The evolutionary history of the Caribbean magnolias (Magnoliaceae): Testing species delimitations and biogeographical hypotheses using molecular data

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ABSTRACT

The Caribbean islands provide an ideal setting for studying biodiversity, given their complex geological and environmental history, and their historical and current geographical proximity to the American mainland. *Magnolia*, a flagship tree genus that has 15 endemic and threatened taxa (12 species and 3 subspecies) on the Caribbean islands, offers an excellent case study to empirically test Caribbean biogeographical hypotheses. We constructed phylogenetic hypotheses to: (1) reveal their evolutionary history, (2) test the current largely morphology-based classification and assess species limits, and (3) investigate major biogeographic hypotheses proposed for the region. Nuclear and chloroplast DNA sequence data of all 15 Caribbean *Magnolia* taxa are included, supplemented by a selection of American mainland species, and species representing most major clades of the Magnoliaceae family. We constructed phylogenetic hypotheses in a time-calibrated Bayesian framework, supplemented with haplotype network analyses and ancestral range estimations. Genetic synapomorphies in the studied markers confirm the species limits of 14 out of 15 morphologically recognizable Caribbean *Magnolia* taxa. There is evidence for four colonization events of *Magnolia* into the Caribbean from the American mainland, which most likely occurred by overwater dispersal, given age estimates of maximum 16 mya for their presence on the Caribbean islands.

1. Introduction

The Caribbean islands, also known as the West Indies, have a rich endemic biodiversity (Mittermeier et al., 2011; Myers et al., 2000; Smith et al., 2004) and complex geological and environmental history (Draper, 2008; Pindell et al., 2012), inspiring different biogeographic hypotheses on the evolutionary history of their present and past biodiversity (e.g. Graham, 2003b; Hedges, 2006; Iturralde-Vinent, 2006; Maunder et al., 2011; Ricklefs and Bermingham, 2008). There are three main hypotheses explaining the distribution of the Caribbean biodiversity regarding

related species on the American mainland: (1) vicariance (Rosen, 1975, 1985), (2) land bridges (Iturralde-Vinent and MacPhee, 1999), or (3) overwater dispersal (Hedges, 1996). The first hypothesis, the vicariance model, proposes that the "Proto-Antilles" volcanic archipelago that existed between North and South America in the Mesozoic moved by plate tectonics in the Late Cretaceous (ca. 100–66 mya), resulting in the separation of biota. The second hypothesis, called the GAARlandia hypothesis: Greater Antilles + Aves Ridge, proposes a "landspan" between South America and the Greater Antilles for a short time during the Latest Eocene–Early Oligocene (35–33 mya) (Iturralde-Vinent, 2006; Iturralde-

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Vinent and MacPhee, 1999). The third hypothesis, overwater dispersal, suggests that organisms dispersed by flying or flotsams from the mainland to the Caribbean islands during the Cenozoic (65.5 mya–present day) (Hedges, 1996). Most of the framework and testing of these biogeographic hypotheses were executed on empirical data of vertebrates, which have distinct dispersal limitations (e.g. Alonso et al., 2012; Hedges, 2006). Data on the biogeography of the Caribbean flora, however, support all three hypotheses (Francisco-Ortega et al., 2007; Nieto-Blázquez et al., 2017; Santiago-Valentin and Olmstead, 2004). This is not surprising as seed dispersal greatly enhances the probability and geographical extent of plant colonization (Cano et al., 2018; Gugger and Cavender-Bares, 2013). Other than investigating relationships between species native to the Caribbean islands *versus* continental species to explain present day distribution of the Caribbean biodiversity, biogeographical research in the region also studies the historical connectivity

and availability of the island masses (Oleas et al., 2013; Santiago-Valentin and Olmstead, 2004).

A flagship tree genus that offers an excellent case study to empirically test the array of Caribbean biogeographical hypotheses is *Magnolia* L. (Magnoliaceae). This because its high endemic biodiversity, i.e. 15 accepted *Magnolia* taxa (i.e. 12 species and 3 subspecies) occur in the Caribbean (Fig. 1, Table 1); and its animal-mediated reproductive biology, i.e. seed dispersal by birds (Testé, 2018) and pollen dispersal by (large) beetles (Thien, 1974).

Of the 15 Caribbean *Magnolia* taxa, 10 make up the complete subsection *Cubenses* (Figlar and Nooteboom, 2004), represented in previous family-wide phylogenetic studies by inclusion of 1–2 representatives (Azuma et al., 2001; Kim and Suh, 2013; Wang et al., 2020). Four out of the 15 Caribbean *Magnolia* taxa belong to subsection *Talauma*, which in the abovementioned studies was inferred as a well-supported sister clade
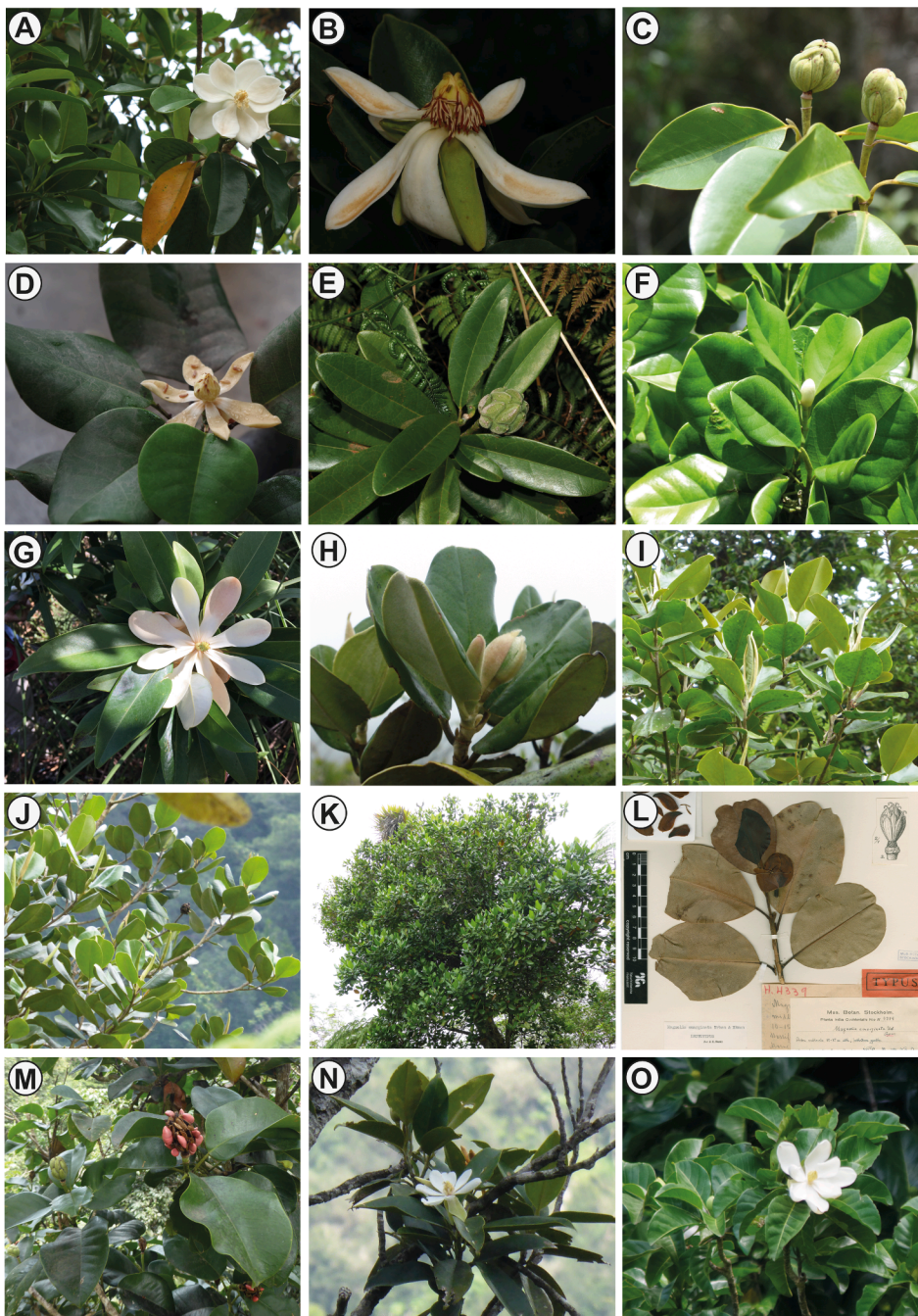

Fig. 1. *Magnolia* diversity of the Caribbean. (A) *M. cubensis* subsp. *acunae* (Cuba). Photo: Alejandro Palmarola Bejerano; (B) *M. cubensis* subsp. *cubensis* (Cuba). Photo: Mikhail S. Romanov; (C) *M. cristalensis* (Cuba). Photo: Banessa Falcón; (D) *M. orbiculata* (Cuba). Photo: Emily Veltjen; (E) *M. oblongifolia* (Cuba). Photo: Alejandro Palmarola Bejerano; (F) *M. minor* (Cuba). Photo: Ernesto Testé; (G) *M. virginiana* subsp. *oviedoae* (Cuba). Photo: Ernesto Testé; (H) *M. domingensis* (Dominican Republic). Photo: Emily Veltjen; (I) *M. hamorii* (Dominican Republic). Photo: Emily Veltjen; (J) *M. pallescens* (Dominican Republic). Photo: Emily Veltjen; (K) *M. ekmanii* (Haiti). Photo: Emily Veltjen; (L) *M. emarginata* (Haiti). Scan: S herbarium. Herbarium abbreviation follows Index Herbariorum (Thiers, continuously updated); (M) *M. portoricensis* (Puerto Rico). Photo: Carlos Rodríguez, Arbonautas; (N) *M. splendens* (Puerto Rico). Photo: Emily Veltjen; (O) *M. dodecapetala* (Lesser Antilles: Saint-Vincent, Saint-Lucia, Martinique, Dominica and Guadeloupe). Photo: Emily Veltjen.
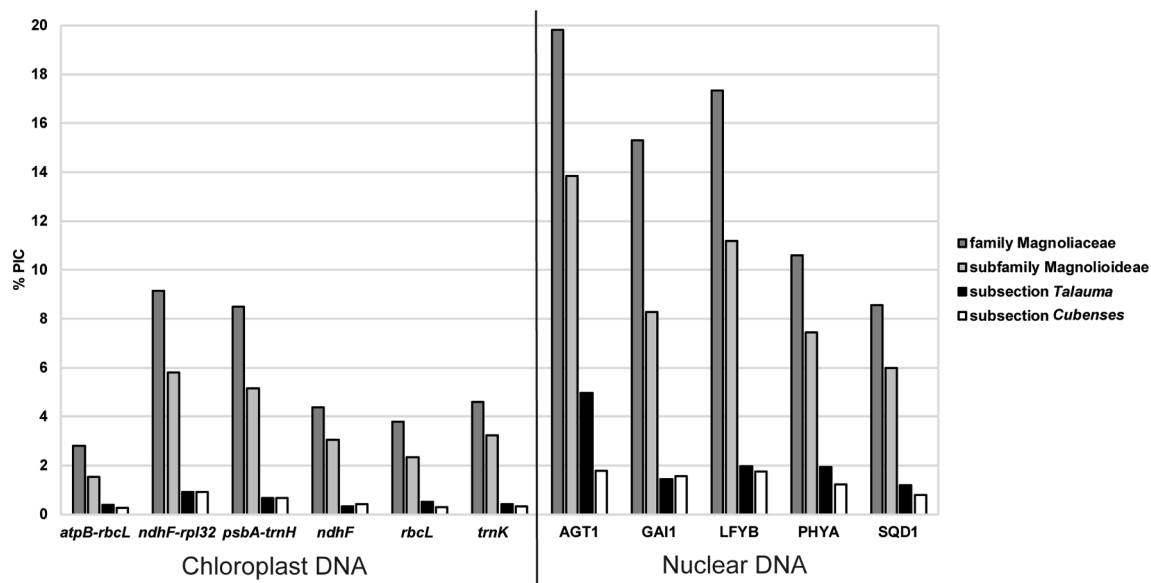
Fig. 2. Parsimony informative characters (PIC) of the Magnoliaceae Sanger sequencing alignments used in this study. This count includes both parsimony informative substitutions, gaps and inversions.

to subsection *Cubenses* (Kim and Suh, 2013; Wang et al., 2020). Subsections *Talauma* and *Cubenses*, together with subsections *Dugandiodendron* and *Chocotalauma* (Pérez et al., 2016), make up section *Talauma* (Figlar and Nooteboom, 2004). The fifteenth Caribbean *Magnolia* taxon, *M. virginiana* subsp. *oviedoae*, is placed in section *Magnolia*, not closely related to the other 14 Caribbean magnolias (Wang et al., 2020). Two time-calibrated phylogenetic hypotheses on the Magnoliaceae generated in previous studies provide insufficient data to answer questions on Caribbean biogeography. The study by Nie et al. (2008) included one Caribbean species and very few potential mainland relatives (two additional species of section *Talauma*). The study by Azuma et al. (2001) was based on data from seven intergenic or intron regions of chloroplast DNA.

Howard (1948) revised the Caribbean *Magnolia* diversity and provided detailed information on 11 native species. The eight *Magnolia* species of Hispaniola, Puerto Rico and the Lesser Antilles are still delineated as such. In contrast to the straightforward taxonomic history of these eight species, the taxonomical history of the Cuban magnolias is more complicated (Bisse, 1988; Imchanitzkaja, 1991; Imchanitzkaja, 1993). The number of Cuban taxa recognized raised to eleven in the studies of Imchanitzkaja (1991, 1993), i.e. five species and six heterotypic subspecies. After the work of Imchanitzkaja, other authors have expressed a different opinion about the number of Cuban *Magnolia* taxa, and several names have been placed in synonymy (e.g. Acevedo-Rodríguez and Strong, 2019, Rivers et al. 2016). The most recent revisions (Greuter & Rankin Rodríguez, 2017; Palmarola et al., 2016) recognize six native Cuban *Magnolia* species, comprising seven native Cuban taxa (see Table 1). These include a recently found population of *M. virginiana* from the Majaguillar Swamp in the north of the Cuban province of Matanzas (Oviedo Prieto et al., 2006), that was described as a subspecies due to its distinctive morphology: *M. virginiana* subsp. *oviedoae* (Palmarola-Bejerano et al., 2008).

Caribbean magnolias have been distinguished based on morphological characters such as leaf size, shape and texture; leaf margin type; absence or presence of pubescence in leaves, branches, gynoecia, stipules and/or fruit; stipules deciduous or not; number of perianth parts; and number of carpels (Howard, 1948; Imchanitzkaja, 1991; Imchanitzkaja, 1993). Although the morphological characters have been defined as distinct in species descriptions and identification keys, variation in many of the distinguishing characters has been reported (Howard, 1948; Palmarola et al., 2016; Stehlé and Marie, 1947).

Each morphologically defined taxon is endemic to one Caribbean island, with the exception of *Magnolia dodecapetala* which is a multiple island endemic (White et al., 2020). *Magnolia* species are found on Cuba, Hispaniola, Puerto Rico and the Lesser Antilles. Interestingly, there are no records of the genus on Jamaica, which, for other taxonomic groups with a similar biogeographic pattern, is often reasoned to be linked to its submergence in the middle Eocene to the middle Miocene (Hedges, 1996). When multiple *Magnolia* species occur on one Caribbean island, they occur on different mountain chains within that island, whereby populations of adjacent species are roughly between 30 km and 400 km apart. There are two exceptions: *M. cubensis* subsp. *cubensis* and *M. orbiculata* in the Sierra Maestra Mountain Range, and *M. cristalensis*, *M. minor* and *M. oblongifolia* in the Nipe-Sagua-Baracoa Massif occur in sympatry.

By generating phylogenetic hypotheses including all the Caribbean and a subset of mainland American magnolias, based on both nuclear and chloroplast markers, this study aims to test (1) species delimitations: Do chloroplast and nuclear DNA regions support the 15 Caribbean *Magnolia* taxa?; (2) classification: Does the classification in which 14 Caribbean magnolias are placed in section *Talauma* hold? Are there two (sister) clades, following subsections *Talauma* and *Cubenses*?; (3) phytogeography of the mainland *versus* the Caribbean: Which of the three biogeographical hypotheses is most likely for the Caribbean magnolias: vicariance, land bridges or overwater dispersal? Which are the most likely source areas for the Caribbean *Magnolia* species?; and (4) phytogeography within the Caribbean islands: Did the historic dispersal of *Magnolia* species follow any of the known Caribbean phytogeographic patterns?

2. Materials and methods

2.1. Taxon and data sampling

DNA sequence data were obtained from leaf samples collected from wild populations and *ex situ* collections dried in silica gel, supplemented by GenBank accessions (www.ncbi.nlm.nih.gov/genbank/, Clark et al., 2016). The molecular phylogenetic analyses comprise 62 Magnoliaceae taxa represented by 100 accessions. An overview of the (sub)species, populations, herbarium vouchers and reference publications is summarized in Appendix A. To provide a good outgroup sampling, we aimed to represent all the different sections of *Magnolia* by at least two

Table 1

List of the 15 Caribbean *Magnolia* taxa (i.e. 12 species and 3 subspecies) and the currently accepted classification (at sectional and subsectional level) by Figlar and Nooteboom (2004).

Accepted taxa	Synonymized names
Section *Talauma* Baill. subsection *Cubenses* Imkhan.	**Section *Talauma* subsection *Splendentes* (Dandy ex A. Vázquez) Figlar & Noot.**
M. cristalensis Bisse	*M. cacuminicola* Bisse
	M. cacuminicola Bisse subsp. *cacuminicola*
	M. cacuminicola subsp. *bissei* Imkhan.
	M. cristalensis Bisse subsp. *cristalensis*
	M. cristalensis subsp. *baracoana* Imkhan.
	M. cristalensis subsp. *moana* Imkhan.
	M. cubensis subsp. *cacuminicola* (Bisse) G. Klotz
	M. cubensis var. *baracoensis* Imkhan.
M. cubensis Urb. subsp. *cubensis*	*M. cubensis* subsp. *turquinensis* Imkhan.
M. cubensis subsp. *acunae* Imkhan.	
M. domingensis Urb.	
M. ekmanii Urb.	
M. emarginata Urb. & Ekman	
M. hamorii R.A. Howard	
M. pallescens Urb. & Ekman	
M. portoricensis Bello	
M. splendens Urb.	
Section *Talauma* subsection *Talauma*	
M. dodecapetala (Lam) Govaerts	
M. minor (Urb.) Govaerts	*Talauma minor* Urb.
	Svenhedinia minor (Urb.) Urb.
M. oblongifolia (Léon) Palmarola	*Talauma minor* subsp. *oblongifolia* (Léon) Borhidi
	Talauma minor var. *oblongifolia* Léon
	Talauma oblongifolia (León) Bisse
	Talauma opithicola Bisse
M. orbiculata (Britton & P. Wilson) Palmarola	*Talauma minor* subsp. *orbiculata* (Britton & P. Wilson) Borhidi
	Talauma orbiculata Britton & P. Wilson
	Svenhedinia truncata Mold.
	Talauma truncata (Mold.) Howard
Section *Magnolia*	
M. virginiana subsp. *oviedoae* Palmarola, M.S. Romanov & A.V. Bobrov	

Note 1: Figlar and Nooteboom (2004) used the name *Magnolia* section *Talauma* subsection *Splendentes* instead of the nomenclaturally correct name subsection *Cubenses*. Imchanitzkaja (1991) published the name *Cubenses* before Vázquez-G (1994) published the name *Splendentes* at subsectional level. Hence, according to the International Code of Nomenclature for algae, fungi and plants Article 11.3 (Turland et al., 2018), subsection *Cubenses* has priority. This information was unaccounted for in the publication of Figlar and Nooteboom (2004), who changed the rank of section *Splendentes* Dandy ex A. Vázquez to the rank of subsection *Splendentes* (Dandy ex A. Vázquez) Figlar & Noot. The classification of Figlar and Nooteboom (2004) has since been updated on the website of the Magnolia Society International (Magnolia Society International, 2012). Here, we employ the correct name: subsection *Cubenses* Imkhan. – Type: *Magnolia cubensis* subsp. *cubensis*.

Note 2: Wang et al. (2020) propose some minor adjustments to the deeper Magnoliaceae classification, based on their chloroplast genome phylogenetic hypothesis and preliminary results of this study presented at the Third International Symposium on the Family Magnoliaceae in 2016 (Veltjen et al., 2016). In their classification, the subsections within *Talauma* are revoked and they recognize sections *Talauma* and *Splendentes* Dandy ex A. Vázquez.

representatives where possible, given that there is no consensus on relationships between *Magnolia* sections yet. However, herbarium vouchers and *ex situ* collections of section *Talauma,* usable for Sanger Sequencing, proved to be scarce. Hence in the final sampling, all species of subsection *Cubenses* were represented at the population level, subsection *Dugandiodendron* was represented by three accessions, subsection *Talauma* by 13 accessions (of which four are Caribbean species) and subsection *Chocotalauma* has no representative in this study.

DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle and Doyle, 1987) with Mag-Attract Suspension G solution (Qiagen, Germantown, USA) (Xin and Chen, 2012) mediated cleaning (Larridon et al., 2015). Sequences were obtained via Sanger sequencing using forward and reverse primers summarized in Appendix B. New primers were developed using the 1KP transcriptome data from *Magnolia maudiae*: XQWC and *Magnolia grandiflora*: WBOD (Matasci et al., 2014); and the transcriptome data of *Liriodendron tulipifera* from the Floral Genome Project (Liang et al., 2006). Eleven DNA regions were targeted and amplified: five (partial) nuclear genes: AGT1, GAI1, LEAFY, PHYA, SQD1; three (partial) chloroplast genes: *ndhF, rbcL* and *trnK*; and three chloroplast intergenic spacers: *atpB-rbcL, ndhF-rpl32* and *psbA-trnH*. GenBank accession numbers per DNA region can be found in Appendix C. The nuclear regions were reconfirmed to be single copy in the *Magnolia* genome by BLAST searches against the *Magnolia kobus* genome (Park et al., 2017). PCRs were performed on a total volume of 25 μl under the following

conditions: 2 min at 95 °C; 35 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 90 s; 72 °C for 6 min. The PCRs contained 0.2 μM forward primer, 0.2 μM reverse primer, approximately 5 ng/ml of DNA (suspended in 1 × TE buffer) and 2 × DreamTaq MasterMix (Thermo Fisher Scientific, Waltham, MA, USA) per reaction. PCR products were run on a 1% agarose gel, stained with ethidium bromide and visualized under UV-light. Sanger sequencing was executed by Macrogen Europe (Amsterdam, the Netherlands).

2.2. Phylogenetic analyses and divergence time estimation

The raw abi-files obtained from Macrogen were assembled in Geneious v. 8.1.9 (https://www.geneious.com, Kearse et al., 2012). The forward and reverse reads were trimmed with an error probability limit of 0.01, sequence read direction was set, and the reads were assembled *de novo*. Ambiguous regions were annotated using the "Find Heterozygotes" plugin set to a peak similarity of 50%. Visual inspection of the assembly was executed while checking the disagreements between forward and reverse strand, as well as annotated heterozygous sites. The sequences were aligned using the MUSCLE plugin in Geneious. The previously marked inversions in *trnK* and *psbA-trnH* (Azuma et al., 1999; Kim and Suh, 2013) were replaced by their reverse complement and coded as binary characters to acknowledge their presence, while not biasing the result given the low sequence divergence of the family overall (Kim and Suh, 2013). The chloroplast genes were concatenated

in Geneious as these fragments are linked on the circular chloroplast genome. The distance matrix of each multiple sequence alignment was extracted using Geneious, meaning that ambiguous sites and gaps (in their full length) were taken into account. PartitionFinder v. 2.1.1 (Lanfear et al., 2017) was used to partition data. Candidate data blocks in the partitioning analyses respected coding and non-coding regions, including the three codon positions within the coding regions. Branch lengths were set to linked and the comparison of partitioning schemes used the greedy algorithm (Lanfear et al., 2012). Gaps were coded using Seqstate v. 1.4.1 (Müller, 2005), with IndelCoder set to Modified Complex Indel Coding (MCIC). Summary statistics for each of the eleven separate fragments were obtained using PAUP v. 4.0a164 (Swofford, 2002). To acquire the separate gene trees, phylogenetic analyses for each of the six alignments (i.e., a single concatenated chloroplast and five nuclear alignments) were run with MrBayes v. 3.2.6 (Ronquist et al., 2012) through the CIPRES web portal (Miller et al., 2010) and visualized using TreeGraph2 v. 2.15 (Stöver and Müller, 2010). *Liriodendron tulipifera* was used as outgroup and partitions followed those found using PartitionFinder. Substitution models for each data partition were estimated during the MCMC runs (so-called "model jumping") by sampling across model space and integrating out over all possible models (Huelsenbeck et al., 2004). Two independent runs were performed, each with four MCMC chains of 10 000 000 generations, of which every 5000th generation was sampled. MCMC diagnostics of the gene trees were run using the package RWTY v. 1.01 (Warren et al., 2017) in R v.3.6.1. (R Core Team, 2019). The first 25% of the sampled trees were discarded as burn-in (i.e. 500 out of 2001 trees).

The six alignments (i.e., a single concatenated chloroplast and five nuclear alignments) were also used to infer a calibrated phylogenetic hypotheses using BEAST v. 2.5.2. (Bouckaert et al., 2019) for all 100 accessions. Given the incongruences found among the six alignments that generated the six gene trees, the package *BEAST2 (Ogilvie et al., 2017) was used to estimate the underlying species tree. The total of 24 partitions (AGT1: 3 partitions; chloroplast: 11 partitions; GAI1: 3 partitions; LEAFY: 2 partitions; PHYA: 3 partitions; SQD1: 2 partitions) were unlinked for substitution parameters and linked per alignment for clock and tree parameters, estimating six clocks and six gene trees that underlie the species tree. Substitution models for each data partition were estimated by model jumping using bModelTest in *BEAST2 (Bouckaert and Drummond, 2017): all the site model parameters were allowed to vary. All six clock models were estimated using random local clocks (Drummond and Suchard, 2010).

Both the *Magnolia* stem node and crown node were calibrated. We used fossils of seeds and fructifications because they are the most diagnostic and reliably identified (Azuma et al., 2001; Hebda and Irving, 2004). Firstly, a uniform prior was placed on the *Magnolia* crown node using *Magnolia tiffneyi*, described from fossilized seeds of the Oligocene Clarno Formation of Oregon (Manchester, 1994). This fossil taxon shares seed morphology synapomorphies with the extant *Magnolia grandiflora* and extinct *Magnolia septentrionalis* (Manchester, 1994; Tiffney, 1977). However, because the sister group of section *Magnolia* remains unresolved in the family-wide phylogeny (Azuma et al., 2001; Kim and Suh, 2013; Nie et al., 2008; Wang et al., 2020), the stem node of section *Magnolia* coincides with the crown node of the genus *Magnolia*. Hence, for the crown node of the genus *Magnolia* we used a uniform prior with the minimum set to 44 mya. The maximum bound of this uniform prior for *Magnolia* was set to be 70 mya, the estimated age for Magnoliaceae by Wikström et al. (2001). Secondly, a prior was set on the stem node of *Magnolia* using the *Archaeanthus* fossil (Dilcher and Crane, 1984), which is (one of) the oldest, well-documented and studied fossil collections assigned to Magnoliaceae (Doyle and Endress, 2010; Romanov and Dilcher, 2013), placed in the uppermost Albanian-mid-Cenomanian of the Cretaceous (ca. 98 mya). Because the fossil is most convincingly placed as a sister lineage to the Magnoliaceae (Doyle and Endress, 2010; Massoni et al., 2015), its age was set as the maximum age for the crown node of the family Magnoliaceae. To allow for younger ages, the

minimum bound for this split was set to 44 mya, again conforming to the oldest, morphologically well-studied fossil linked to the extant members of *Magnolia*.

*BEAST runs were set to continue indeterminately, and the resulting parameter values were tested periodically for convergence as indicated by the effective sample sizes (ESS) using Tracer v. 1.7.1 (Rambaut et al., 2018). A final number of 2 000 000 000 generations was needed to reach ESS values > 100 and a burn-in of 10% was shown to be necessary. To study the topology, the species trees were visualized using DensiTree v. 2.5.2 (Bouckaert and Heled, 2014) for which a resampling of 20 000 was allowed using LogCombiner v. 2.5.2 (Rambaut and Drummond, 2019), due to memory constrains of the DensiTree software. To visualize the estimated age of each node, the 2 000 000 000 species trees were summarized using TreeAnnotator v. 1.8.2 (Rambaut and Drummond, 2015) with a burn-in of 10% as found by Tracer, into a maximum-clade-credibility summary tree, whereby the node heights represent the mean heights. The summary tree was visualized using Figtree v. 1.4.2 (Rambaut, 2014).

2.3. Testing of biogeographical hypotheses

Ancestral range estimation of *Magnolia* in the Caribbean was conducted using the R package 'BioGeoBEARS' (Matzke, 2013; Matzke, 2014). We used the calibrated subtree only with the members of subsections *Cubenses* and *Talauma*, excluding the *M. virginiana* subsp. *oviedoae* accession of Cuba. This exclusion was executed because of two reasons. Firstly, to keep focus on the Caribbean taxa. Secondly, the sampling of *M. virginiana* was too incomplete to answer questions on the ancestral range of *M. virginiana* subsp. *oviedoae* (Azuma et al. 2011). We defined six geographic areas: from North to South: Mesoamerica (M), Cuba (C), Hispaniola (H), Puerto Rico (P), the Lesser Antilles (L) and South America (S). We analysed our dataset under three models: "DEC" (Dispersal-Extinction-Cladogenesis; Ree et al., 2005; Ree and Smith, 2008), "DIVALIKE" and "BAYAREALIKE". The models with the "jump dispersal" or founder-event speciation (+J) were interpreted with caution, given the ongoing debate on its usage (Matzke, 2014; Ree and Sanmartín, 2018). The fit of the models to the dataset was compared using the AICc criterion (Burnham and Anderson, 2002).

2.4. Haplotype network analyses

We conducted a network analysis of the (derived) haplotypes using the R package pegas (Paradis, 2010) to investigate the species delimitation of the sympatrically occurring *M. minor* and *M. oblongifolia* in greater detail. *Magnolia cristalensis*, also occurring in sympatry with *M. minor* and *M. oblongifolia*, is morphologically and phylogenetically distinct from the latter two (see Figs. 3 and 4 and Appendix D), and was thus not included in the investigation. *Magnolia orbiculata*, the third *Magnolia* of the subsection *Talauma* in Cuba, was used as an outgroup in the analysis. This haplotype network analysis was executed, because 1) heterozygous sites represented by ambiguous IUPAC characters were unaccounted for in the Bayesian phylogenetic analyses, yet clearly present in a set of the sequenced DNA regions when looking at the raw alignments; 2) the turbulent taxonomic past of the species and 3) newly observed intermediate morphologies in the field. For the nuclear single copy genes, the genotypes were phased to their haplotypes using DnaSP v. 6.12.03 (Rozas et al., 2017) using the PHASE algorithm (Stephens and Donnelly, 2003; Stephens et al., 2001) run per gene with the default MCMC settings and the assumption of no recombination within one gene. Each of the included samples was coded by its species identification and population. The first consisted of the options *M. minor*, *M. oblongifolia* or mixed morphology, whereas the latter was composed of a three-letter abbreviation representing its collection site: CGU, CMU, CUP, LME, MIB, NDT, PCR, YAM and YUM (Appendix A).
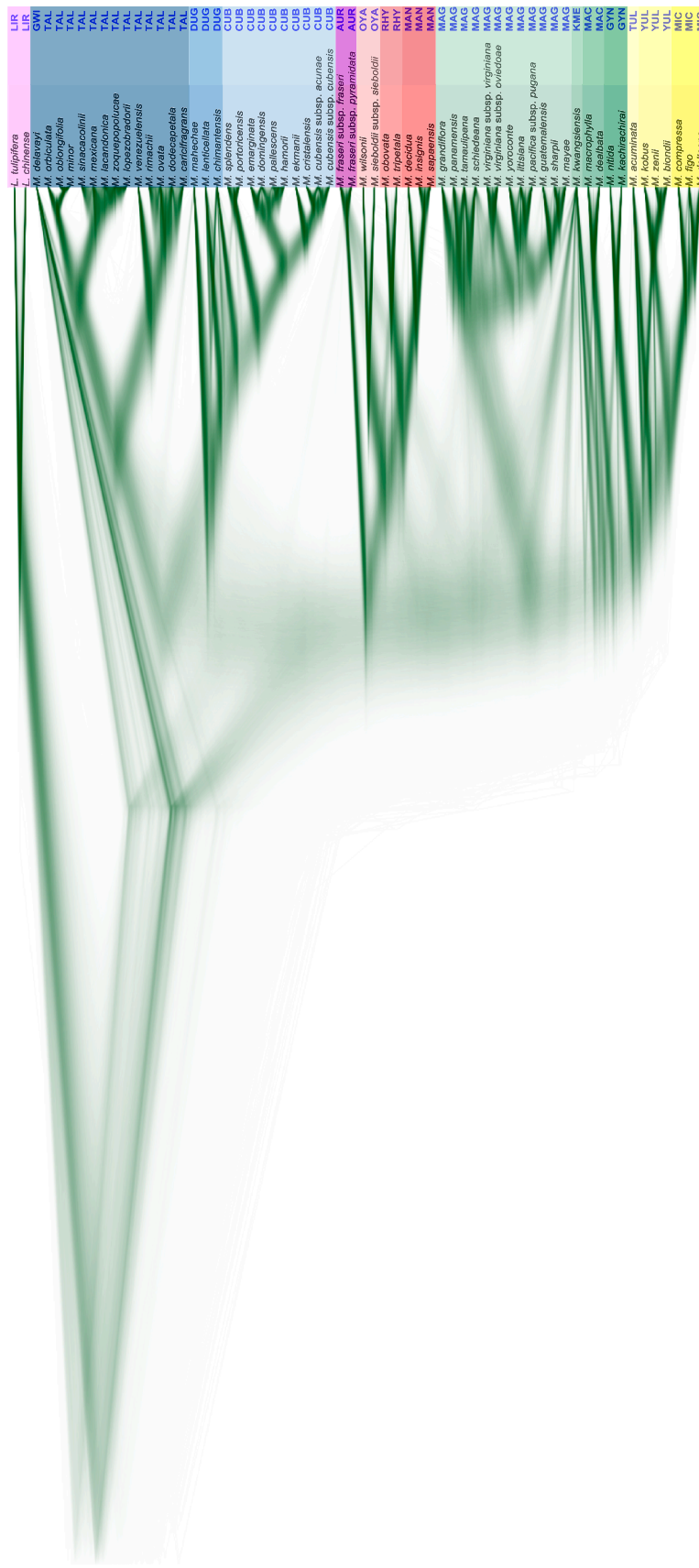
Fig. 3. Species trees of the Magnoliaceae species comprising both the nuclear (i.e. AGT1, GAI1, LEAFY, PHYA, SQD1) and the chloroplast (i.e. *atpB-rbcL, ndhF, ndhF-rpl32, psbA-trnH, rbcL, trnK*) sequences visualized using DensiTree (900001 species trees after resampling). *Liriodendron* and *Magnolia* species are classified according to their lowest possible rank published in Figlar and Nooteboom (2004), represented by a three-letter abbreviation.
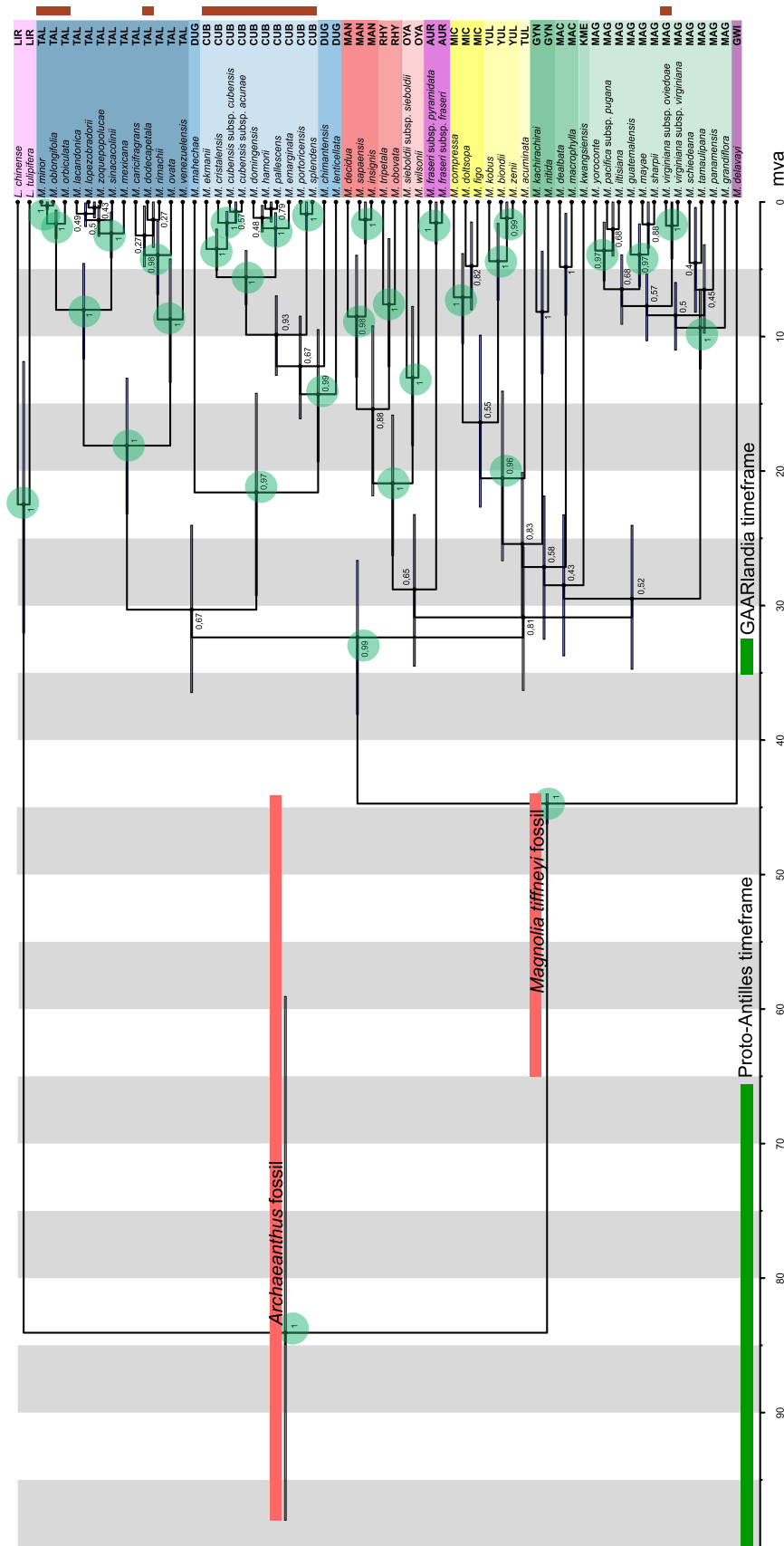
Fig. 4. Calibrated summary tree of the Magnoliaceae species comprising both the nuclear (i.e. AGT1, GAI1, LEAFY, PHYA, SQD1) and the concatenated chloroplast (i.e. *atpB-rbcL, ndhF, ndhF-rpl32, psbA-trnH, rbcL, trnK*) sequences. Node labels represent the posterior probabilities; when considered supported (i.e. > 0.95) the node label is circled in green. Node bars represent the 95% interval of the age estimates. *Liriodendron* and *Magnolia* species are classified according to their lowest possible rank published in Figlar and Nooteboom (2004), represented by a three-letter abbreviation. Caribbean *Magnolia* species are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

Support values (pp = posterior probability) and age estimates for clades, observed after Bayesian phylogenetic inference of 100 Magnoliaceae accessions representing 62 species. Ages are expressed as mya (million years ago). 95% HPD: 95% Highest Posterior Density intervals. All clades including Caribbean species of *Magnolia* are included. At family level, only the clades supported by pp higher than 0.95 are tabulated. For each clade, the underlining gene trees with pp higher than 0.70 are listed (Appendix D). MRCA = Most Recent Common Ancestor. Asterisks * indicate calibrated nodes.

Clade	pp	Age: mean	Age: 95% HPD	Gene trees
Family Magnoliaceae	1	84.06*	98*–59	D1–6
Genus *Liriodendron*	1	22.49	32–12	D1–6
Genus *Magnolia*: *M. delavayi* most basal	1	44.73*	46–44*	D2, D3
Genus *Magnolia sine M. delavayi*	0.99	32.37	38–27	D2, D3
Section *Talauma*	0.67	30.31	36–24	D1
Subsection *Talauma*	1	18.11	23–13	D1, D3–6
Subsection *Talauma*: split Cuba & Mexico	1	8.02	12–5	D1–2, D4–5
Subsection *Talauma*: Lesser Antilles & South Am. clade	0.98	3.94	7–1	D1
Subsection *Cubenses* + *Dugandiodendron*	0.97	21.59	29–14	D1, (D2, D4)
Subsection *Cubenses*: split from *M. chimantensis*	0.67	12.21	16–8	D1–2, D4
Subsection *Cubenses*: MRCA of Puerto Rico and Hispaniola	0.93	9.87	13–7	D1–3, D5
Subsection *Cubenses*: MRCA of Cuba and Hispaniola	1	5.59	8–4	D1, D4
Subsection *Cubenses*: MRCA of *M. ekmanii* and Cuba	1	3.49	5–2	D1, D5–6
Section *Auriculata*	1	1.56	3–2	D1–6
Section *Rhytidospermum* + Section *Manglietia*	1	20.91	26–16	D3, D5
Subsection *Oyama*	1	13.06	18–8	D1–5
Subsection *Rhytidospermum*	1	7.61	12–3	D1, D3–5
Section *Manglietia*	0.98	8.52	13–4	D1, D3
Section *Macrophylla*	1	4.83	8–1	D1–2, D5
Section *Tulipastrum* + *Yulania* + *Michelia*	0.96	20.54	27–14	D2, D5
Section *Gynopodium*	1	8.17	13–4	D1–3, D5–6
Section *Yulania*	1	4.40	7–2	D1–5
Section *Michelia*	1	7.09	11–4	D1, D3–6
Section *Magnolia*	1	9.35	12–6	D1–2, D5

3. Results

3.1. Phylogenetic analyses and divergence time estimation

The six alignments comprised 12257 base pairs in total. The concatenated chloroplast sequence alignment comprised 8351 base pairs, which corresponds to about 5% of the full *Magnolia* chloroplast genome (Shen et al., 2018). The percentage of parsimony informative characters (PIC) of the different amplified regions are depicted in Fig. 2.

Gene trees for each of the amplified regions and for the concatenated chloroplast alignment are compiled in Appendix D, whereby the pairwise distance matrix of the Caribbean magnolias is tabulated in Appendix E. Partitioning schemes for all analyses are summarized in Appendix F. The DensiTree species tree and the calibrated multi-species coalescent summary tree are depicted in Figs. 3 and 4, respectively. The time calibrations of significant clades, all the main nodes containing Caribbean taxa and the supported nodes of the non-Caribbean taxa (i.e. with a posterior probability higher than 0.95) from Fig. 4 are depicted in Table 2.

3.2. Testing of biogeographical hypotheses

The output of the BioGeoBEARS analysis is tabulated in Appendix G. The lowest AICc score was for the "DEC + J" model. Out of the three models without the + J parameter the "DIVALIKE" model best fits the data on the Caribbean magnolias. Ancestral range estimation results constructed using the "DIVALIKE" model and a schematic overview of the six defined geographic regions are visualized in Fig. 5.

3.3. Haplotype network analyses

Fig. 6 illustrates the relationships among the sequenced chloroplast and simulated nuclear haplotypes present in the data for the *M. minor* and *M. oblongifolia* species complex. In the chloroplast haplotypes we can allocate the H_IV haplotype to *M. oblongifolia* given the pure population of MIB and the H_II to *M. minor* given the pure population of YUM (Fig. 6A). CGU, YAM, LME, all surrounding populations of MIB with individuals morphologically identified as *M. minor*, have the same haplotype as defined for *M. oblongifolia*. In AGT1 (Fig. 6B), two more derived haplotypes are found in the MIB population (i.e., H_VIII and H_VII). For GAI1 (Fig. 6C) the haplotype of the MIB population is found in all populations around (CGY, YAM, CUP. LME, NDT). For PHYA (Fig. 6D) there is one haplotype only found in MIB (i.e., H_III).

4. Discussion

4.1. Species delimitations

We find genetic synapomorphies delimiting 14 out of 15 Caribbean taxa across the different gene trees (Appendix D), except for the *M. minor* and *M. oblongifolia* species complex. Although the number of genetic synapomorphies is low (Appendix E), the data support the 14 taxa to be considered separately evolving metapopulation lineages (de Queiroz, 2007); even a single genetic synapomorphy can result in monophyly and hence be defined as a species under the general lineage species concept (de Queiroz, 2007).

For all the Caribbean taxa we included at least one accession per population in our sampling, which in most cases rendered no to very little intraspecific variation (Appendix E). Most of the chloroplast intraspecific variation that shows high pairwise distance numbers denoted in Appendix E represents gaps, especially in the non-coding DNA such as the poly-A sequence in *psbA-trnH*. An exception is *Magnolia dodecapetala*: the genetic differences between the populations of Martinique and Guadeloupe in all six alignments, even for the conserved chloroplast sequences, show a similar extent as differences between within-island sister species pairs (Appendix E).

The relationships between *M. domingensis, M. emarginata, M. hamorii* and *M. pallescens,* all occurring on Hispaniola, show a very clouded network (Fig. 3) and low posterior probabilities in the summary tree (Fig. 4, Table 2). This could partly be due to the species circumscription of *M. domingensis* which needs revision: the Haitian accession identified as *M. domingensis* MA2167 (*Ekman 2810,* B herbarium) from the Massif du Nord (part of the Hispaniolan central mountain massif) is not inferred as sister to the *M. domingensis* accessions from the Cordillera Central
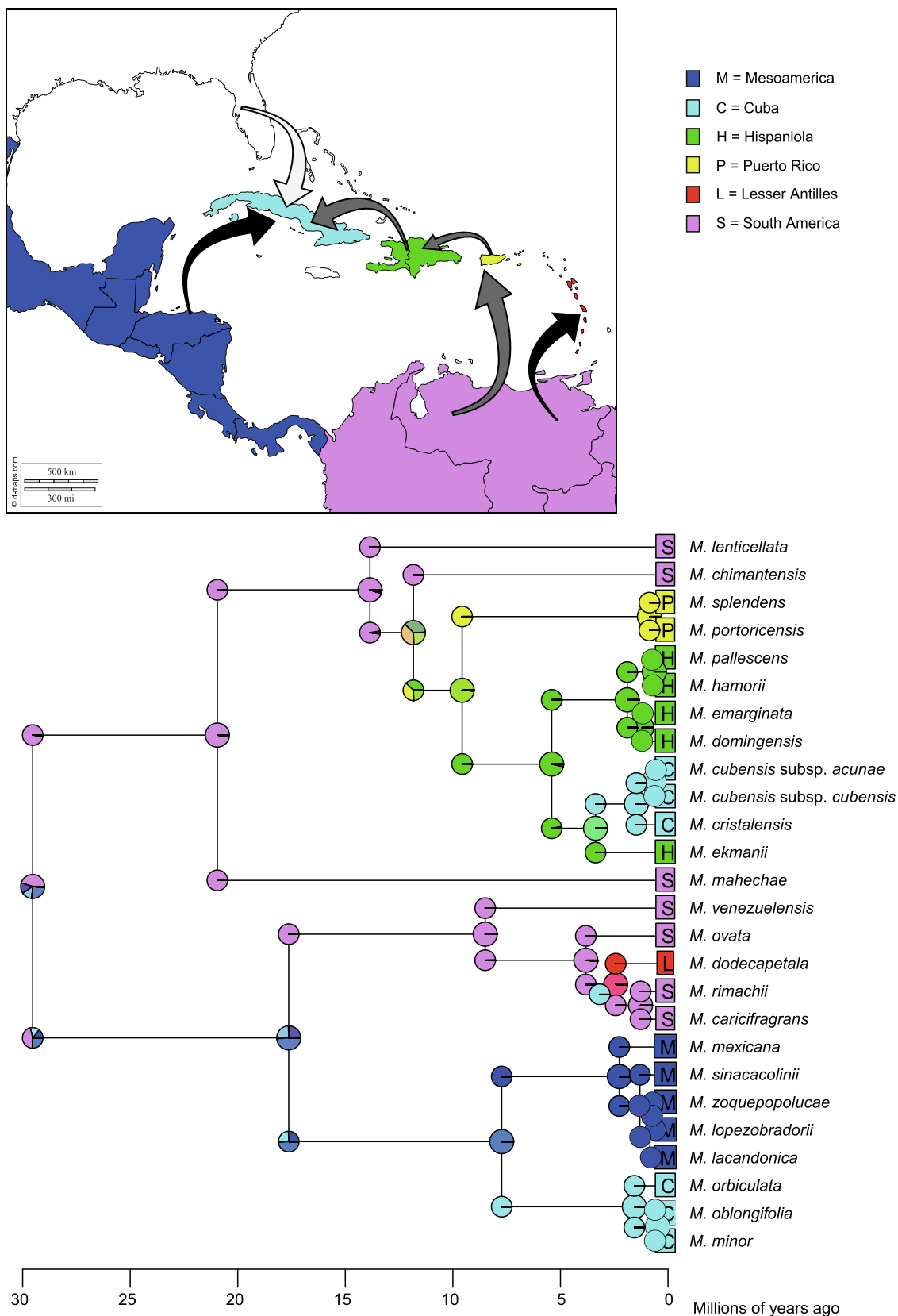
Fig. 5. Ancestral range estimation results of *Magnolia* taxa from the subsection *Cubenses* and subsection *Talauma* subtree, constructed in BioGeoBEARS using the "DIVALIKE" model. Each colour represents one of the six defined geographic regions, illustrated by the map and the legend in the top of the figure. Most likely dispersal routes are illustrated on the map by different coloured arrows. The black arrows illustrate the two colonization events for *Magnolia* of the section *Talauma* subsection *Talauma*. The grey arrows illustrate the trajectory of *Magnolia* of the section *Talauma* subsection *Cubenses*. The white arrow illustrates the colonization of *Magnolia virginiana* subsp. *oviedoae* from the section *Magnolia*. For each of the four colonization events, the exact source areas are unknown.
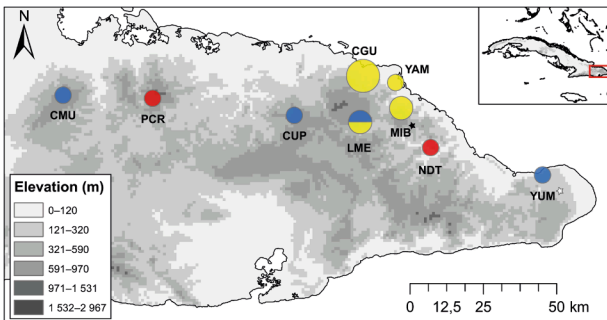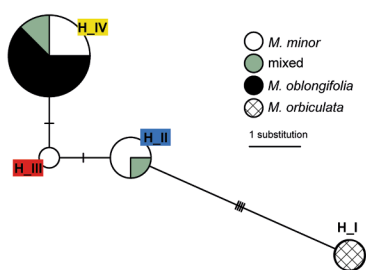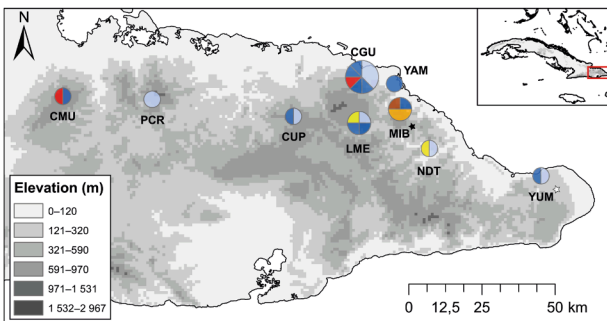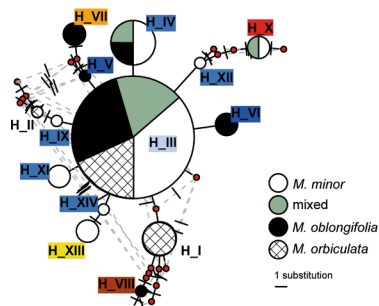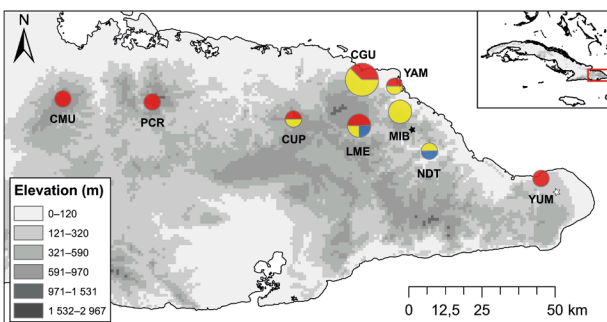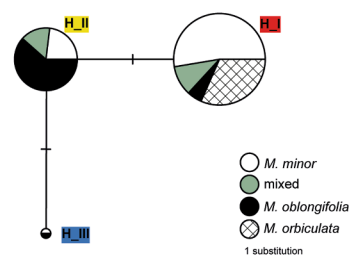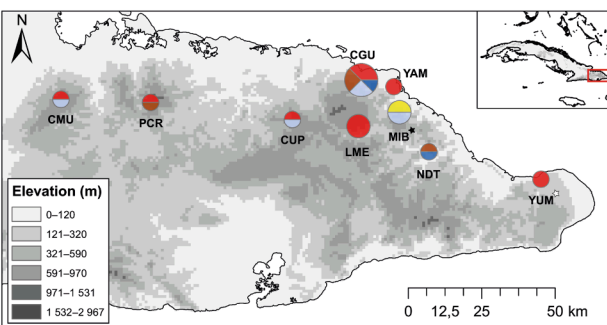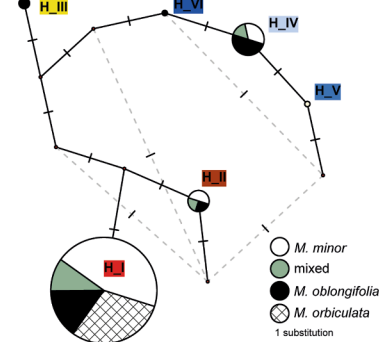
Fig. 6. Haplotype networks and haplotype pie charts per population based on the parsimony informative characters found in four alignments of the Cuban sympatric species *Magnolia minor* and *Magnolia oblongifolia*. The size of the pie charts corresponds to the sample size of that haplotype or population. *Magnolia orbiculata* was included as an outgroup in the haplotype analyses, as its species delimitation is not questioned due to its geographical, phylogenetic and morphological distinctness. The MIB population (black star) is labelled to have individuals only with a *M. oblongifolia* morphology and the CMU, YAM and YUM populations were labelled to have only individuals with the *M. minor* morphology, of which YUM is the most isolated (white star). For the chloroplast (A), GAI1 (C) and PHYA (D) haplotypes, each haplotype is assigned a different colour. For the AGT1 haplotypes (B) H_III was the most frequent found haplotype (light blue). The AGT1 haplotypes that differ with only one substitution from H_III are given shades of blue (medium blue for those with the *M. minor* morphology and dark blue for those with the *M. oblongifolia* morphology). The AGT1 haplotypes that differ with more than one base pair (i.e. H_XIII, H_VII, H_VIII and H_X) are coloured from yellow to dark red with increasing redness according to increased number of substitutions compared to H_III. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(also part of the Hispaniolan central mountain massif) in the Dominican Republic (Appendix D1, D2, D4). Unfortunately, the DNA extracted from the Haitian type specimen (*Nash 1081*, BM herbarium) was fragmented and could not be used for this study to support this proposed taxonomic change further with molecular data. Since the collections of Ekman in 1925, no new collections of *Magnolia* have been made in the Massif du Nord, nor in the Montagnes Noires of Haiti. It is clear from both this study and a previous one using microsatellite markers (Veltjen et al., 2019) that the four species evolved recently.

Similarly, the relationships among the three Cuban taxa of subsection *Cubenses* show a clouded network in Fig. 3. Interestingly, based on chloroplast data (Appendix D1), *M. cubensis* subsp. *acunae* and *M. cristalensis* appear to be sister taxa – a signal of six genetic synapomorphies shared between *M. cubensis* subsp. *acunae* and *M. cristalensis*, of which four are in the *ndhF* gene. This signal outweighs the single synapomorphy found between the two *M. cubensis* subspecies and the single synapomorphy that brings together *M. cubensis* and *M. cristalensis* (Appendix E). One synapomorphy in the nuclear gene GAI1 (Appendix D3) in favour of the two *M. cubensis* subspecies being sister taxa without any contrasting signals, outweighs the chloroplast data in the summary tree, albeit with low support (Fig. 4). It was expected that there would be a great genetic similarity between the two subspecies given the morphological similarity (Hernández Rodríguez, 2014; Imchanitzkaja, 1991; Imchanitzkaja, 1993) and phytogeographical patterns (Borhidi, 1996). As the current data cannot give us a convincing answer on their sister relationship, they do show that the genetic discrimination among the two subspecies is similar to that between other recognized sister species pairs (Appendix E). This information, together with the large geographical distance between them (ca. 400 km), does call for a revision of their subspecies status to be lifted to the species level.

Magnolia minor and *M. oblongifolia* show a very recent, yet supported, sister species relationship in the summary tree (Figs. 3, 4), but this is not expressed as a clear alignment of genetic synapomorphies and morphology in any of the gene trees (Appendix D, E). This contrasts with the morphological differentiation between the two, which is at least as equally distinct as between most of the other sister-species pairs of Caribbean magnolias (see Fig. 1): *M. oblongifolia* has a distinct oblong-elliptic leaf shape, compared to the orbicular or obovate leaves from the other Cuban magnolias; a rhombic fruit shape, compared to the ellipsoid fruit of *M. minor*; and the small tree size, in contrast with the large tree size of *M. minor* (Palmarola et al., 2016). The high number of ambiguous characters in the sequences of the species' representatives did also not translate into a clear haplotype pattern that aligns with their morphological identifications (Fig. 6). The occurrence of a range of different haplotypes (Fig. 6) and the long branches of the accessions of these species in many of the gene trees (Appendix D3, D4, D5, D6) do show that this species complex has a high amount of genetic variation compared to the other Caribbean accessions; yet most of this genetic variation does not align with the currently defined morphological differences. Two possible explanations for this genetically variable species complex are: 1) they are two former species that are now hybridizing successfully for already more than one generation (Schley et al., 2020), or 2) sympatric speciation is in process, whereby only the genes under selection will give a clear differentiation for the two morphological entities we observe (Smadja and Butlin, 2011).

An intraspecific study of *M. virginiana* (Azuma et al., 2011) proved that *M. virginiana* subsp. *oviedoae* did not have a specific chloroplast haplotype linked to its population, in contrast to other chloroplast haplotypes found in the wider distribution of the species. In our study, when comparing LEAFY and *ndhF-rpl32* sequences from the Cuban sample with those from *M. virginiana* of Florida (Appendix A) we do find one and two substitutions, respectively. A more profound sampling of the species and, preferably, comparative genomic data would be necessary for a more conclusive answer on its taxon delimitation. At this point we cannot exclude the possibility that the difference in morphology, and thus the status of a subspecies, is merely phenotypic

plasticity in response to the colonized marsh habitat (Oviedo Prieto et al., 2006; Palmarola-Bejerano et al., 2008; Testé, 2018).

4.2. Classification

Talauma is the section of interest when discussing magnolias in the Caribbean. In all the nuclear alignments (i.e., Appendix D2–D6) this section does not hold as a supported clade, which results in a low posterior probability in the summary tree (Fig. 4, Table 2). This questions the current classification (Figlar and Nooteboom, 2004) and is not in line with the previous studies executed on chloroplast DNA only (Kim and Suh, 2013, Wang et al., 2020).

The results also demonstrate that subsection *Cubenses* is nested within a paraphyletic subsection *Dugandiodendron* (Fig. 3, Fig. 4). This was formalized by the merger of subsections *Dugandiodendron* and *Cubenses* into section *Splendentes* by Wang et al. (2020), who took into consideration our preliminary results (Veltjen et al., 2016). It is expected that the support of the clade comprising subsection *Dugandiodendron* and *Cubenses* would be even higher, should *M. mahechae* be resequenced. We expect this because we observe a conflicting position of this sequenced herbarium type specimen between the gene tree of *ndhF* (Kim et al., 2001), where it is in the clade comprising members of subsection *Talauma*, and the gene tree of *matK* (Azuma et al., 2001), where it is in the clade comprising of members of subsection *Dugandiodendron*. Nonetheless evident from our data, the paraphyletic relationship should be considered a preliminary result due to few accessions of subsection *Dugandiodendron* sequenced so far (Rivers et al., 2016), and the limited number of genetic regions sequenced for the included taxa of subsection *Dugandiodendron* (Appendix B). With more intensive sampling, both of taxa and genes, it is expected that either subsection *Cubenses* will be the crown group of subsection *Dugandiodendron* or that a genetic synapomorphic signal for the members of subsection *Dugandiodendron* will be recorded and that the two subsections become sister clades. The first would be supported by the morphological synapomorphy of anther tip embedment (Figlar and Nooteboom, 2004). The latter would be supported by the difference in woodiness of the fruit pericarp (Figlar and Nooteboom, 2004).

Within subsection *Talauma*, the sampling across the family is far from complete (Rivers et al., 2016), yet this dataset separates the Mesoamerican taxa from the South American ones in two well-supported clades.

In Magnoliaceae, sister-relationships among well-supported and recognized clades (Table 2) other than section *Talauma* are inconsistently placed when comparing the different gene trees (Appendix D), hence, their relationships remain unresolved in the summary tree (Fig. 4). This result agrees with previous Sanger sequencing based studies (Azuma et al., 2011 and precursors; Kim and Suh, 2013 and precursor; Nie et al., 2008). The inconsistent topology of the clades in Magnoliaceae depending on the genetic region studied contrasts with the robustness of the separate clades. This puts forward the hypothesis that either we are dealing with such a low sequence divergence that genetic homoplasies combined with few gene fragments quickly disturb the analyses to recover the true relationships between taxa, or that evolutionary novelties defining the clades evolved in a rapid evolutionary timespan resulting in incomplete lineage sorting. With the era of phylogenomics (McKain et al., 2018, Young and Gillung, 2019) knocking on the door of the Magnoliaceae phylogeny research (Park et al., 2017; Veltjen et al., 2018; Wang et al., 2020), solving the overall relationships among the clades is within reach. The phylogenomic results of Wang et al. (2020) based on chloroplast data are promising in terms of solving the deeper relationships, but our study indicates that the inclusion of nuclear genomic data will be key.

4.3. Justification of the fossil calibrations

When calibrating a phylogenetic hypothesis, the priors can greatly

influence the results, as they, together with the sequence data, determine the range in which the posterior values can be found. In the calibrated summary tree (Fig. 4) we observe that for both calibrated nodes the set maximum for the crown node of the Magnoliaceae, and set minimum for the crown node of *Magnolia*, are within the 95% HPD (Table 2). The Magnoliaceae node, calibrated by the prior setting *Archaeanthus*, has a large 95% HPD, while the *Magnolia* node, calibrated by the prior setting of *M. tiffneyi*, shows a very short 95% HPD. For the latter, the data push the posterior towards younger ages, towards the minimum bound of the prior distribution.

We placed *Archaeanthus* on the crown node of the Magnoliaceae, representing the maximum age of the node, following the placement of the fossil being sister to the Most Recent Common Ancestor (MRCA) of the extant Magnoliaceae (Doyle and Endress, 2010). This reasoning is followed by other authors, however, translated differently given distinct research questions and sampling design: other studies often position *Archaeanthus* on the stem node of the Magnoliaceae, representing the minimum age of the node (e.g. Massoni et al., 2015; Pirie and Doyle, 2012). In contrast, a detailed morphological study of the fruit pericarp, executed by Romanov and Dilcher (2013), considers *Archaeanthus* to be a stem relative of *Liriodendron*, rather than of Magnoliaceae, which implies the fossil could be placed on the crown node of the Magnoliaceae family as a minimum age, not a maximum age. According to Massoni et al. (2015), this conclusion was the result of redundant characters usage and inappropriate outgroups, and hence invalid. A last argument in the debate on the fossil calibration of the Magnoliaceae node is a less commonly used Cretaceous fossil: *Liriodendroidea*, which is reported to be reliably linked to the family (Nie et al., 2008) and estimated to be 93.5 mya (Frumin and Friis, 1996; Frumin and Friis, 1999). The fossil is associated with *Liriodendron* which implies it could be used as a minimum age for the Magnoliaceae crown node as well. However, given the more in-depth study of the morphology of *Archaeanthus*, the higher amount of fossil structures available and the more frequent usage by a range of researchers who each assessed its reliability for fossil calibration linked to the Magnoliaceae or its related plant-families, we decided to use *Archaeanthus* instead of *Liriodendroidea*.

The crown node age of *Magnolia*, calibrated with the minimum age of 44 mya, represented by the fossil *M. tiffneyi*, is being pushed towards the minimum bound of the prior distribution, apparent from its narrow confidence interval (Fig. 4, Table 2). This could either be due to a discrepancy between the placement and/or age of the fossil and the sequence data, or an unforeseen interaction with the first calibrated node which forces a younger age downstream in the tree, given that 98 mya was set as a maximum bound, rather than a minimum bound for the node calibration as discussed in the previous paragraph. Considering the potential discrepancy in sequence diversity and the fossil placement/date: although morphologically resembling the extant *M. grandiflora*, it is possible that the fossil belongs to an extinct *Magnolia* stem lineage with homoplasious seed morphology characters. Considering the potential influence of the two calibration points: it is possible that the two calibrations interact, yet when the analysis was run sampled from the prior only (hence the sequence data are empty alignments), the ages of the nodes have 95% HPD ranges that encompass their full prior range. It is also possible that the fossil placement and dating is correct, and that there is no interaction between the set priors, whereby the low sequence evolution is in such extent that we obtain underestimations of the clade ages based on the sequence data alone (Barba-Montoya et al., 2018). The placement of the *M. tiffneyi* fossil ideally should be on the bifurcation between the section *Magnolia* and its sister clade. However, in the most recent plastid phylogenomic analysis (Wang et al., 2020) and in our analysis (Fig. 4) this relationship is not supported. Older relationships would result from placing this fossil on a more recent node in the family phylogeny, yet we would expect that the sequence data would still push the age of this node towards the minimum of this prior (Table 2).

Lastly, Nie et al. (2008) also used the *Archaeanthus* fossil in their calibration, however, in combination with the Miocene *M. latahensis* fossil (Golenberg et al., 1990). We decided not to use the latter in our analysis, given the conflicting results retrieved from two amplified regions of this fossil, casting doubt on its placement (Golenberg et al., 1990; Kim et al., 2004).

4.4. Biogeographical history of the Caribbean Magnolia: Mainland vs. Islands

The obtained molecular phylogenetic hypotheses (Fig. 3, Fig. 4) illustrate four different colonization events of *Magnolia* from the mainland to the Caribbean islands (Fig. 5) that occurred since 16 mya (Table 2), i.e. 12.21 (16–8) mya for subsection *Cubenses*, 8.02 (12–5) mya for the Cuban species of subsection *Talauma*, 3.94 (7–1) mya for *M. dodecapetala* and 1.76 (4–1) mya for *M. virginiana* subsp. *oviedoae*. In this time frame, simulations of the position and state of submergence of the Caribbean islands resemble that of the current geography (Iturralde-Vinent, 2006). The found young ages coincide with the view of Gentry (1982) which lists the Magnoliaceae as Laurasian-derived taxa, which are primarily montane, higher altitude plant groups that are not very species-rich in the Caribbean, a fact interpreted by Gentry as evidence of their recent (Late Tertiary-Quaternary) arrival.

Section *Talauma* subsection *Talauma* has colonized the Caribbean islands twice from two different source areas (Fig. 5). A first colonization is inferred from the sister relationship between the Cuban species of subsection *Talauma*, i.e. *M. orbiculata*, *M. oblongifolia* and *M. minor*, and Mexican species of that subsection. A second colonization event, that of *M. dodecapetala* to the Lesser Antilles from South America, is supported by its nested position in a well-supported clade of South American species: *M. venezuelensis*, *M. ovata*, *M. caricifragrans* and *M. rimachii*. The young age of the dispersal of *M. dodecapetala* from the South American mainland seems plausible, given a) that the Panama isthmus was already formed (Bacon et al., 2015; Montes et al., 2015; Graham, 2003a; Iturralde-Vinent, 2006) allowing the ancestral lineage of subsection *Talauma* to cross from Mesoamerica to the South American mainland – after which it colonized the Lesser Antilles, and b) age estimates of the formation of the Lesser Antilles range from the Middle Miocene: ca. 15 mya until present day (Draper et al., 1994). With this reasoning the estimates of Azuma et al. (2001) that date the MRCA of *M. ovata* and *M. dodecapetala* to be around 24.5 mya would imply the presence of subsection *Talauma* in South America at that time, which is an older age than the oldest ages associated with the Panama isthmus formation (Bacon et al., 2015).

A third colonization of the Caribbean islands by the genus *Magnolia* is inferred from the sister relationship between species of subsection *Cubenses* and South American species of subsection *Dugandiodendron*. Interestingly, the estimated age (Fig. 4, Table 2) of this clade is 21.59 (29–14) mya, or when excluding the doubtful *M. mahechae* 14.29 (19–9) mya, suggesting *Magnolia* to be present in South America at that time. Assuming that the formation of the Panama isthmus is a prerequisite for *Magnolia* to disperse to the South American mainland, this phylogenetic hypothesis coincides with the more ancient timing (i.e., Middle Miocene ca. 15 mya) of the formation of the Panama isthmus (Bacon et al. 2015, Montes et al., 2015), rather than the relatively younger estimations (i.e., 3 mya) of, for example, Graham (2003a) and Iturralde-Vinent (2006). Our results are in contrast with the time-calibrated study of Azuma et al. (2001), where the crown node of the *Cubenses* clade is estimated at ca. 30 mya and its stem node at ca. 35 mya, implying a long-distance overwater dispersal event from Mesoamerica to South America for *Magnolia*, and coinciding with the timing of the GAARlandia hypothesis (Iturralde-Vinent, 2006).

Magnolia. virginiana subsp. *oviedoae* represents a fourth colonization of *Magnolia* to the Caribbean islands, estimated between 4 mya to the present time. The well-documented botanical records of Cuba did not record the species prior to 2006 (Oviedo Prieto et al., 2006) and it is not assumed to have been in Cuba prior 1950 (pers. comm. Ernesto Testé).

The four colonization events (Fig. 4, Fig. 5) significantly postdate

ages that would corroborate the vicariance theory or the GAARlandia hypothesis. By exclusion, overwater dispersal remains the most likely candidate for all four colonization events of *Magnolia* on the Caribbean islands. This seems highly plausible given that magnolias are associated with seed dispersal by birds. For the Caribbean *Magnolia* species there are some observations on current seed dispersers published (Alemañy-Merly 1999, Martínez 1996, Testé 2018), yet a comprehensive study of the Caribbean *Magnolia* seed disperser community would greatly improve the understanding of present and past genetic dispersal patterns (Veltjen et al., 2019).

The inferred dispersal ages are young compared to former biogeographic analyses of the family (Azuma et al., 2001; Nie et al., 2008). Similarly, our age estimates of *Magnolia* dispersal into the Caribbean (Fig. 4, Table 2) fall within the younger ranges of dispersal dates in the review of Nieto-Blázquez et al. (2017), such as the stem node age of 11.12 mya of the endemic cactus genus *Leptocereus* sister to lineages from South America, or the stem node age of 8.64 mya of the endemic legume genus *Stahlia* with sister lineages from Central America. The arrival of the genus *Buxus* to the Caribbean from Central America (Mexico), at ca. 12.3 mya (González Gutiérrez, 2014), the genus *Amphilophium* at ca. 10 mya from Central America (Thode et al., 2019), and of different Euphorbiaceae genera from either Central or South America are also situated around the Miocene (Cervantes et al., 2016).

4.5. Biogeography within the Caribbean islands

Within the Caribbean islands, the phytogeographic relationships between the *Magnolia* species of subsection *Cubenses* illustrate a stepping-stone dispersal (MacArthur and Wilson, 1967) colonization trajectory (Figs. 4 and 5). A first over-water dispersal occurred from South America to Puerto Rico (between 16 and 8 mya), a second from Puerto Rico to Hispaniola (between 13 and 7 mya), followed by a last over-water dispersal from Hispaniola (between 8 and 4 mya) to Cuba (between 5 and 2 mya) (Fig. 4, Table 2). With the relatively young colonization times found in the calibrated summary tree (Fig. 4), the absence of *Magnolia* in Jamaica cannot be attributed to the island being submerged at the time active dispersal between islands was happening. Possible explanations for the absence of *Magnolia* on this island are: ecological constraints (i.e. suitable habitat was not present), or that the genus did not colonize this island by chance.

The Caribbean magnolias from subsection *Cubenses* within each island are more closely related to each other than to magnolias from the other islands, with the exception of *Magnolia ekmanii*, occurring in the Massif de La Hotte of Haiti, that has a well-supported sister group relationship with the Cuban magnolias of subsection *Cubenses*, rather than with the other *Magnolia* species belonging to that section, residing in Hispaniola. The higher affinity of *M. ekmanii* with *M. cubensis* rather than the Dominican magnolias could either be a back-colonisation from a MRCA of *M. ekmanii* and *M. cubensis* from Cuba, or the colonisation route from Hispaniola to Cuba being via the Massif de La Hotte in Haiti towards Cuba. The overall simple colonization pattern suggests that, although overwater dispersal has played an important role for the colonization of *Magnolia* on each different island, afterwards, dispersal between islands was apparently limited, at least up to the present day. This apparent paradox of island monophyly is commonly found in (plant) island biogeographical studies (Herben, 2005) and can be attributed either to niche pre-emption (Silvertown, 2004): (periods of) dispersal probably did occur more than once, yet the new migrants did not establish in competition with the former, established population(s); or alternatively it is also possible that later arrivals did occur but hybridized with the former, yet that this is undetected in the current populations due to loss of alleles by genetic drift or low sampling of individuals or genes (Herben, 2005). Given the tight link of the (semi-)frugivorous dispersal due to the fleshy outer seed coat of *Magnolia,* this island monophyly is most likely be driven by the evolutionary dynamics of the seed disperser populations that enabled the migration between the islands.

5. Conclusion

In conclusion, the generated phylogenetic hypotheses provide genetic synapomorphies supporting the taxon limits of 14 of the 15 Caribbean magnolias; the exception being the *M. minor* and *M. oblongifolia* species complex, which occur sympatrically in Cuba. Furthermore, genetic differences were found between the two subspecies of *M. cubensis* and between the two included populations of *M. dodecapetala* to the same extent as between other Caribbean *Magnolia* species. Therefore, we advise for the taxonomy of the two *M. cubensis* subspecies to be revised, whereas *Magnolia dodecapetala* requires further investigation over its full geographic extent to re-evaluate its diversity and taxonomy. The classification within section *Talauma* is unsupported due to the discrepancies between gene trees, yet the clades representing subsection *Talauma* and subsection *Cubenses* + *Dugandiodendron* are well-supported. The data support four colonization events of *Magnolia* from the mainland to the Caribbean islands since 16 mya, which indicate overwater dispersal to be most likely. Within subsection *Cubenses*, we see an south to north stepping-stone dispersal migration pattern and within island diversification. The exception to this pattern is *Magnolia ekmanii* that is sister to the Cuban magnolias of subsection *Cubenses*. Future studies will benefit from using phylogenomic data of both the chloroplast and the nuclear DNA to elucidate the continued problem of low support between the well-supported clades within the Magnoliaceae family, and need to incorporate a broader mainland taxon sampling. Similarly, genomic-level data may help elucidate whether or not we have sympatric speciation or hybridization in the, for now, genetically undistinguishable species complex of *M. minor* and *M. oblongifolia*.

CRediT authorship contribution statement

Emily Veltjen: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition. **Ernesto Testé:** Resources, Visualization, Writing – review & editing. **Alejandro Palmarola Bejerano:** Conceptualization, Resources, Writing – review & editing. **Pieter Asselman:** Methodology, Supervision, Writing – review & editing. **Majela Hernández Rodríguez:** Resources, Writing – review & editing. **Luis Roberto González Torres:** Conceptualization, Resources. **Lars W. Chatrou:** Writing – review & editing. **Paul Goetghebeur:** Supervision, Writing – review & editing. **Isabel Larridon:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Marie-Stéphanie Samain:** Conceptualization, Investigation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

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