Geneious.

Sliding window analysis of plastomes

To detect evolutionary hotspots in the plastomes for phylogeographic and phylogenetic studies, the nucleotide diversity (π) values were calculated based on two datasets using sliding window analysis. The first dataset comprised the six newly assembled plastomes of the *Broussonetia* alliance, aiming to find high divergence hotspots for recent speciation and phylogeographic studies. The second dataset

comprised 13 plastomes of the urticalean rosids for family level phylogenetic studies. Plastome sequences were aligned using MAFFT (Katoh and Standley 2013). The sliding window analysis was performed using PopGenome R package (Pfeifer et al. 2014) to calculate π (Nei and Li 1979) for the window length of 800 bp and step size of 400 bp.

Phylogenomic analyses

A preliminary phylogenomic analysis of the urticalean rosids were conducted using 16 plastomes (Fig. S1). Because MW465960 ("*Broussonetia kazinoki*"), MF496038 ("*Broussonetia kazinoki* × *Broussonetia papyrifera*"), and MH223642 ("*Broussonetia kazinoki*") are almost identical and appear to be conspecific with *B. monoica* (MH189567) that was newly assembled in the current study (Fig. S1), the former three plastomes were not included in our final analysis. Phylogenomic relationships of the remaining 13 plastomes and eight nrDNA sequences of urticalean rosids were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) methods. Plastome sequences were aligned using MAFFT version 7 with default parameters (Katoh and Standley 2013). For plastome sequences, phylogenomic relationships were reconstructed based on LSC, SSC and a single IR. The nucleotide substitution rates and distribution shapes were estimated by bModeltest (Bouckaert and Drummond 2017), with the model GTR+Γ selected for all partitions and all analyses. The concatenated plastid data were divided into four partitions: protein-coding sequences, tRNAs, rRNAs, and other non-coding sequences. Model parameters were unlinked across partitions. The nrDNA phylogeny was reconstructed based on ITS1, ITS2, and rRNA (18 S, 5.8 S, and 26 S) regions. Two partitions (i.e., ITS regions and rRNA regions) with unlinked model parameters were applied. ML analyses were performed using RAxML version 8 (Stamatakis 2014). Twenty heuristic searches with 1,000 bootstrap replicates were conducted, and the congruence was checked manually. BI tree was reconstructed by BEAST2 (Bouckaert et al. 2014) with 100,000,000 generations and sampling every 5,000 generations. The effective sample sizes (ESS) of final posterior probability were confirmed in Tracer version 1.7 (Rambaut et al. 2018). If the BI tree topology was congruous with the ML tree, the posterior probabilities greater than 0.80 were mapped to the backbone of the ML tree at corresponding nodes.

Phylogeographic analyses

Based on the sliding window analysis, *matK-rps16*, *rps16-psbK*, *rps4-ndhJ*, *ndhF-rpl32*, *rpl32-ccsA*, and *ycf1* were identified as the most variable regions suitable for phylogeographic analyses (see Results). However, because our samples included substantial herbarium materials (Table S2) with compromised DNA quality, we first tested the suitability of the six potential markers for easiness of PCR amplification and Sanger sequencing. Consequently, *ndhF-rpl32* intergenic spacer (IGS), which was also adopted by Won (2019) based on Chang et al. (2015), was chosen for phylogeographic analyses.

In combination with Won (2019)'s samples, a total of 81 samples were sequenced, including 9 *kōzo* from 9 *washi* workshops of 3 prefectures of Japan, 9 *daknam*u from 7 provinces of South Korea, and 63 *B. monoica* from China (14 samples from 7 provinces), Japan (32 samples from 17 prefectures), South Korea (4 samples from 3 provinces), and Taiwan (13 samples from 7 counties). Table S2 details the collecting information of the 81 accessions.

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol. Conditions for PCR amplification and sequencing were detailed in Won (2019) using primers listed in Table S3. Sequences were assembled in Geneious software version 11 (Kearse et al. 2012). The assembled sequences were aligned by MAFFT version 7 (Katoh and Standley 2013) with default parameters. The haplotype network was reconstructed and visualized using the R package pegas (Paradis 2010). Because pegas cannot handle indel and single nucleotide polymorphism (SNP) at the same time, two long indels (9-bp and 77-bp) were replaced with SNPs to maximize sequence information. Gaps, ambiguous nucleotides, mononucleotide repeats, and microsatellite-like sequence variation at the junctions of the 77-bp indel (Fig. S2) were ignored due to uncertainty of objective alignment. The most parsimonious links in the haplotype network were determined by the default settings in pegas following the algorithm of Templeton et al. (1992). The sequences of unique haplotypes are available in Online Resource 3. The distribution map of haplotypes was drawn using the R package ggplot2. The nucleotide diversity, haplotype diversity, analysis of molecular variance (AMOVA), and pairwise G_{ST} were calculated in R package pegas and MMOD (Winter 2012). The source code is available in Online Resource 4.

Results

Characteristics of plastomes

The six newly assembled plastomes display the typical circular, quadripartite structure of angiosperms (Kwon et al. 2020; Ruhlman and Jansen 2021), composed of an LSC, an SSC, and a pair of IRs (Fig. 3). For assembly quality (Table 1), 1,379,002 (*Broussonetia papyrifera*) to 2,208,733 (*Allaeanthus kurzii*) trimmed reads were mapped to the final plastomes, with the mean average coverage for each base pair ranging between 171.4 ± 58.7 (*Malaisia scandens*) and

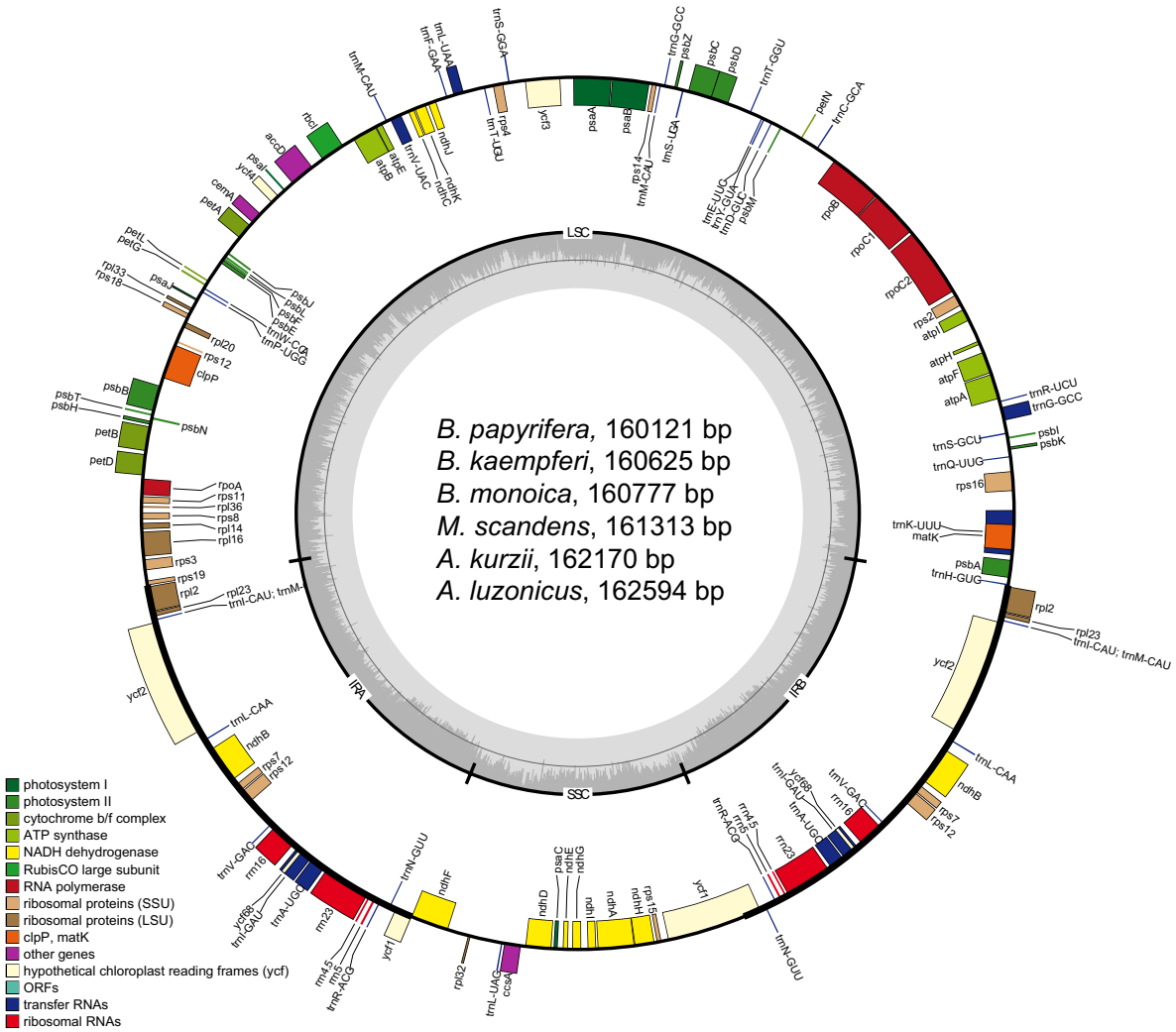

Fig. 3 Gene map of the plastomes of the *Broussonetia* alliance. The genes drawn outside of the circle are transcribed clockwise, while those insides are counterclockwise. Small single copy (SSC), large single copy (LSC), and inverted repeats (IRa and IRb) are indicated

566.5 ± 153.9 (*A. kurzii*). The plastomes of *Broussonetia* (*B. kaempferi*: 160,625 bp; *B. monoica*: 160,777 bp; *B. papyrifera*: 160,121 bp) are slightly smaller than the plastomes of *Malaisia* (*M. scandens*: 161,313 bp) and *Allaeanthus* (*A. kurzii*: 162,170 bp; *A. luzonicus*: 162,594 bp). Gene contents of the six plastomes of the *Broussonetia* alliance are identical, composed of 111 unique genes including 4 rRNA genes, 30 tRNA genes, and 77 protein-coding genes (Table S4). Among the 30 unique tRNA genes, the sequence of the *trnM*-CAU gene in the LSC differs from the *trnM*-CAU gene in IRs and thus they are regarded as different. Introns were found in 18 genes, including 12 protein-coding genes and 6 tRNA genes. Among the 111 unique genes, 17 genes are completely duplicated in IRs. In addition, the trans-splicing *rps12* gene is partially duplicated (2nd and 3rd exon) in IRs. Based on plastome sequence alignment using Mauve, rearrangements were not detected among the six plastomes nor in the other urticalean rosids sampled in the present study (Fig. S3). Comparison of 12 plastomes of urticalean rosids also shows that the IR boundaries are highly conserved within the *Broussonetia* alliance, with a slight IR contraction in the LSC-IRA boundary in *Malaisia* and an expansion of ca. 600 bp in the *ycf1* gene in *(A) luzonicus* in the SSC-IRB boundary (Fig. 4). However, as shown in Fig. 5, a premature stop codon was identified in the *rpl22* gene of plastomes of *(B) papyrifera*, *Malaisia scandens*, *Allaeanthus kurzii*, and *(A) luzonicus*, indicating that the gene has pseudogenized. Additionally, this locus was completely lost in *(B) monoica* and *B. kaempferi*.

Evolutionary hotspots in plastomes

The *Broussonetia* alliance dataset shows that the nucleotide diversity (π) ranges from 0 to 0.04132 with a mean ±

Table 1 GenBank accession numbers, HAST ID, and main features of the six newly assembled plastomes and nrDNA sequences

	HAST ID	Plastome								nrDNA			
		NCBI	Length (bp)	LSC (bp)	SSC (bp)	IR (bp)	% GC	av. Cov. (×)	# reads	NCBI	Length (bp)	av. Cov. (×)	# reads
Broussonetia kaempferi	145520	MH189569	160,625	88,956	19,965	25,852	35.6	432.8 ± 110.4	2,017,983	MH135783	5,866	1844.1 ± 831.7	43,383
Broussonetia monoica	145521	MH189567	160,777	88,993	20,076	25,854	35.8	358.1 ± 78.4	1,506,872	MH135780	5,866	785.6 ± 376.9	18,868
Broussonetia papyrifera	143675	MH189570	160,121	88,517	19,904	25,850	35.9	450.7 ± 125.8	1,379,002	MH135784	5,850	1218.5 ± 682,1	27,204
Malaisia scandens	143832	MH189568	161,313	89,852	19,883	25,819	35.9	171.4 ± 58.7	1,570,807	MH135781	5,841	1275.8 ± 444.9	26,938
Allaeanthus kurzii	139867	MH118529	162,170	90,174	20,150	25,923	35.7	566.5 ± 153.9	2,208,733	MH135782	5,813	1512.3 ± 169.3	35,402
Allaeanthus luzonicus	142809	MH189566	162,594	89,980	19,560	26,527	35.7	204.7 ± 51.1	1,092,569	MH135779	5,813	447.2 ± 177.3	11,424

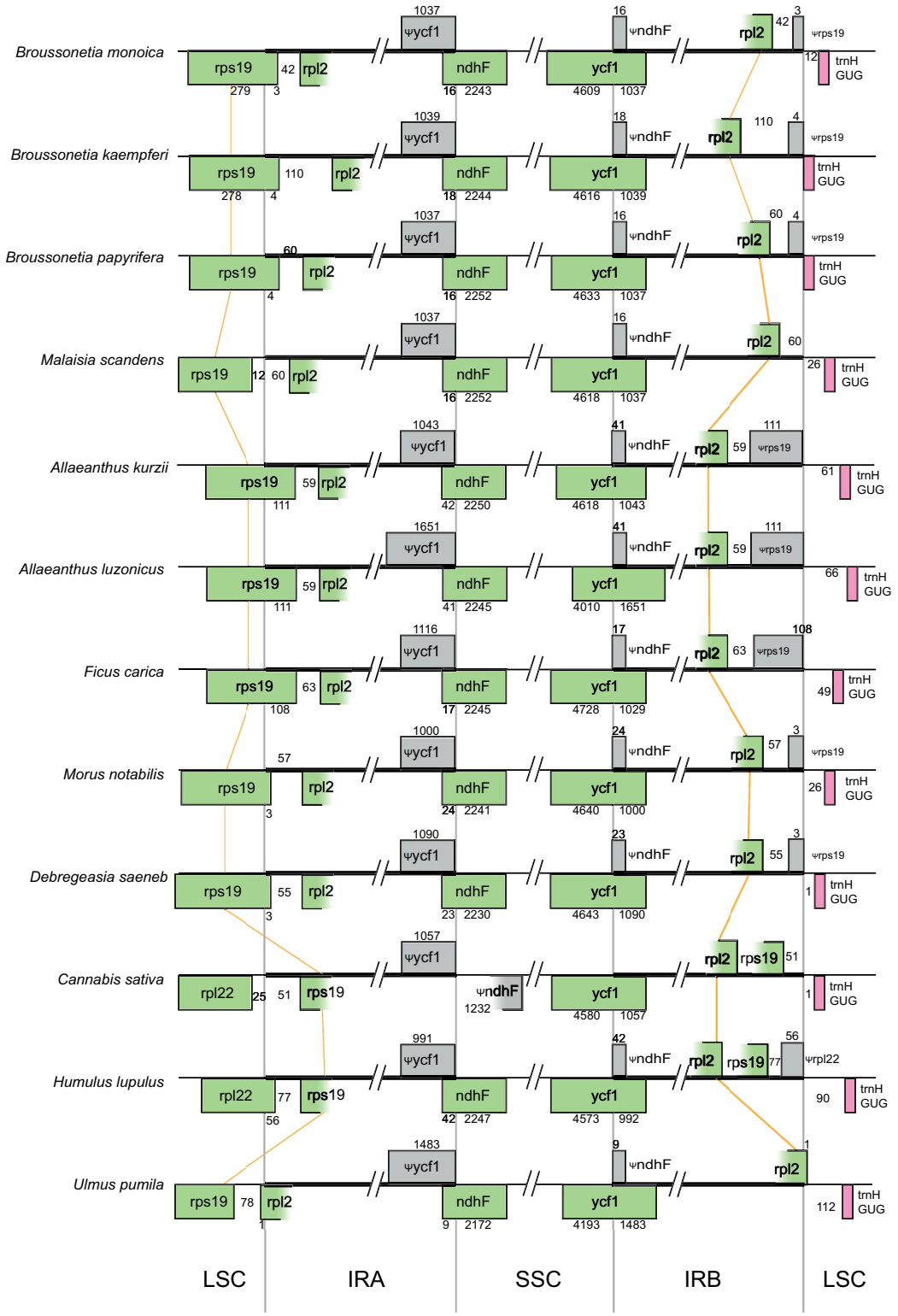

Fig. 4 Comparison of inverted repeat (IR) boundaries of the plastomes of 12 urticalean rosids

standard deviation (SD) of 0.01287 ± 0.00906. Six highly variable regions ($\pi > 0.03$) including *matK-rps16*, *rps16-psbK*, *rps4-ndhJ*, *ndhF-rpl32*, *rpl32-ccsA*, and *ycf1* were identified (Fig. S4a). Three of these regions lie in the LSC region and three in the SSC region. Five out of six regions are non-coding regions located within intergenic spacers. In

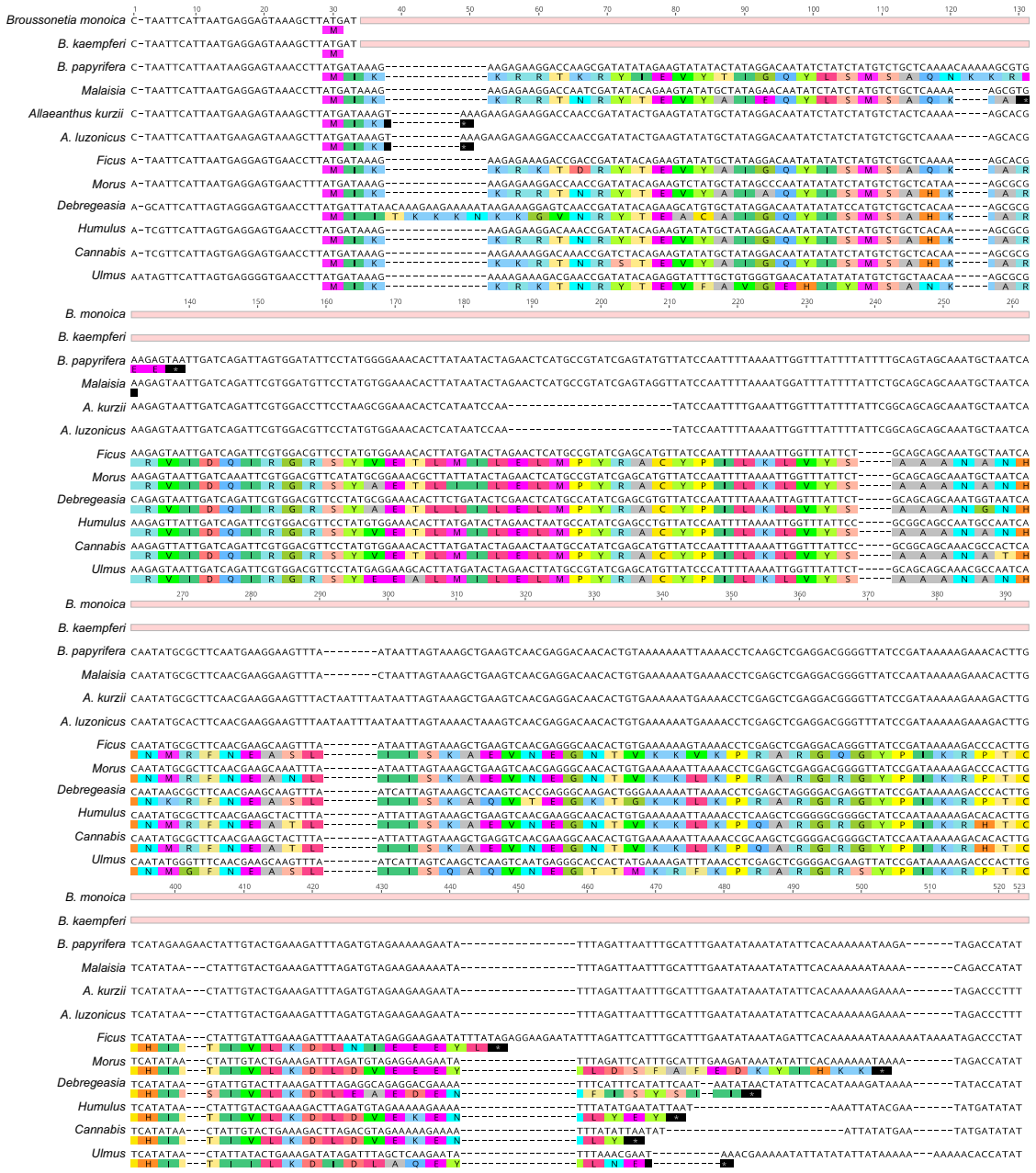

Fig. 5 Alignment of the *rpl22* gene and protein sequences of the 12 urticalean rosids

the urticalean rosids dataset, the nucleotide diversity ranges from 0.00116 to 0.06988 with a mean ± SD of 0.02345 ± 0.01328. The identified hotspot regions [i.e., *matK*, *rpoC2*, *ndhF*, and *ycf1* ($\pi > 0.045$)] are all protein-coding genes (Fig. S4b). In both datasets, π is much lower in IR regions than LSC and SSC.

Phylogenomic analyses

The alignment of the 13 plastomes of urticalean rosids is 151,354 bp (LSC + SSC + a single IR), with 12,424 parsimony-informative sites (8.21%), 16,913 parsimony-uninformative sites (11.17%), and 122,017 constant sites (80.62%). Phylogenetic analyses using both ML and BI methods resolved Moraceae, Urticaceae (*Debregeasia saeneb*), Cannabaceae (*Cannabis sativa* and *Humulus lupulus*), and Ulmaceae (*Ulmus pumila*) forming successively sister and increasingly more inclusive monophyletic groups with full support values (Fig. 6a), congruent with previous studies using Sanger sequencing (Sytsma et al. 2002; Zhang et al. 2011). Within Moraceae, the *Broussonetia* alliance and *Ficus* form a clade sister to *Morus*, also congruent with early

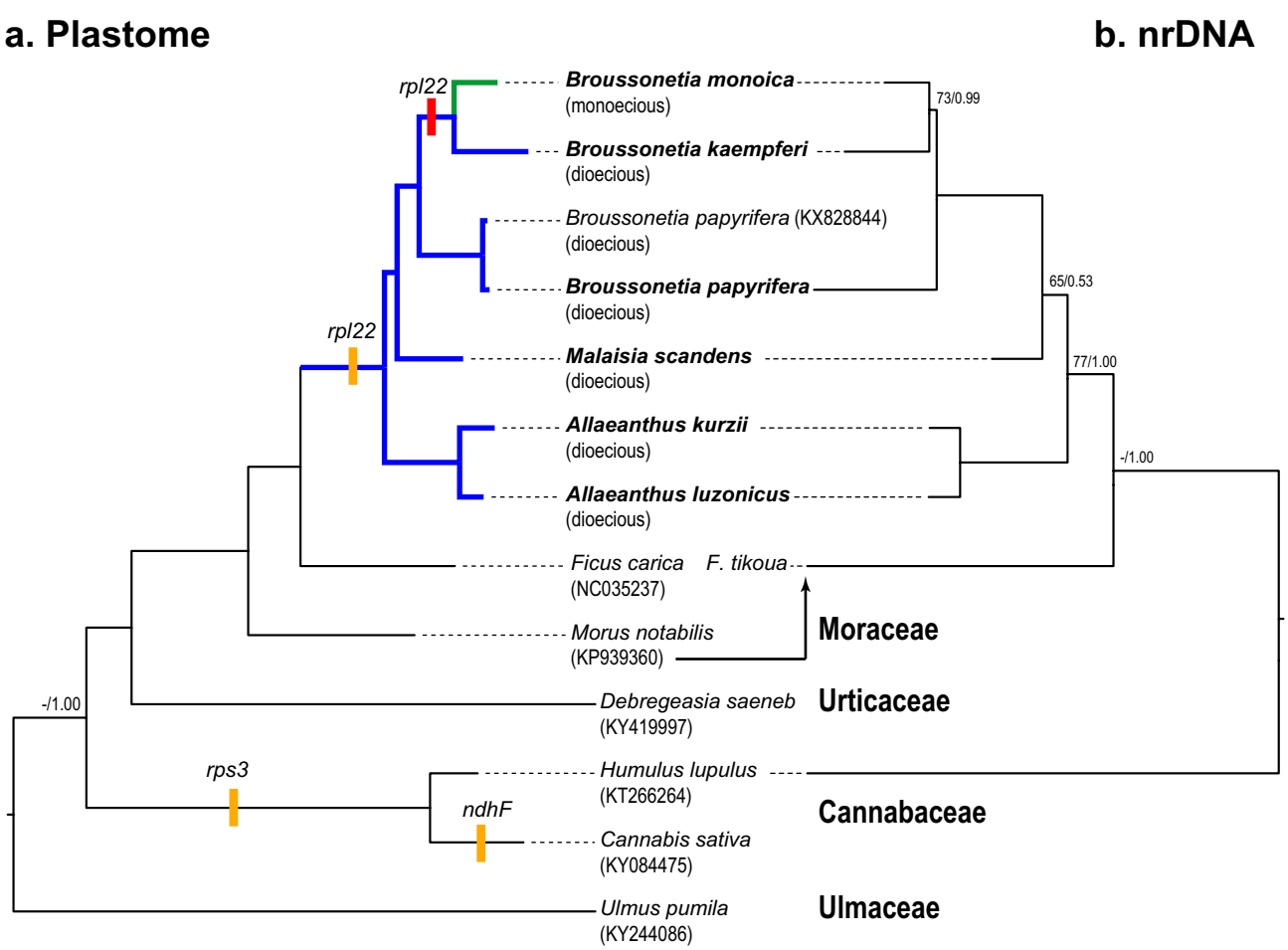

Fig. 6 Comparison of plastome and nrDNA phylogenies. (**a**) Plastome phylogeny of 13 urticalean rosids. Numbers at each node are bootstrap support value/posterior probability. Nodes without number indicates full support (100/1.0). Premature stop codons in genes are indicate as yellow bars. Gene deletions are indicated as red bars. Within the *Broussonetia* alliance, all species are dioecious (blue) except for *B. monoica* (green). (**b**) nrDNA phylogeny of the *Broussonetia* alliance and the out groups. Numbers at each node are bootstrap support value/posterior probability. Nodes without number indicates full support (100/1.0)

studies (Chung et al. 2017; Clement and Weiblen 2009). Within the *Broussonetia* alliance, *Broussonetia* and *Malaisia* form a clade sister to *Allaeanthus*, identical to relationships revealed in Chung et al. (2017). Within *Broussonetia*, *B. kaempferi* and *B. monoica* form a clade sister to *B. papyrifera*, as revealed in Chung et al. (2017).

The alignment of the eight nrDNA is 5,899 bp, with 200 parsimony-informative sites (3.39%), 336 parsimony-uninformative sites (5.70%), and 5,363 constant sites (90.91%). The topology of the nrDNA phylogeny (Fig. 6b) is completely congruent with that of the plastome phylogeny (Fig. 6a), though supporting values at the *Broussonetia* + *Malaisia* clade is low (BS = 65%, PP = 0.53).

Phylogeographic analyses

The network depicting evolutionary relationships among the 16 haplotype is shown in Fig. 7. Based on the calculation of pegas, the 77-bp indel (Fig. S2) appears to have evolved independently between haplotypes cp-1 and cp-9, cp-2 and cp-4, and cp-10 and cp-12 (Fig. 7), likely triggered by the presence of the microsatellite-like sequences (Fig. S2). Of the 81 accessions of *B. monoica* and *B.* × *kazinoki* sampled (Table S2), 16 haplotypes were detected, with two major haplotypes (cp-1 and cp-2) carried by a majority (ca. 63%) of samples (Fig. 7). Of the 16 *ndhF-rpl32* haplotypes, 15 were detected in *B. monoica* ($N = 63$), while only five haplotypes were found in *B.* × *kazinoki* ($N = 18$). Of the 18 accessions of *B.* × *kazinoki* sequenced, five haplotypes were detected in *daknamu* ($N = 9$), while *kōzo* ($N = 9$) carried only cp-1.

Overall, continental populations of China and Korea have higher nucleotide and haplotype diversities than their adjacent island populations of Taiwan and Japan (Table 2). According to AMOVA, haplotype compositions between regions (China, Korea, Japan and Taiwan) are highly

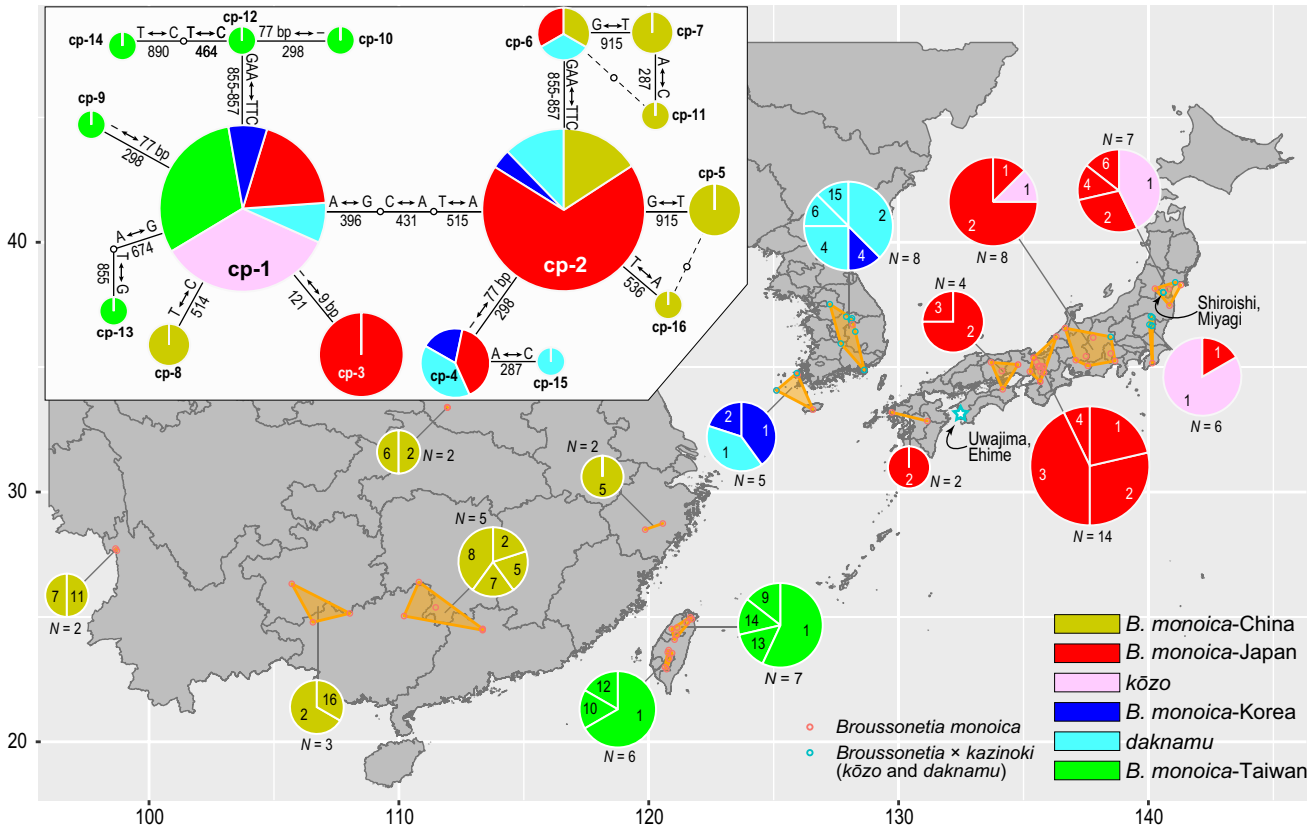

Fig. 7 Distribution of *ndhF-rpl32* haplotypes and haplotype network of *Broussonetia monoica* and *B.* × *kazinoki* (*kōzo* and *daknamu*). The numbers on the network correspond to the haplotype sequence alignment in the Online Resource 3

Table 2 Nucleotide diversity and haplotype diversity of *ndhF-rpl32* intergenic spacer in *Broussonetia monoica* and *B. monoica*

Regions	Nucleotide diversity (π)	Haplotype diversity
CHINA ($N = 14$)	0.003261835	0.879120879
KOREA ($N = 13$)	0.002519487	0.807692308
JAPAN ($N = 41$)	0.001722545	0.535365854
TAIWAN ($N = 13$)	0.001889615	0.628205128
Total ($N = 81$)	0.002651099	0.725000000

Table 3 Analysis of Molecular Variance

	SSD	MSD	df	σ	P value[a]
Between regions	7.926025	2.6420084	3	0.11136	< 0.001***
Within regions	50.073975	0.6503114	77	0.65031	
Total	58.000000	0.7250000	80		

[a]From1000 times permutation bootstrap

Table 4 Pairwise G_{ST} calculated based on Nei (1973)(upper diagonal) and Hedrick (2005) (lower diagonal)

	CHINA	KOREA	JAPAN	TAIWAN
CHINA	–	0.05564099	0.11608003	0.15942529
KOREA	0.5578286	–	0.03524862	0.08095313
JAPAN	0.6619250	0.1947902	–	0.0954929
TAIWAN	1.0000000	0.4819686	0.4011602	–

differentiated (Table 3). However, the high within-region variance suggests the presence of population structure within regions, though a comprehensive sampling is required to give detailed information. Estimates of pairwise G_{ST} (Nei 1973) and G''_{ST} (Hedrick 2005) also indicate the highest level of population differentiation between China and Taiwan, while the lowest values of both G_{ST} and G''_{ST} are found between Korea and Japan (Table 4).

Discussion

Taxonomy of *Broussonetia* alliance

Using the genome skimming approach, complete plastome and nrDNA sequences of six species of the *Broussonetia* alliance were assembled and their phylogenomic relationships were reconstructed. Given the identical relationships between plastome and nrDNA trees among genera of the *Broussonetia* alliance (Fig. 6), our current data clearly show that *Broussonetia s.l.* is paraphyletic, results that are also congruent with those of Chung et al. (2017) and Gardner et al. (2021). To rectify the paraphyly of *Broussonetia s.l.*, *Malaisia* can either be synonymized under *Broussonetia s.l.* or the generic status of Sect. *Allaeanthus* must be resurrected as proposed by Chung et al. (2017). To achieve an objective generic delimitation, Backlund and Bremer (1998), Linder et al. (2010), Heenan and Smissen (2013), and Hsieh et al. (2022) advocated five criteria: (1) prioritizing primary (i.e., family, genus, and species) over secondary ranks (i.e., subgenus, section, etc.), (2) maximizing phylogenetic information and reducing redundancy in a classification, (3) recognizing evolutionarily equivalent (i.e., clade age, phylogenetic distance, and morphology) groups as the same rank, (4) delimiting genus that is morphologically, ecologically, and geographically homogenous, and (5) taking into account the full taxonomic history of the group and minimizing name changes to maintain nomenclatural stability. Although our phylogenetic relationships of the *Broussonetia* alliance is also compatible with an expanded *Broussonetia* that synonymizes both *Allaeanthus* and *Malaisia* as sections, we support Chung et al. (2017)'s proposition because *Malaisia* is morphologically distinct (Corner 1962) and has never been subsumed under *Broussonetia*. Maintaining the generic status of *Malaisia* and resurrecting *Allaeanthus* also prioritizes the primary rank genus over the secondary rank section, maximizes our phylogenomic conclusions (Fig. 6), delimitates three genera that are morphologically (Fig. 1), ecologically, and geographically homogenous, reflects the full taxonomic histories of the three groups, and avoids an unnecessary name change.

Character evolution and origin of *B. monoica*

By mapping gene loss/deletion on the robust and congruent phylogenomic relationship, the *rpl22* gene appears to have been pseudogenized prior to the diversification of the *Broussonetia* alliance and lost completely in the clade composed of *B. kaempferi* and *B. monoica* (Fig. 6). Additionally, our analyses also detected premature stop codons in the *rps3* gene in Cannabaceae and the *ndhF* gene in *Cannabis sativa* (Fig. 6a) not previously known in the family (e.g., Zhang et al. 2018).

The robust and congruent phylogenomic relationships among species of the *Broussonetia* alliance also allow us to track the evolution of reproductive systems in the *Broussonetia* alliance. Except for *B. monoica* which is monoecious (Fig. 1j), all other species of the *Broussonetia* alliance are dioecious (Chung et al. 2017), indicating that the monoecy in *B. monoica* is a derived state (Fig. 6a) and suggesting that dioecy is not an evolutionary dead end (Schaefer and Renner 2010; Zhang et al. 2019) as commonly assumed (Heilbuth 2000). Volz and Renner (2008) surmised that transitions between monoecy and dioecy in Cucurbitaceae might correlate with polyploidy, resulted from hybridization between dioecious diploids and hermaphroditic polyploids. In hindsight, the vegetative morphology of the monoecious *B. monoica*, such as its shrubby habit (Chung et al. 2017), and the highly variable and oblique leaves (Fig. 2b) appear to be intermediate between the lianaceous and oblong-leaved *B. kaempferi* (Fig. 2a) and the arborescent and ovate-leaved (but often divided) *B. papyrifera* (Fig. 2c), suggesting that *B. monoica* could have resulted from hybridization of the former two dioecious species (Ďurkovič et al. 2012; Gil and Kim 2016; Liu et al. 2019; Tamaki et al. 2021). Cytologically, while chromosome numbers of both *B. kaempferi* (Narita and Yosinaga 1955) and *B. papyrifera* (Oginuma and Tobe 1995) are $2n = 26$, Seki (1950) reported $2n = 26$ and 39 for *B. monoica* (as *B. kazinoki*). If *B. monoica* did originate through hybridization between *B. kaempferi* and *B. papyrifera*, the cytological data suggest that *B. monoica* is either a homoploid hybrid or a triploid, supporting Volz and Renner (2008)'s speculation that changes in ploidy level could trigger the transition of sexual system. Under the hybridization scenario, the sister relationship between *B. kaempferi* and *B. monoica* in the plastome tree (Fig. 6a) would also indicate that the former should be the maternal parent of the latter species. The proposition on the hybrid origin of *B. monoica* is currently under investigation using genomic approaches.

Phylogeography of *B. monoica* and origin of *B.* × *kazinoki*

Although the main islands of Japan and Taiwan had been intermittently connected to the Asian Continent to various degrees by land bridges over recurrent glacial periods, the Korea Strait and Taiwan Strait have both been effective geographic barriers for gene flow since the end of last glacial maximum as revealed by phylogeographic studies (Huang 2011; Park et al. 2000; Qiu et al. 2009, 2011). However, while the current study reveals that *B. monoica* displays a marked phylogeographic pattern between populations of China and Taiwan, the low values of both G_{ST} and G''_{ST} and

largely overlapping haplotypes carried by *B. monoica* and *B.* × *kazinoki* of Korea and Japan indicate that the Korean Strait has been a porous barrier for the dispersal of the two species (Table 4; Fig. 7).

Because plastids are maternally inherited in *Broussonetia papyrifera* (Zhang et al. 2003), the hypervariable *ndhF-rpl32* IGS (Fig. S4a) provides an ideal marker for investigating maternal origins of *kōzo* and *daknamu* (*B.* × *kazinoki*). In our analysis, all haplotypes (except for cp-15 which is one mutation away from cp-4) detected in *B.* × *kazinoki* are also carried by *B. monoica* (Won 2019). Although our sampling of both *B. monoica* and *B.* × *kazinoki* is far from comprehensive, the presence of five haplotypes in *kōzo* and *daknamu* clearly shows multiple origins of the hybrid (Londo et al. 2006; Miller and Schaal 2005) and an asymmetrical hybridization (Hamzeh et al. 2007; Zha et al. 2010) between *B. monoica* and *B. papyrifera*.

Despite the limited geographic sampling of both *B. monoica* and *B.* × *kazinoki* in Korea, high nucleotide and haplotype diversities comparable to the Chinese populations were detected (Table 2). Additionally, *daknamu* carries five haplotypes (Fig. 7), indicating recurrent and multiple origins of the hybrid. On the contrary, hybridization between *B. monoica* and *B. papyrifera* has never been reported in China or Taiwan where *B. monoica* and *B. papyrifera* are both common and often sympatrically distributed, except for the synthetic 'hybrid paper mulberry' (i.e., *B. kazinoki* × *B. papyrifera*) bred for the poverty alleviation project in China (Ni et al. 2020; Peng et al. 2014). Even in this 'hybrid paper mulberry', *B. monoica* is the maternal parent (Fig. S1), corroborating our inference that hybridization between *B. monoica* and *B. papyrifera* is possible only when the former species serves as the maternal parent. In Korea, *B. papyrifera* is restricted to islands and coastal areas while *B. monoica* is generally an inland species (Won 2019). Although the type specimen of *B.* × *hanjiana* was collected from the natural broadleaved forest of Gageo Island and likely originated naturally (Yun and Kim 2009), the largely non-overlapping distribution, the rarity of natural hybridization between *B. monoica* and *B. papyrifera* elsewhere, and asymmetrical hybridization between the two species suggests that the multi-origin *daknamu* might not have originated naturally but instead have been the result of deliberate and artificial hybridizations, possibly generated and selected for making *hanji* in Korea.

On the contrary, the proposition of a single origin of *kōzo* in Japan cannot be rejected given that all sequenced individuals of *kōzo* carry cp-1 (Fig. 7). Although our current sampling of *kōzo* is restricted to three prefectures (Miyagi, Nagano, and Tochigi) of Honshu, *kōzo* used by the *washi* workshop of Shiroishi, Miyagi was introduced from Uwajima, Ehime of Shikoku during the early 17th Century by Hidemune Date (Kahoku News, 25 February 2018) for encouraging *washi* production, indicating that *kōzo* carrying cp-1 might have a much wider distribution. The ca. 900 km translocation of *kōzo* from Ehime to Miyagi during the Edo period also indicates that the current distribution of *kōzo* cannot reflect the precise geographic origin of this economically important fiber crop in ancient Japan. Nevertheless, because *B. monoica* carrying cp-1 is also distributed in Japan (Fig. 7), our sampled *kōzo* could have originated indigenously, either naturally or artificially. On the other hand, the relatively low population differentiation between Japan and Korea (Table 4) and absence of genetic variation of *kōzo* suggests that a Korean origin of *kōzo* along with the introduction of the papermaking technique (Mizumura et al. 2017; Song and Munn 2004; Yun and Kim 2009) cannot be ruled out completely. Future studies using genomic approaches and collaborations with traditional *washi* and *hanji* makers are required to fully understand the origins of the intriguing *kōzo* and *daknamu*.

Given the importance of *kōzo* for *washi* and *daknamu* for *hanji*, our study not only addresses important evolutionary questions but also has profound value for the manufacture of traditional Korean and Japanese paper, which are also highly relevant to the understanding of the historical paper material and paper conservation. Because of the short histories of the introduction of papermaking to Korea and Japan, *kōzo* and *daknamu* also represent a unique study system to understand how cultural and societal developments and biological process of hybridization (Diamond 2002; Zeder 2015) could have shaped the genetic diversity of a hybrid domesticated crop species.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10265-022-01369-w.

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