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Investigation

Phylogenomics resolves deep subfamilial relationships in Malvaceae *s.l.*

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Abstract

Malvaceae *s.l.*, the most diverse family within Malvales, includes well-known species of great economic importance like cotton, cacao, and durian. Despite numerous phylogenetic analyses employing multiple markers, relationships between several of its nine subfamilies, particularly within the largest lineage /Malvadendrina, remain unclear. In this study, we attempted to resolve the relationships within the major clades of Malvaceae *s.l.* using plastid genomes of 48 accessions representing all subfamilies. Maximum likelihood and Bayesian analyses recovered a fully resolved and well-supported topology confirming the split of the family into /Byttneriina (/Grewioideae +/Byttnerioideae) and /Malvadendrina. Within /Malvadendrina, /Helicteroideae occupied the earliest branching position, followed by /Sterculioideae, /Brownlowioideae, /Tiliodeae, and /Dombeyoideae formed a clade sister to /Malvatheca (/Malvoideae +/Bombacoideae), a grouping morphologically supported by the lack of androgynophore. Results from dating analyses suggest that all subfamilies originated during hot or warm phases in the Late Cretaceous to Paleocene. This study presents a well-supported phylogenetic framework for Malvaceae *s.l.* that will aid downstream revisions and evolutionary studies of this economically important plant family.

Keywords: phylogenomics; Malvaceae *s.l*; next-generation sequencing; plastomes; historical diversification

Introduction

Malvaceae is a diverse and economically important family distributed throughout the tropical and temperate areas of both hemispheres (Bayer and Kubitzki 2003). Members of this family are widely used in agriculture, forestry and horticulture. Well-known examples are cotton (*Gossypium* spp.), cacao (*Theobroma cacao*), okra (*Abelmoschus esculentus*), durian (*Durio zibethinus*), cola (*Cola* spp.), baobab (*Adansonia* spp.), and the highly valued ornamental species of *Hibiscus* and *Alcea* (Bayer and Kubitzki 2003). Malvaceae account for about 70% of the diversity of the order Malvales. The family comprises 244 genera and over 4225 accepted species (Stevens 2001; Bayer and Kubitzki 2003; Christenhusz and Byng 2016), with new ones being regularly added (Alverson 1991; De Carvalho-Sobrinho and De Queiroz 2008; Bovini 2016; Areces-Berazain and Vega-Lopez 2019; Ganesan et al. 2020).

Earlier morphology-based studies (*e.g.*, Bentham and Hooker 1867; Schumann 1890; Hutchinson 1926; Edlin 1935a,b) recognized just four of the ten families now included in the order Malvales: Bombacaceae, Malvaceae, Sterculiaceae, and Tiliaceae. However, the boundaries and relationships among the four groups were long considered problematic due to the difficulty in interpreting morphological traits that now appear to be homoplasious (Alverson *et al.* 1999; Le Péchon and Gigord 2014). Phylogenetic studies based on both morphological (Judd and Manchester 1997) and molecular data (Alverson *et al.* 1998, 1999; Baum *et al.* 1998; Bayer *et al.* 1999) confirmed the Malvalean identity of these families but revealed that each was nonmonophyletic. The traditional families were merged into an expanded Malvaceae, which was subdivided into nine subfamilies:/Bombacoideae, /Brownlowioideae, /Byttnerioideae, /Dombeyoideae, /Grewioideae, /Helicteroideae, /Malvoideae, /Sterculiodeae, and /Tilioideae (Bayer *et al.* 1999).

In most molecular phylogenetic analyses of Malvaceae *s.l.* (*e.g.*, Baum *et al.* 1998; Alverson *et al.* 1999; Whitlock *et al.* 2001; Richardson *et al.* 2015; Hernández-Gutiérrez and Magallón 2019) subfamilies Grewioideae and Byttnerioideae, which include species previously placed in Tiliaceae and Sterculiaceae, respectively, form a well-supported clade that has been named /

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Byttneriina. The remaining subfamilies are grouped in / Malvadendrina (Baum *et al.* 1998; Alverson *et al.* 1999; Nyffeler *et al.* 2005). Within /Malvadendrina, /Malvoideae, and / Bombacoideae form another well-supported monophyletic group known as /Malvatheca (Baum *et al.* 1998, 2004). In contrast, the positions of /Brownlowioideae, /Dombeyoideae, /Helicteroideae, / Sterculioideae, and /Tilioideae vary greatly between different datasets and studies and remain unresolved (Alverson *et al.* 1999; Bayer and Kubitzki 2003; Nyffeler *et al.* 2005). The lack of a well-resolved framework in /Malvadendrina has not only limited progress on classification, but also impaired our understanding of the evolution of morphological characters and the family's biogeographic history (Alverson *et al.* 1999; Nyffeler *et al.* 2005).

Molecular studies in Malvaceae *s.l.* have typically relied on a small number of markers, which have failed to provide an adequate resolution at the subfamily level, particularly within / Malvadendrina. A recent study employing eight markers (six plastid, one nuclear, and one mitochondrial) (Hernández-Gutiérrez and Magallón 2019) retrieved /Helicteroideae sister to the rest of /Malvadendrina with high support, but relationships between the other subfamilies within the clade remained poorly supported. Also, relationships among tribes and early-branching genera within /Malvoideae and /Bombacoideae were notably different from those found in previous phylogenetic analyses (*e.g.*, Baum *et al.* 2004; Areces-Berazain and Ackerman 2017).

The most straightforward way to address relationships that have not been resolved with small datasets is to increase sequence data. Analyses based on tens to hundreds of genes (*i.e.*, genomic data) have provided resolution among major clades of Angiosperms, and successfully clarified difficult relationships within many orders and families (Soltis *et al.* 2013). In Malvaceae, the potential of comparative genomics was recently explored by Conover *et al.* (2019) to infer the number and location of whole-genome multiplication events. Their phylogenetic analysis, based on 67 plastid genes for 8 species and transcriptome data for 11 species representing eight of the nine subfamilies, showed improved resolution within /Malvadendrina, albeit with moderate to low support. The extremely short branches at the base of / Malvadendrina recovered in this and previous studies (*e.g.*, Alverson *et al.* 1999; Nyffeler *et al.* 2005) suggest rapid radiation of the subfamilies and contribute to explain prior difficulties in resolving subfamilial relationships (Conover *et al.* 2019).

Here, we assembled a plastome dataset of 35 species of Malvaceae *s.l.* to investigate the subfamilial relationships and establish a preliminary temporal framework for the evolution of the family. We used a genome-skimming approach to generate plastome sequences for 28 species in 25 genera, representing all subfamilies in Malvaceae *s.l.* and two outgroup families, Thymelaeaceae and Dipterocarpaceae.

Our objectives were (1) to assess whether this expanded plastome dataset can clarify the backbone phylogeny and relationships among subfamilies of Malvaceae *s.l.*, especially at the base of /Malvadendrina; (2) to estimate molecular divergence times of subfamilies and major lineages (/Byttneriina, /Malvadendrina, and /Malvatheca).

Materials and methods

Taxon selection

We obtained samples from 28 species representing all nine subfamilies in Malvaceae *s.l.*, plus species from two malvalean families, Thymelaeaceae and Dipterocarpaceae. Samples were collected during field work in Yunnan (China), Gabon, Cameroon, and Singapore Botanic Gardens, or retrieved from silica-gel dried tissue samples from the Naturalis Biodiversity Centre collections (Leiden, The Netherlands). All vouchers were deposited in our herbarium (BGT, Brunei Darussalam). Additionally, we retrieved 19 plastome sequences from GenBank, 15 belonging to Malvaceae, three to Brassicaceae, and one to Thymelaeaceae (for accession numbers and voucher information see Supplementary Appendix S1).

DNA extraction, sequencing, and phylogenomic analyses

Standard DNA extraction protocols were followed with minor modifications (Doyle and Doyle 1990). Genomic DNA was purified from either fresh/frozen or silica-dried, then a 350 bp paired-end sequencing library was built and sequenced on an Illumina HiSeq2500 platform (San Diego, California, USA) with 150 bp reads. Library construction and sequencing were performed by Novogene (Beijing, China).

Raw sequence reads were assembled into circular contigs using Novoplasty v2.7.0 (Dierckxsens *et al.* 2016) and ORG.Asm v0.2.05 (ORG.ASM 2016) with default settings. The contigs were then imported in Geneious R10 v.10.0.5 (http://www.geneious.com; Kearse *et al.* 2012), for further assembly and refinement, as described by Cvetković *et al.* (2019). Plastomes were annotated with cpGAVAS (Liu *et al.* 2012) and GeSeq (Tillich *et al.* 2017), followed by manual curation, and aligned using the MAFFT v7 (Katoh and Standley 2013) plugin in Geneious R10 with default settings, following Hinsinger and Strijk (2015) and Cvetković *et al.* (2017). The complete plastome sequences were submitted to DRYAD (https://datadryad.org/stash/share/LkbLwUlzW_GJ5rBDMHYZz69S19HzkNWY6fPOySH9tBQ).

We used ModelTest-NG v0.1.5 (Darriba *et al.* 2019) to select the best-fitting nucleotide substitution model for the plastome dataset [with one inverted repeat (IR) removed]. No partition was defined because, in our experience with plastome data, we have not observed an effect of partitioning on tree inference (see Areces-Berazain *et al.* 2020). A maximum likelihood (ML) tree was built with RAxML-NG v0.9.0 (Kozlov *et al.* 2019) using the GTR+I+G model. We performed an 'all in one' analysis with 20 parsimony starting trees and 1000 bootstrap replicates for the evaluation of branch support. The best-scoring ML tree was edited using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Molecular divergence time estimation

We performed a divergence time estimation analysis with the plastome dataset in BEAST v2.6.3 (Drummond and Rambaut 2007; Suchard and Rambaut 2009). We used an uncorrelated relaxed clock model (Drummond *et al.* 2006) and the Birth–Death model (Nee *et al.* 1994) as tree prior. The two hyperparameters of the clock model were assigned exponential distributions with an *ucldMean.c*: mean = 10.0, and *ucldStdev.c*: mean = 0.333 as specified in Areces-Berazain and Ackerman (2016).

Eight fossil calibrations were selected following a survey of the relevant literature (see Supplementary Appendix S2). The root node (corresponding to the crown node of Malvales) was constrained to a minimum age of 82 Ma based on the malvalean fossil wood *Bombacoxylon langstoni* from the late Cretaceous (Campanian) of Texas (Wheeler and Lehman 2000). We applied a log-normal prior to this node age with a mean of 1.0 and SD of 1.25. The other fossil constraints were used to calibrate seven ingroup nodes representing major clades of Malvaceae *s.l.* Five of these fossils were considered in the study of

Hernández-Gutiérrez and Magallón (2019) although three of them were here assigned to different nodes (Supplementary Appendix S2).

The stem *Sterculia* (or crown Sterculiodeae) was calibrated at 70 Ma based on fossil leaves of several species of *Sterculia* (*S. washburnii* and *S. patagonica*) described from the late Cretaceous of South America (Berry 1938). Together with *Sterculiaephyllum australis* from the Cretaceous of Antarctica (Dutra and Batten 2000), these leaf impressions, which resemble those of modern *Sterculia*, are among the oldest records of Sterculioideae.

Two palynomorphs were used to constrain the age of stems Tilioideae and Brownlowioideae, respectively. For Tilioideae, we used *Tilia*-type pollen from the Late Cretaceous (72-66 Ma) of Canada (Rouse *et al.* 1970). The stem Brownlowioideae was calibrated with *Discoidites borneensis*, a palynomorph from the Paleocene (66-56 Ma) of Malaysia, similar to pollen of modern *Brownlowia* and *Pentace* (Muller 1968).

The stem Grewioideae was assigned a minimum age of 65 Ma based on the fossil wood *Grewinum canalisum*. The combined occurrence of tile cells and radial canals allows us to confidently assign this fossil wood to this subfamily (Wheeler *et al.* 2017). Another fossil wood, *Guazuma santacrucensis* from the middle Eocene (39 Ma) of Peru, and which shares many anatomical characteristics with the New-world genus *Guazuma* (Woodcock et al. 2019), was used to constrain the age of the crown group of Byttnerioideae.

Within /Malvatheca, the age of modern bombacoids was calibrated with *Bombacacidites annae*, a fossil pollen from the Mid- to Late Palaeocene (66–56 Ma) of Colombia very similar to the *Bombax*-type pollen of most Bombacoideae (Van Der Hammen 1954; Carvalho *et al.* 2011). The age of crown eumalvoid was constrained with *Malvaciphyllum macondicus*, found in Colombian mid- to late Palaeocene (61.6–56 Ma) deposits. Leaves of this species exhibit several architectural features of modern malvoids and appear to be the oldest fossils assignable to this clade (Carvalho *et al.* 2011).

All calibrated ingroup nodes were assigned exponential priors with a mean of 1.0 and offset values corresponding to the fossil age. Two independent runs were conducted in the CIPRES Science Gateway (Miller *et al.* 2010) for one billion generations, sampling trees every 4000 generations. Effective sample size values and convergence of the runs were assessed in Tracer v1.7.1 (Rambaut *et al.* 2018). The sampled trees were combined with LogCombiner 2.6.0 (Rambaut and Drummond 2017a), but due to the large number of trees generated, we discarded 88% of them. The maximum clade credibility (MCC) tree was constructed with the remaining 60,000 trees using TreeAnnotator v 2.6.0 (Rambaut and Drummond 2017b).

Data availability

Complete plastome sequences are available through the Dryad Digital Repository, at https://datadryad.org/stash/share/LkbLwUlzW_GJ5rBDMHYZz69S19HzkNWY6fPOySH9tBQ. Supplemental material available at figshare: https://doi.org/10.25387/g3.14245967.

Results

Plastome size and genomic data

We assembled 24 plastomes of Malvaceae *s.l.* species, three of Thymelaeaceae and one of Dipterocarpaceae. These plastomes varied in length from 151,071 bp (*Sterculia lanceolata*) to 172,707 bp (*Edgeworthia chrysantha*), and had the typical organization of a large single copy (~90 kb, 34.5% GC), small single copy (~20 kb, 31.5% GC) and two IRs (~25 kb, 42.7% GC). The length of the alignment, including the 20 sequences retrieved from Genbank, was 182,323 bp, with 43,475 (23.8%) of identical sites and 84.7% pairwise identity.

Phylogenomic analyses

The best-scoring ML tree based on plastomes was highly supported (BS $\geq$ 99), except for the clade formed by /Malvatheca plus subfamilies /Brownlowioideae, /Dombeyoideae, and /Tilioideae which was moderately supported (BS = 72). Within subfamilies, only two nodes in Bombacoideae had moderate support (BS = 66; 73; Figure 1). Monophyletic /Byttneriina (/Grewioideae and /Byttnerioideae) was retrieved as sister to /Malvadendrina, a clade composed of all other Malvaceae *s.l.* subfamilies (BS = 100; Figure 1). Within /Malvadendrina, /Helicteroideae was retrieved as sister to a clade including the other /Malvadendrina subfamilies (/Sterculioideae, /Brownlowioideae, /Dombeyoideae, /Tilioideae, /Bombacoideae, and /Malvoideae). The latter clade consists of /Sterculioideae which is sister to the rest, and a /Brownlowioideae- /Dombeyoideae- /Tilioideae clade that is sister to the /Malvatheca clade (/Bombacoideae and /Malvoideae).

Dating analyses

The Bayesian MCC tree resulting from the analysis of the plastome dataset in BEAST2 was well-supported and fully congruent with the ML tree (Figure 2). The molecular dating analysis placed the origin of Malvaceae in the Late Jurassic, about 153 Ma, albeit with a very wide highest posterior density (HPD) interval (95–208; 95% HPD; Figure 2 and Supplementary Appendix S3). The split of /Malvadendrina and /Bytneriina (corresponding to the crown node of Malvaceae *s.l.*) was dated to the late early Cretaceous (110 Ma, 86–143; 95% HPD). Diversification of /Malvadendrina was estimated to have begun around 93 Ma (79–112; 95% HPD) with the separation of Helicteroideae. Sterculioideae diverged shortly after (about 84 Ma, 75–100; 95% HPD), followed by the split of /Malvatheca from its sister clade formed by /Brownlowioideae-/Dombeyoideae-/Tilioideae about 80 Ma (72–95; 95% HPD). As shown in Figure 2 (see also Supplementary Appendix S3), differentiation of the lineages leading to the nine subfamilies occurred in the late Cretaceous, between 93 and 66 Ma.

Discussion

Genomics has increasingly enabled the use of phylogenomics to address phylogenetic relationships in Malvales over the last decade (Argout *et al.* 2011; Heckenhauer *et al.* 2018; Conover *et al.* 2019; Cvetković *et al.* 2019; Abdullah *et al.* 2020). Here, we used a genome skimming strategy for the inference of phylogenomic relationships in Malvales, with a focus on subfamily relationships in Malvaceae *s.l.* Plastome analyses are largely consistent with the results obtained in previous studies that used a limited number of loci (Alverson *et al.* 1998, 1999; Bayer *et al.* 1999; Nyffeler and Baum 2000; Whitlock *et al.* 2001; Baum *et al.* 2004; Nyffeler *et al.* 2005). However, plastome data clarified some subfamilial relationships that remained unresolved in previous analyses (see below) relying on single or few plastid loci (Stevens 2001; Smith and Baum 2003; Le Péchon and Gigord 2014) providing the best estimate of the plastome phylogeny of Malvaceae *s.l.* to date.

The results of our phylogenomic analyses provided strong support for the monophyly of the three previously recognized major clades within Malvaceae *s.l.*, viz. /Byttneriina, /

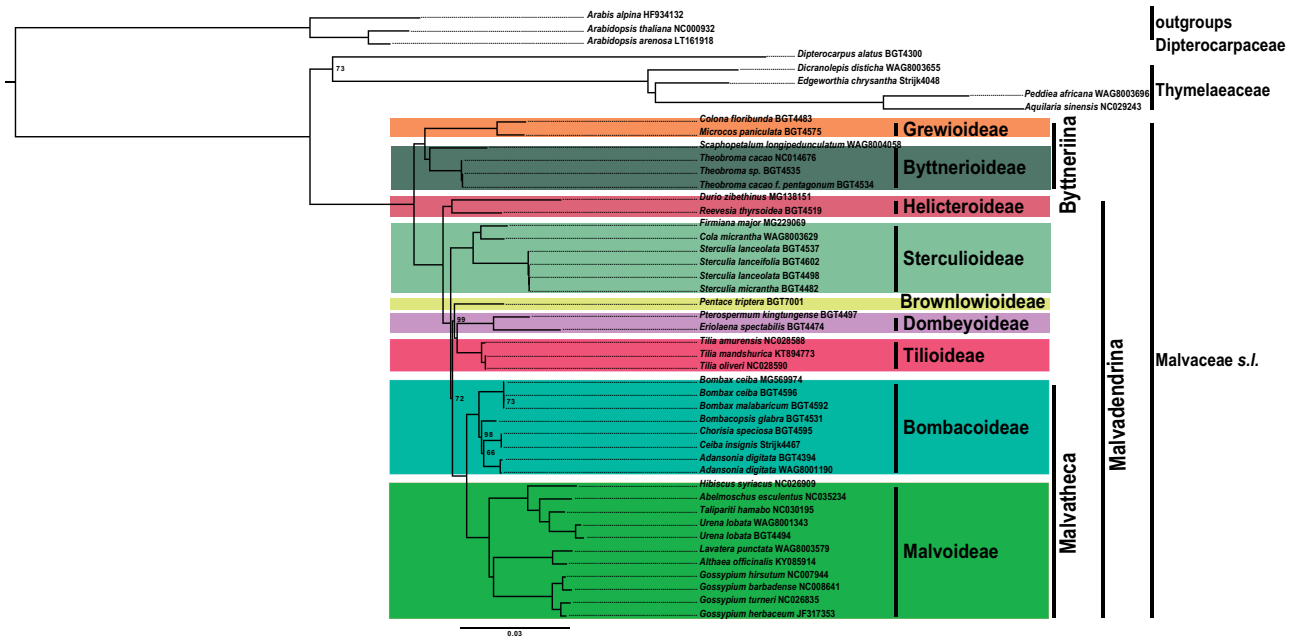

Figure 1 ML phylogenomic tree using a plastome dataset inferred by RAxML. Bootstrap branch support shown at the nodes, unlabeled branches = 100 BS.

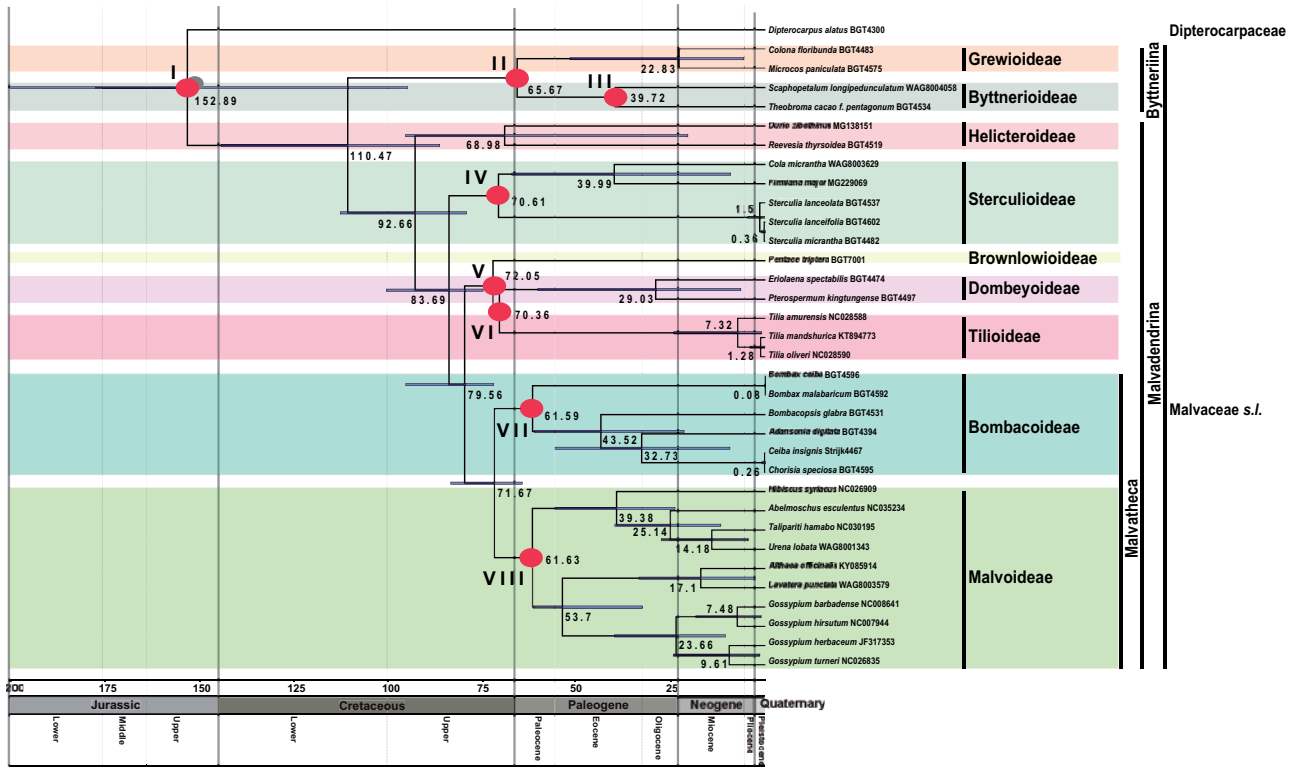

Figure 2 Chronogram using a plastome dataset inferred using BEAST 2. Node ages (in Ma) shown at nodes, with the 95% HPD intervals (blue bars). All nodes have posterior probabilities (PP) 1, except a node with gray circle (PP = 0.65). Calibrations used in this study (red circles): I: *B. langstoni* (84–74 Ma, Wheeler and Lehman 2000); II: *G. canalisum* (67–64 Ma, Wheeler *et al.* 2017); III: *Guazuma santacruzensis* (39 Ma, Woodcock *et al.* 2019); IV: *S. patagonica* and *S. washburnii* (72–66 Ma, Berry 1938; Dutra and Batten 2000); V: *D. borneensis* (66–56 Ma, Muller 1968); VI: *Tilia* sp. (72–66 Ma, Rouse *et al.* 1970); VII: *B. annae* (62–56 Ma, Van Der Hammen 1954, Carvalho *et al.* 2011); VIII: *M. macondicus* (62–56 Ma, Carvalho *et al.* 2011). Geological time scale in millions of years shown.

Malvadendrina, and /Malvatheca (Baum *et al.* 1998; Alverson *et al.* 1999; Smith and Baum 2003), which are discussed below. The characteristic morphological features and potential synapomorphies of the subfamilies and some major clades, based on our inferred plastome phylogenomic tree were presented in Figure 3.

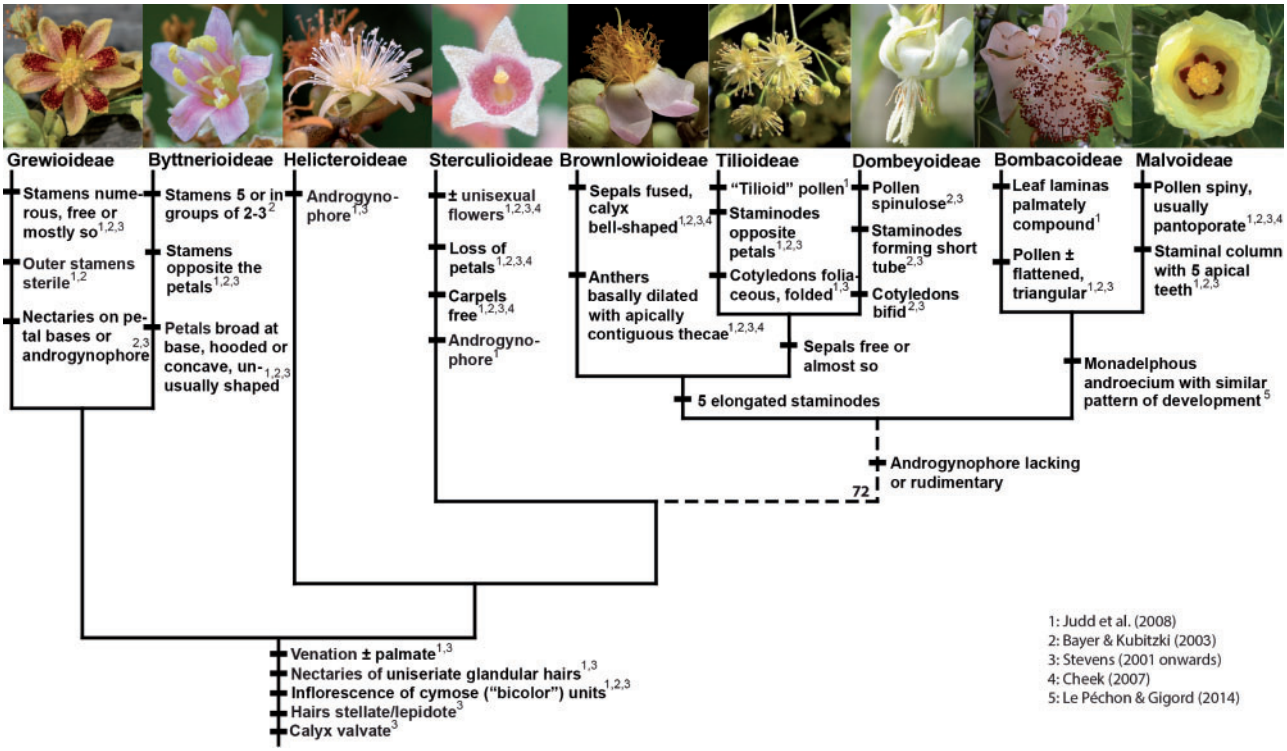

Figure 3 Cladogram of Malvaceae indicating putative synapomorphies of major clades. Tree topology corresponds to ML plastome phylogeny (see Figure 1). The broken line indicates weak support (all other nodes ≥ 98 bootstrap support). 1: Judd *et al.* 2008; 2: Bayer and Kubitzki (2002) 2003; 3: Stevens 2001; 4: Cheek (2007); 5: Le Péchon and Gigord 2014. Images (left to right): (A) *Colona serratifolia*, (B) *Melochia umbellata*, (C) *Durio griffithii*, (D) *Firmiana malayana*, (E): *Brownlowia peltata*, (F) *Tilia cordata*, (G) *Pterospermum lanceifolium*, (H) *Adansonia digitata*, and (I) *Thespesia populnea*. Photo credits: (A–D), (G and I): Santhana K. Ganesan, (E) Zaki Jamil, (F) cropped from photograph by Ivar Leidus, distributed under a Creative Commons Attribution-Share Alike 4.0 International license, and (H) Paul Leong.

New subfamilial phylogenomic relationships at the base of /Malvadendrina

Among Malvaceae *s.l.*, the phylogenomic relationships at the base of /Malvadendrina (Brownlowioideae, Dombeyoideae, Helicteroideae, Sterculioideae, and Tilioideae) are arguably the most problematic as morphology-based and molecular classifications differ (Bayer *et al.* 1999; Whitlock *et al.* 2001; Nyffeler *et al.* 2005; Le Péchon and Gigord 2014). The boundaries between the former families of the "Core Malvales": Sterculiaceae-Tiliaceae, Sterculiaceae-Bombacaceae, and Bombacaceae-Malvaceae "remain nebulous" (Baum *et al.* 1998), and would require substantial revision to accommodate the molecular-based subfamilies (Baum *et al.* 1998; Kubitzki and Chase 2003; Baum *et al.* 2004; Wilkie *et al.* 2006).

Traditional taxonomic placement of /Sterculioideae was uncertain, as it is a group with a highly variable morphology, anatomy and palynology (Wilkie *et al.* 2006; Takhtajan 2009). The phylogenetic placement of /Sterculioideae within Malvadendrina was previously unclear (Wilkie et al. 2006); it was retrieved as sister to /Brownlowioideae (Alverson *et al.* 1999; Bayer *et al.* 1999) or to /Malvatheca (Nyffeler *et al.* 2005; Hernández-Gutiérrez and Magallón 2019). In the study by Conover *et al.* (2019), /Sterculioideae formed a moderately supported sister clade with /Tilioideae, but /Brownlowioideae were not included in their dataset. Our analyses of the plastome dataset indicate that /Sterculioideae may be sister to a clade comprising a /Brownlowioideae-/Dombeyoideae-/Tilioideae subclade, and /Malvatheca (/Bombacoideae and /Malvoideae), but this relationship was only weakly supported (BS = 72). The /Brownlowioideae-/Dombeyoideae- /Tilioideae+ /Malvatheca clade can be characterized by the absence of androgynophore (Figure 3), although this structure is uniquely present in *Pterospermum* (Dombeyoideae) and is rudimentary in a few genera of Brownlowioideae (Bayer and Kubitzki 2003).

/Dombeyoideae were strongly supported as sister to /Tilioideae (BS = 100) in our analysis of the plastid dataset, corroborating previous studies (Nyffeler *et al.* 2005). In Hernández-Gutiérrez and Magallón (2019), a sister clade of /Dombeyoideae and /Brownlowioideae was unsupported. A putative synapomorphy for this grouping might be the presence of free (or nearly free) sepals, which are typically connate in the rest of /Malvadendrina (Figure 3). The phylogenetic relationship of /Tilioideae within /Malvadendrina has remained elusive despite detailed studies of pollen morphology (*e.g.*, Chambers and Godwin 1961, 1971) and various molecular phylogenetic studies [*e.g.*, basal position in Malvadendrina (Bayer *et al.* 1999); close to /Malvatheca (Alverson *et al.* 1999); sister to /Brownlowioideae-/Sterculioideae-/Malvatheca (Nyffeler *et al.* 2005); moderately supported (BS = 80) sister clade of /Sterculioideae (Conover *et al.* 2019); poorly supported sister to a clade comprising /Sterculioideae and /Malvatheca (Hernández-Gutiérrez and Magallón 2019)]. Previous studies comparing the anatomy of the petiole's vascular system, placed polyphyletic /Tiliaceae close to /Sterculiaceae (Dehay 1941, 1942; Takhtajan 2009).

/Brownlowioideae were strongly supported (BS = 99) as sister to the /Dombeyoideae-/Tilioideae clade in our plastome analysis, resolving polytomous or poorly supported relationships from previous studies (*e.g.*, Alverson *et al.* 1999; Le Péchon and Gigord 2014; Hernández-Gutiérrez and Magallón 2019). The three subfamilies share the presence of five elongate staminodes, although

it is unclear whether these structures are homologous. Staminodes can be free or integrated into the staminal tube (Dombeyoideae) and can vary from linear to ovate or petaloid (Bayer and Kubitzki 2003). Such staminodes are absent in the sister /Malvatheca and in /Sterculioideae.

Dating analyses

Our estimates of divergence times based on eight fossil calibrations (Supplementary Appendix S2) revealed a relatively old origin for Malvaceae (152 Ma) compared with the estimate of 98 Ma inferred by Hernández-Gutiérrez and Magallón (2019). Similarly to their study, we assigned the oldest fossil, *B. langstoni*, to the stem (rather than crown Malvaceae) to provide a minimum age constraint of 82 Ma for the origin of this family. We used a log-normal distribution for this node as it places the highest probability on ages slightly older than the fossil.

Hernández-Gutiérrez and Magallón (2019), however, used a different strategy to calibrate the root of their tree. They employed a uniform prior with maximum and minimum age bounds of 103.2 and 89.44 Ma based on the age of the stem Malvales estimated in a previous study (Magallón *et al.* 2015). As a result, their time estimate for the origin of Malvaceae was younger than that obtained in this study. Our main reason for not following their approach to calibrate the root of our tree was to avoid the use of secondary calibrations, which has been shown to produce unreliable estimates and "a false impression of precision" (Schenk 2016).

The crown age of Malvaceae inferred in our study was 110 Ma (Figure 2), about 20 Ma older than that estimated by Hernández-Gutiérrez and Magallón (2019). However, the differentiation of the subfamilies, here estimated to have occurred in the upper Cretaceous-Paleocene interval (93–66 Ma; Figure 2 and Supplementary Appendix S3), was relatively consistent with the ages reported in their study (between 82 and 72 Ma). In addition to the different calibration approaches for the tree root, it is also very likely that our scarce taxon sampling plays a role in the different divergence time estimates reported. It is clear that an increased taxon sampling is needed in order to obtain robust age estimates as it has been shown that undersampling affects the accuracy of phylogenetic estimation (in terms of branch lengths) and thus the molecular divergence dates (Heath et al. 2008; Schulte 2013).

The stem group age estimates for the Malvaceae *s.l.* subfamilies suggest early onset of Malvaceae diversification occurred. Stem groups of most subfamilies originated during humid and hot conditions of the Late Cretaceous or a subsequent warm phase in the Paleocene. Most Malvaceae subfamilies are predominantly tropical, except for /Tilioideae, which is predominantly temperate, and /Malvoideae, which is widespread in both temperate and tropical habitats (Zachos et al. 2001; Huber *et al.* 2018; Hernández-Gutiérrez and Magallón 2019). Additional analyses incorporating a denser taxon sampling and fossil distributions are needed to infer ancestral geographic ranges and clarify where and how often niche shifts between tropical-humid, tropical-semiarid, and temperate habitats occurred.

/Byttneriina

Our analyses confirmed the placement of /Byttneriina, the clade comprising /Grewioideae and /Byttnerioideae, as sister to all remaining Malvaceae *s.l.* subfamilies (/Malvadendrina; Figures 1 and 2 and Supplementary Appendix S3). The plastome dataset strongly supports the monophyly of /Byttneriina, which is in contrast to some traditional classifications based on morphology [*e.g.*, /Grewioideae included in /Tiliaceae (Hutchinson 1967; Takhtajan 1997)]. No morphological synapomorphies of /Byttneriina have been identified to date.

In this clade, /Byttnerioideae can usually be characterized by a reduction in stamen number, stamens in multiple of five, arranged in groups opposite the petals, and by the distinctive hooded petals, often with a broad or cucullate base and an apical appendage (Whitlock *et al.* 2001; Judd *et al.* 2008), although the apical appendages may be homoplasious (Whitlock *et al.* 2001; Le Péchon and Gigord 2014).

/Grewioideae are supported by numerous, mostly free stamens, staminodes that are modified outer stamens and by the nectaries on petal bases or androgynophore (Whitlock *et al.* 2001; Judd *et al.* 2008; Brunken and Muellner 2012).

/Malvadendrina

No morphological synapomorphies characterizing /Malvadendrina have been identified.

/Helicteroideae is strongly supported as sister clade to the remaining taxa of /Malvadendrina (Alverson *et al.* 1999; Bayer *et al.* 1999; Conover *et al.* 2019; Hernández-Gutiérrez and Magallón 2019; this study). The presence of an androgynophore has been indicated as a potential synapomorphy for the subfamily (Stevens 2001; Judd *et al.* 2008). The placement of *Durio* within Bombacaceae based on morphology (Kostermans 1958, Fryxell 1968; Takhtajan 2009) was previously not supported by both chromosome numbers (Baum and Oginuma 1994) and molecular studies (Bayer *et al.* 1999; Nyffeler and Baum 2000, 2001; Nyffeler *et al.* 2005; Hernández-Gutiérrez and Magallón 2019). Our plastome-based molecular phylogeny placed *Durio* among /Helicteroideae.

Monophyly of /Sterculioideae is supported by a suite of unique characters including unisexual or polygamous flowers, coloured calyx of petaloid sepals and a loss of petals, secondary apocarpy, presence of an androgynophore, and woody follicle fruits (Figure 3; Stevens 2001; Bayer and Kubitziki 2003; Judd *et al.* 2008; Le Péchon and Gigord 2014) and was confirmed in several previous studies (Judd and Manchester 1997; Wilkie et al. 2006).

/Dombeyoideae are characterized by bilobed cotyledons, a short staminode tube, and large-sized, echinate pollen grains with 3-zonoporate apertures (Falque *et al.* 1992; Stevens 2001; Bayer and Kubitzki 2003; Perveen and Qaiser 2009; Hamdy and Shamso 2010; Silveira Júnior *et al.* 2017). /Tilioideae have been characterized by their pollen structure ("tilioid pollen type"; Chambers and Godwin 1961, 1971; Bayer and Kubitzki 2003; Le Péchon and Gigord 2014), and the presence of folded cotyledons and staminodes opposite the petals have also been highlighted as potential synapomorphies (Figure 3; Stevens 2001; Bayer and Kubitzki 2003; Judd *et al.* 2008).

Potential synapomorphies of /Brownlowioideae include a strongly fused, bell-shaped calyx, and basally dilated and divergent anthers with apically contiguous anther sacs (Figure 3; Stevens 2001; Bayer and Kubitzki 2003; Cheek 2007; Judd *et al.* 2008).

/Malvatheca

The monophyly of /Malvatheca (/Bombacoideae and /Malvoideae) in our plastome analysis is consistent with numerous previous studies (Alverson *et al.* 1999; Bayer *et al.* 1999; Baum *et al.* 2004; Nyffeler *et al.* 2005; De Carvalho-Sobrinho *et al.* 2016). Anthers with transversely septate locules (in most species modified to 1-loculed half-anthers) were indicated as synapomorphy in some studies (*e.g.*, Judd *et al.* 2008), but this character is likely

homoplasious in /Malvatheca (Le Péchon and Gigord 2014). Le Péchon and Gigord (2014) indicate that the aspects of the androecial development as described in detail by von Balthazar *et al.* (2004, 2006) may be synapomorphic, and Stevens (2001) additionally indicates the absence of a root hypodermis and stamen filaments forming a tube as potential synapomorphies (Figure 3).

/Bombacoideae are characterized by palmately compound leaf laminas, the triangular, nonspiny pollen, a unique chromosome number ($n = 36$), embryo morphology, seed anatomy and the absence of mucilaginous substances (Fuchs 1967; Hutchinson 1967; Fryxell 1968; Singh and Chauhan 1984; Baum and Oginuma 1994; El Naggar 2001; Bayer and Kubitzki 2003; Takhtajan 2009).

/Malvoideae, by far the largest (~1800 species) of the nine subfamilies in Malvaceae *s.l.*, have been characterized by the frequent presence of spiny, usually pantoporate pollen grains and a staminal column with five apical teeth (Bayer and Kubitzki 2003; Judd *et al.* 2008).

We present a detailed, well-supported phylogenetic framework for Malvaceae *s.l.* that will aid downstream revisions and evolutionary studies of this iconic and economically important plant family.

Conclusions

Resolving the subfamilial phylogenomic relationships at the base of /Malvadendrina is a crucial step for further evolutionary analyses. The inclusion of additional nuclear data in particular could be of interest for further evolutionary and taxonomic studies in this complex group. Incorporating new techniques [*e.g.*, MIG-seq (Suyama and Matsuki 2015); high-throughput sequencing of target-enriched libraries (Jones and Good 2016; Bossert and Danforth 2018; Johnson *et al.* 2019)] could provide valuable data from rare or unplaced (Binh *et al.* 2018), extinct (Feigin *et al.* 2018), or herbarium specimens (Strijk *et al.* 2020).

Our study provides a phylogenetic framework with robust support at deep levels, suitable for downstream revisions and evolutionary studies of this iconic and economically important plant family. As such, it serves as a valuable genomic resource to further investigate the complex evolutionary history of the family at lower taxonomic levels. The nine subfamilies were retrieved as previously circumscribed confirming the promising potential of using next-generation sequencing data in studies of Malvales.

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Author contributions

T.C. performed the experiment and analyses and collected samples. D.D.H. and J.S.S. designed the experiment and collected samples. F.A.B. and D.C.T. performed the analyses. J.J.W, S.K.G., and D.C.T. contributed sampling materials. All authors contributed to the writing, editing, and review of the article, and approved the final version for submission.

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