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Phylogenetic origin of dioecious *Callicarpa* (Lamiaceae) species endemic to the Ogasawara Islands revealed by chloroplast and nuclear whole genome analyses

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ABSTRACT

Oceanic islands offer excellent opportunities to study the ecology, evolutionary biology, and biogeography of plants. To uncover the genetic basis of various evolutionary trends commonly observed on these islands, the origins and phylogenetic relationships of the species being studied should be understood. *Callicarpa glabra*, *Callicarpa parvifolia*, and *Callicarpa subpubescens* are evergreen woody plants endemic to the Ogasawara Islands, which are remote oceanic islands located off of the Japanese Archipelago. These species are ideal for studying evolutionary changes on oceanic islands because of their adaptive radiation and shift toward dioecious sex expression. We used a phylogenomic perspective to determine the evolutionary relationship of the three species within the genus and infer their colonization time. Based on the analysis of both chloroplast genomes and 86 nuclear single-copy genes, we found that these three species were monophyletic and embedded in a backbone clade that included multiple East Asian species. The phylogenetic tree based on over 10,000 nuclear genes placed the insular species in the East Asian clade, although the topology did not entirely correspond to the chloroplast tree, probably because of incomplete lineage sorting and interspecific hybridization. The three endemic species were estimated to have diverged from continental species approximately three million years ago (Mya). The results of this study suggested that the ancestor of the Ogasawara endemic species originated from long-distance dispersal from East Asia mainland in the late Pliocene, and then progressively speciated within the islands.

1. Introduction

Oceanic islands are considered “natural laboratories”, providing simple and repeated systems studying ecology, evolutionary biology, and biogeography (Whittaker and Fernández-Palacios, 2007; Whittaker et al., 2017). Many biologists have been fascinated by adaptive radiation, often observed on oceanic islands with unoccupied diverse environmental niches, and the various evolutionary trends common to the flora of oceanic islands called “island syndrome,” such as, evolution of dioecy, woodiness and seed gigantism (Carlquist, 1974; Cody and McC, 1996; Losos and Ricklefs, 2009). During the initial stages of oceanic islands formations, when submarine volcanoes rose above the sea surface, there would have been no terrestrial life present. Organisms on oceanic islands must have established through long-distance dispersal from surrounding continents. This founder effect during immigration limits the amount of genetic diversity within populations on the oceanic islands. In addition, repeated inbreeding within small islands is expected to reduce population genetic diversity (Frankham, 1997). Nevertheless, dramatic phenotypic changes and high degrees of diversification have been observed in the endemic taxa of several islands’ (Carr, 1985; Francisco-Ortega et al., 1996), posing an open question of why such diversification occurs despite limited genetic variation. The first step to examine this paradox is to identify continental species that are most closely related to species groups endemic to oceanic islands, because island syndromes develop from the eco-evolutionary processes that operate on mainlands (Patino et al., 2017). DNA sequences of closely

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related species and ancestor species can also help infer the origin of adaptive polymorphisms in species groups within oceanic islands (Choi et al., 2021).

The Ogasawara Islands are oceanic islands of volcanic origin located approximately 1,000 km from the nearest continental island, Honshu, Japan (Fig. 1A). The Ogasawara Islands formed during the Paleogene and appeared above the sea level before the Middle Pleistocene (Imaizumi, 1984; Kaizuka, 1977). The Ogasawara Islands consist of Chichi-jima, Haha-jima, Muko-jima, and the Volcano Islands. They are located in the subtropical region between 24- and 27-degrees north latitude. Their total area is approximately 80 km^2, which is small in comparison to other oceanic islands worldwide. Nevertheless, approximately 40 % of the native vascular plants [125/280 species (Toyoda, 2014)] are recognized as endemic. Based on the morphology and commonality of the flora, there are assumptions that the ancestors of the plant species on the Ogasawara Islands originated mainly from three regions: the tropical or subtropical regions of Southeast Asia and East Asia, the temperate regions of the Japanese Archipelago, and the South Pacific islands, such as Micronesia and Polynesia (Ono, 1975). This hypothesis has been tested in recent molecular phylogenetic studies to show that *Pittosporum* (Kawakita and Setoguchi, 2020) and *Ochrosia* (Noda et al., 2022) originated from the subtropical East Asian origin, *Rubus* (Yang et al., 2019) from temperate regions of Japan and South Korea, and *Santalum* (Harbaugh and Baldwin, 2007) from Polynesia.

The genus *Callicarpa* L. (Lamiaceae) commonly known as 'beautyberry', consists of approximately 140 perennial woody plants. This genus is distributed from temperate to tropical regions in East Asia, Southeast Asia, Australia, the Pacific Islands, Madagascar and North and Central America (Fig. 1B) (Bramley, 2013; Ohashi et al., 2016). The number of species is particularly high in the temperate forests of East

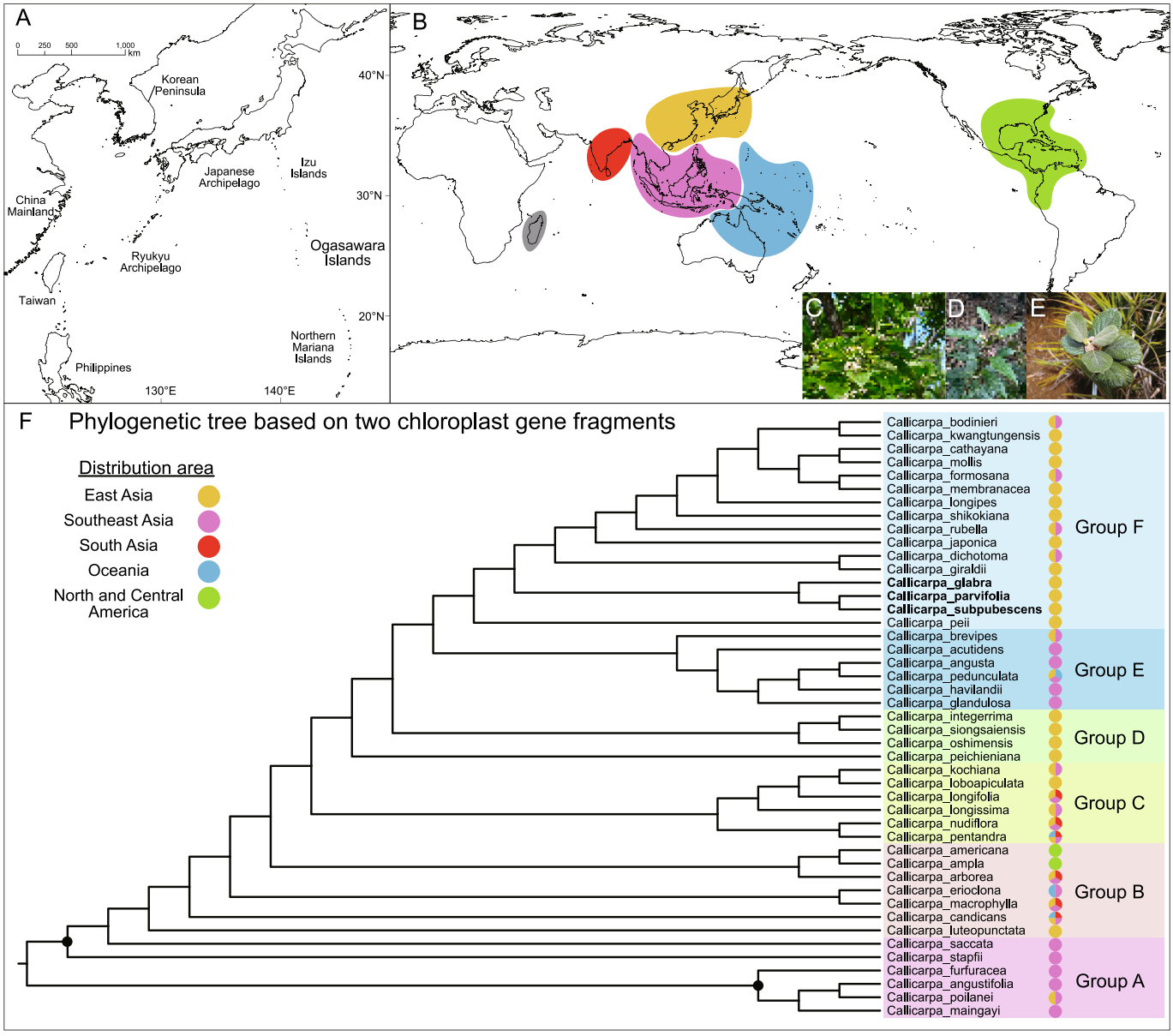

Fig. 1. (A) A map showing the location of the Ogasawara Islands and the surrounding continents and islands. (B) Global distribution map of *Callicarpa*. Images of the three *Callicarpa* species endemic to the Ogasawara Islands: (C) *C. subpubescens*, (D) *C. glabra* and (E) *C. parvifolia*. (F) Phylogenetic tree based on two chloroplast gene fragments inferred from maximum likelihood method. Nodes with high support values (Ultrafast bootstrap > 95 % and SH-aLRT > 80 %) are indicated by black dots. Each of the three circled numbers represents a calibration point. The pie chart next to each species name indicates the distribution area of that species. The distribution area of the species was based on Flora of China (http://www.efloras.org [accessed 12 January 2024]) and Global Biodiversity Information Facility (GBIF.org (18 Jan 2024) GBIF Occurrence Download https://doi.org/10.15468/dl.gz2ee2).

Asia and tropical forests of Southeast Asia (Bramley, 2013). Three species of *Callicarpa* endemic to the Ogasawara Islands are distributed on the islands: *Callicarpa subpubescens* Hook. et Arn. (Fig. 1C) and *Callicarpa glabra* Koidz. (Fig. 1D) and *Callicarpa parvifolia* Hook. et Arn (Fig. 1E). These three species are subtropical semi-evergreen plants endemic to the Ogasawara Islands, with differing ecological and habitat preferences. *Callicarpa glabra* and *C. parvifolia* are narrowly endemic to the Chichijima Islands, with the former growing in the understory of dry scrub and the latter growing in open dry dwarf scrub on rocky ground. *Callicarpa subpubescens* is more widely distributed on the Ogasawara Islands, however, it has been suggested that cryptic species exist on Haha-jima Island because of the variations seen in leaf morphology and flowering time (Kawakubo, 1986; Setsuko et al., 2024b; Sugai et al., 2019). According to a recent population genetic study (Setsuko et al., 2024a), diverse ecotypes, including three endemic *Callicarpa* species found in the Ogasawara Islands, and cryptic species of *C. subpubescens*, may have evolved through adaptive radiation in the late Pleistocene. Although plants in the genus *Callicarpa* are predominantly hermaphrodites, only three morphospecies occurring on the Ogasawara Islands have been reported to be dioecious (Kawakubo, 1990a). Sexual dimorphism, such as dioecy, on oceanic islands at first glance contradicts Baker's Law, that it is more likely for self-compatibility than for self-incompatibility individuals to establish a sexually reproducing colony after long distance dispersal (Baker, 1967; Pannell and Barrett, 1998). It has been suggested that resolving this contradiction requires determining an ancestral sexual strategy (Pannell et al., 2015). Examining the degree of inbreeding between closely related island and continent species that exhibit different sexual expression may be one way to test this hypothesis (Miller et al., 2019). In this way, the *Callicarpa* species endemic to the Ogasawara Islands are considered a taxonomic group suitable for studying adaptive radiation and dioecy that likely evolved on the oceanic islands. Molecular phylogenetic analyses within *Callicarpa* have been based on a small number of nuclear and chloroplast markers (e.g., Cai et al., 2023; Liu et al., 2023; Nihara, 2016), and whole chloroplast genomes (e.g., Phan et al., 2023). However, the three species endemic to the Ogasawara Islands have not been included in any of the studies. Therefore, the phylogenetic positions of these the species remain unclear.

We ultimately aim to understand the phylogenetic background and evolutionary time scale of the *Callicarpa* species endemic to the Ogasawara Islands, which has undergone sexually expressive evolution and adaptive radiation. To this end, we performed molecular phylogenetic analyses and divergence time estimation using whole chloroplast and nuclear whole genomes. Our objectives in this study were (1) to reveal the phylogenetic and monophyletic relationships of the Ogasawara Island endemic *Callicarpa* species, *C. subpubescens*, *C. glabra*, and *C. parvifolia*, and (2) to estimate the ages when their ancestors migrated to the islands and when these three species diverged within the islands.

2. Materials and methods

2.1. Taxon sampling, DNA extraction, and sequencing

We collected leaves of 12 *Callicarpa* species from wild and cultivated individuals at the Botanical Garden of the University of Tokyo. Silica gel-dried leaf materials stored in a deep freezer (MDF-C8V1-PJ, Panasonic, Japan) at −80 °C were pulverized using a TissueLyser (QIAGEN, Hilden, Germany). After removing the polysaccharides from the powder using the wash medium described by Setoguchi & Ohba (1995), total DNA was extracted using the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1987). Whole-genome sequence libraries were created using the KAPA Hyper Prep Kit (F. Hoffmann-La Roche, Ltd., Basel, Switzerland). The prepared libraries were sequenced using a DNBSEQ-G400 (MGI Tech Co., Ltd., Shenzhen, China). The prepared libraries were sequenced using DNBSEQ-G400 at paired-end 150 bp or 300 bp to an expected coverage greater than ten. Detailed sequencing conditions for each sample can be found on DDBJ BioSample (SAMD00633046-SAMD00633062).

2.2. Chloroplast genome construction

Low-quality reads and adapter were removed using Trimmomatic 0.39 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP 2:30:10 (seed mismatches = 2, palindrome clip threshold = 30, simple clip threshold = 10) and SLIDINGWINDOW 4:15. The *de novo* assembly of each chloroplast genome was conducted by get_organelle_from_reads.py implemented in the GetOrganelle v.1.7.5.3 (Jin et al., 2020), in which chloroplast-derived reads were extracted from total genomic reads, and then SPAdes v.3.15 (Bankevich et al., 2012) for assembly. We manually removed noisy connections using Bandage (Wick et al., 2015) only in cases where the assemblies could not be solved as a circular path or were too complicated to be solved.

2.3. Phylogenetic analysis

(1) Chloroplast gene fragments

To understand the approximate phylogenetic position of the Ogasawara Islands endemic species, we downloaded two chloroplast gene fragment loci (matK and trnH-psbA) of 40 taxa of *Callicarpa* genus from the NCBI database (Table S1). These taxa were selected to cover the main distribution area of the genus (Fig. 1B). The two loci were chosen because they encompass a large number of species registered in the database. These sequences were aligned for each gene using MAFFT v.7.505 (Katoh and Standley, 2013) with the following parameters: −-maxiterate 1,000 −-adjustdirection –nwildcard and trimmed using TrimAl v.1.4.1 (Capella-Gutiérrez et al., 2009) with the following parameters: −gt 0.7. After trimming, multiple sequence alignments were concatenated for each taxon using AMAS (Borowiec, 2016). A maximum likelihood (ML) phylogenetic tree was constructed by IQ-TREE2 (Minh et al., 2020) using the ModelFinder option (−m MFP) (Kalyaanamoorthy et al., 2017). To assess branch support, the ultrafast bootstrap approximation (UFboot) with 1,000 replicates (Hoang et al., 2018; Minh et al., 2013) and the SH-like approximate likelihood ratio test (SH-aLRT) also with 1,000 bootstrap replicates (Guindon et al., 2010) were adopted.

(2) Chloroplast whole genome

In the phylogenetic analysis using the two chloroplast loci, the resolution of the interspecific relationships was low because there were few polymorphic sites. Therefore, we performed a phylogenetic analysis based on the whole chloroplast (wCp) genome. In addition to the 12 newly constructed individuals in this study, we also downloaded 16 wCp genomes from the *Callicarpa* genus from the National Center for Biotechnology Information (NCBI). Three taxa, *C. kochiana* Makino, *C. cathayana* Chang, and *C. formosana* Rolfe, were included two individuals in the phylogenetic analysis (one in the 12 newly constructed genomes mentioned above and the other in the 16 downloaded from the NCBI database). Therefore, 28 sequences of 25 taxa of *Callicarpa* genus were used for analysis. To root the phylogenetic tree and provide calibration points for estimating divergence time, chloroplast whole genomes for 12 species of Lamiaceae excluding *Callicarpa*, five species of Mazaceae, four species of Bignoniaceae and one species of Verbenaceae, were also retrieved from NCBI (Table S2). Fifty wCp genomes were aligned using HomBlocks pipeline (Bi et al., 2018). In this pipeline, the sequence of each individual was first aligned using progressiveMauve (Darling et al., 2010) to identify the locally collinear blocks (LCBs) shared by input genomes. The LCBs co-existing among the input genomes were extracted and trimmed using Gblocks (Castresana, 2000) to screen phylogenetically informative regions. Finally, the optimal partition schemes of the sequences, which are important in downstream phylogenetic analysis, were constructed using PartitionFinder (Lanfear

et al., 2012). A maximum likelihood (ML) phylogenetic tree was constructed using IQ-TREE2.

(3) Single-copy nuclear genes

To perform phylogenetic inference, including outgroups in the nuclear genome, and to check whether the tree topology and divergence date estimates were consistent in different datasets of nuclear and chloroplast genomes, we used Angiosperms353 (AGS353, Baker et al., 2022), which is a database of single copy protein-coding nuclear orthologs widely conserved in angiosperms. Among the *Callicarpa* genus, only *Callicarpa americana* has sequences registered in AGS353. Thus, we attempted to construct homologous sequences of other *Callicarpa* species using genome re-sequencing data. The chromosome-scale reference genome of *C. americana* (Hamilton et al., 2020) and its general feature format (gff) file containing the coordinates of the predicted protein-coding genes were downloaded and used to obtain the protein sequences of all genes of *C. americana* using gffread (Pertea and Pertea, 2020). BLASTX was run using the protein sequence as a database and the AGS353 sequence of *C. americana* as a query; multiple hit genes with a homology rate of 90 % or higher were excluded from further analysis as they may be potential paralogs and 329 genes remained after this procedure. The re-sequence reads of 12 *Callicarpa* species were then mapped against the *C. americana* reference genome using SNAP v.2.0.1 (Zaharia et al., 2011) with default parameters. Variant calls were made using BCFtools v.1.16 mpileup and call commands (Danecek et al., 2021; Li, 2011). Sites with a mapping quality of less than 20 or read depths of less than 4 were removed. Next, consensus sequences of the genome regions corresponding to the 329 genes remaining in the previous step were output using the SAMtools faidx and Bcftools consensus for each taxon. These output sequences of the *Callicarpa* genus (including *C. americana*) and six species of Lamiaceae excluding *Callicarpa*, two species of Mazaceae, four species of Bignoniaceae, and one species of Verbenaceae downloaded from AGS353 were aligned for each gene using MAFFT v.7.505 (Katoh and Standley, 2013) with following parameter: −-maxiterate 1,000 −-adjustdirection –nwildcard, and trimmed using TrimAl v.1.4.1 (Capella-Gutiérrez et al., 2009) with following parameters: −gt 0.8 −st 0.003. After trimming 86 loci had an alignment length above 100 bp were concatenated using AMAS (Borowiec, 2016). Phylogenetic analysis using the maximum likelihood method was performed in IQ-TREE2 using the same settings as those used for the wCp genome analysis. Estimation of the species phylogenetic tree using the supertree method was performed using ASTRAL III v.5.7.8 (Zhang et al., 2018).

(4) Nuclear whole genome

Because the resolution of phylogenetic relationships within the *Callicarpa* genus was low in the wCp and single-copy (scNr) nuclear results, phylogenetic inference was performed using the genes of the entire genome only for the 13 species of *Callicarpa* genus. Only genes with known functional annotations were included in the analysis. If a gene had multiple transcriptome IDs, the gene with the smallest number of IDs was extracted. The output of the consensus sequence for each gene, were performed using the same method as above. To reduce the time and computational burden of subsequent analysis, short sequences with low informative content (< 999 bp) were removed. A total of 11,538 genes remained and phylogenetic trees were constructed for all of the genes using the same method described above. A species tree was constructed using ASTRAL III software. ML phylogenetic tree estimation between species was performed using IQ-TREE2 for 20, 30, 50, 100, 250, and 500 genes selected using Sortadate (Smith et al., 2018) to ensure topological consistency between subsets with different amounts of data.

2.4. Divergence time estimation

Estimation of divergence time among the three species endemic to the Ogasawara Islands and other *Callicarpa* species was performed using BEAST v.2.7.4 (Bouckaert et al., 2019) on three datasets: wCp, scNr, and Nr (nuclear whole genome). For wCp, for species that contained multiple individuals in the dataset used for phylogenetic analysis, duplicates were removed to leave the newly constructed genome in this study. Two fossil calibration points and one secondary calibration point were used in the analysis using datasets wCp and scNr; crown age of the *Catalpa–Campsis* (Bignoniaceae) clade was constrained with a log-normal prior having an offset of 38.3 Ma, mean and the standard deviation set to 1 (Manchester, 2000; Rana et al., 2021); crown age of Nepetoideae was constrained with a log-normal prior having an offset of 49 Ma with mean set to 2.6 and the standard deviation set to 0.5 (Drew and Sytsma, 2012; Rose et al., 2022); crown age of Lamiales was constrained with an uniform prior of 60–107 Ma (Drew and Sytsma, 2012). The most recent common ancestor (MRCA) prior was added to the crown of Lamiaceae and Mazaceae (Fonseca, 2021). Analysis using the Nr dataset was performed on a subset of 500 clock-like genes selected by Sortadate (Smith et al., 2018) to improve computational tractability. The crown age of the 13 species of *Callicarpa* genus was estimated using the Nr dataset was constrained with a log-normal prior having an offset of 1 with mean set to 2 and the standard deviation set to 0.4, calibrated based on the estimated divergence time of the wCp data set (95 % HPD = 4.43–15.6 Mya). When running BEAST2, the substitution model was estimated using the transition/transversion split models implemented in bModelTest (Bouckaert and Drummond, 2017). We selected Optimized Relaxed Clock (Douglas et al., 2021) as the clock model and the Birth-Deth model (Gernhard, 2008) as tree prior. Markov chain Monte Carlo was run for 100 million generations, with one tree sampled every 10,000 generations, following a burn-in of the initial 20 %. The results of three replicate runs were combined using LogCombiner v.2.7.4. We used Tracer v.1.7.2 (Rambaut et al., 2018) to confirm that all parameters had an estimated sample size (ESS) of more than 200. A maximum clade credibility tree was produced using TreeAnnotator v.2.7.4 and then visualized using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Chronograms were created using the R packages ape (Paradis and Schliep, 2019), deeptime (https://CRAN.R-project.org/package = deeptime), ggplot2 (Wickham, 2016), ggtree (Yu et al., 2017), and treeio (Wang et al., 2020).

3. Results

3.1. Phylogenetic relationships

In the chloroplast gene fragment dataset, 45 sequences and 1257 sites including 96 variant sites, were used for phylogenetic analysis. Although the support values for almost all nodes were low (UFBoot < 95 and SH-aLRT < 90), we observed regional cohesion among species; therefore, we defined five groups based on tree topology. Group A, the base of the *Callicarpa* genus, is composed of species endemic to Southeast Asia. Groups B and C mainly consist of species that are found across multiple regions from the tropics to temperate. Group B in particular includes species from the Americas far away from the Asia-Oceania region. Group D consisted of species endemic to East Asia, whereas group E contained many species endemic to Southeast Asia. Group F, included species endemic to the Ogasawara Islands, was mainly composed of East Asian species (Fig. 1F).

Fifty sequences and 68,435 sites, including 4,962 variant sites, were used in the wCp maximum–likelihood phylogenetic analysis. Unexpectedly, the three *Callicarpa* species for which the duplicate samples were used in the analysis were not monophyletic in the analysis (Fig. S1). This result may be attributed to chloroplast capture due to the different origin of the samples or to human error, such as mistaken identification. To accurately test for monophyly, other markers such as

nuclear should be used or the number of sequences included in the analysis should be increased. In the other *Callicarpa* species, the tree topology estimated by the Bayesian (BI) method implemented in BEAST2 (Fig. 2) and the ML method (Fig. S1) in IQ-TREE2 was consistent. *C. arborea*, which has its distribution centers in South Asia, and *C. saccata,* endemic to Borneo, were located at the basal position. This was followed by the divergence of *C. americana*, a species from the Americas, and the species mainly distributed in East Asia (Fig. 2, S1). The three *Callicarpa* species endemic to the Ogasawara Islands formed a monophyletic group that split at the base of an East Asian clade. This topology showed almost no contradictions, with a few exceptions (e.g., *C. dichotoma* and *C. nudiflora*), compared to the results of the phylogenetic analysis using two chloroplast loci (Fig. 1F).

In scNr gene dataset, 26 taxa and 17,638 sites, including 4,328 variant sites at 86 loci, were used for phylogenetic analysis. The three *Callicarpa* species endemic to the Ogasawara Islands were monophyletic in all data analyses; however, their positions in the phylogenetic tree varied. In the ML and BI phylogenetic trees, these three species were sister groups to *C. japonica* var. *japonica* and are located at one terminal branch of the *Callicarpa* genus. In contrast, in the species tree estimated by the supertree method was located at the base of the East Asian species group, which was the same as the result for the chloroplast datasets (Fig. S2, S3). However, the support values for many nodes in the ML tree were low (UFBoot < 95; SH-aLRT < 90). In the species phylogenetic tree, the support values among the East Asian species tended to be low (ASTRALBoot < 95).

The species tree inferred using 11,538 nuclear genes did not partially match the tree inferred from the wCp and scNr datasets (Fig. 3A, S4). Specifically, *C. kochiana* was located at the base along with *C. saccata*, after which *C. americana* and *C. dichotoma* diverged. The three *Callicarpa* species endemic to the Ogasawara Islands are monophyletic and therefore formed a sister group with *C. japonica* and *C. mollis*. Tree topology was estimated from subsets of different sizes (20, 30, 50, 100, 250 and 500 genes) using the ML method (Fig. S4, as a representative, only a phylogenetic tree using 30 loci was shown) consistently agreed with the topologies estimated by the supertree (Fig. 3A) and BI methods (Fig. 3B).

3.2. Divergence time estimation

Estimation of the divergence age in the wCp and scNr data sets, including outgroups, indicated that the crown age of the *Callicarpa* genes was approximately 10 Mya (Fig. 2, S2).

The mean crown age of three *Callicarpa* species endemic to the Ogasawara islands, whose monophyly was strongly supported by phylogenetic analysis, was estimated to be 0.41 Mya (95 %HPD = 0.07–0.89 Mya) in wCp, 0.49 Mya (95 %HPD = 0.17–0.99 Mya) in scNr and 0.60 Mya (95 %HPD = 0.18–1.19 Mya, Table 1) in Nr.

The mean divergence age of three *Callicarpa* species endemic to the Ogasawara islands from the most closely related species is 2.93 Mya (95 %HPD = 1.52–4.60 Mya) in wCp, 2.03 Mya (95 %HPD = 0.99–3.24 Mya) in scNr and 2.83 Mya (95 %HPD = 0.95–5.30 Mya, Table 1) in Nr. Estimates from the three different datasets ranged from approximately 2 to 3 Mya (Pliocene to early Pleistocene), and their confidence intervals overlapped.

4. Discussion

4.1. Origin of Callicarpa species endemic to the Ogasawara islands

Based on the morphology and commonality of flora, it has been assumed that the ancestors of the plant species distributed on the

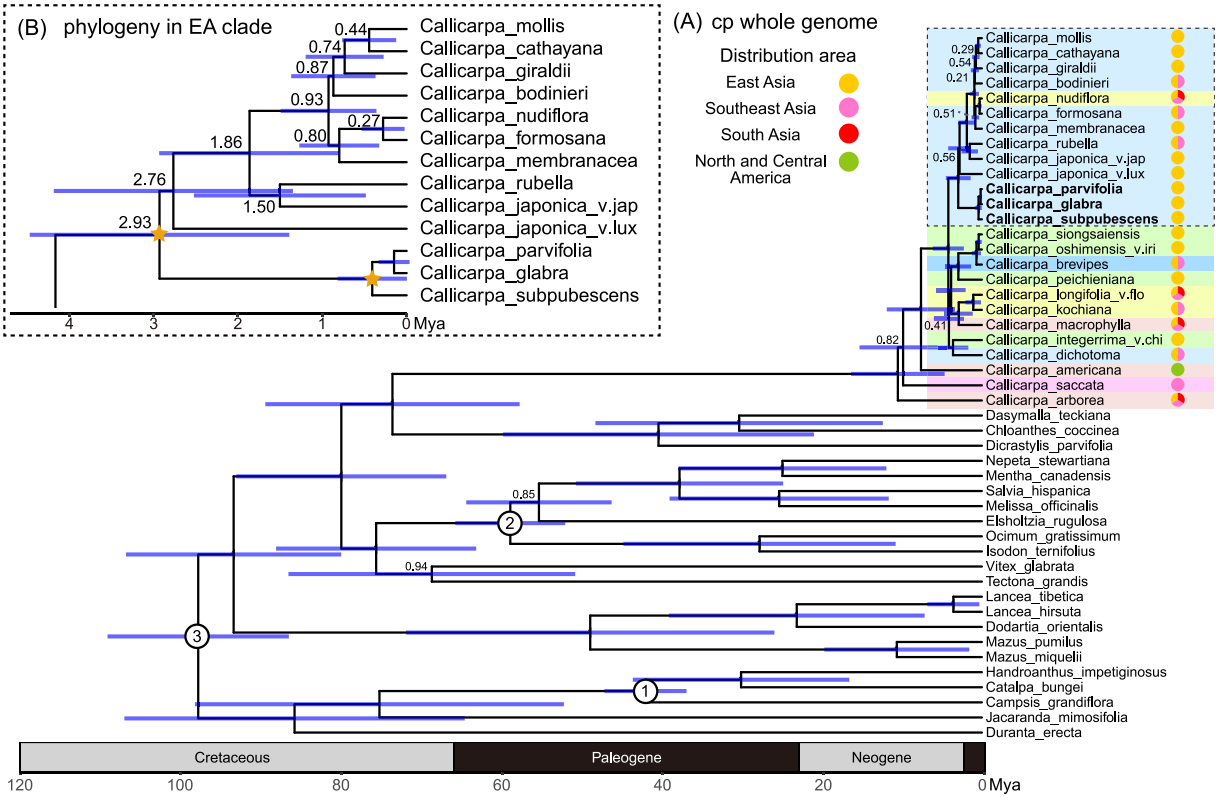

Fig. 2. (A) Chronogram of Callicarpa and outgroups inferred from BEAST2 using chloroplast whole genome. Blue bars above each node indicate the 95% highest posterior densities. Numbers next to each node represent the posterior probability, only nodes less than 0.95 are shown. The background color of the species name corresponds to the group defined in Fig. 2. (B) Chronogram enlarged dash line box in (A). Numbers next to each node represent node age. The yellow star represents the divergence point between the three endemic species of Ogasawara and other species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

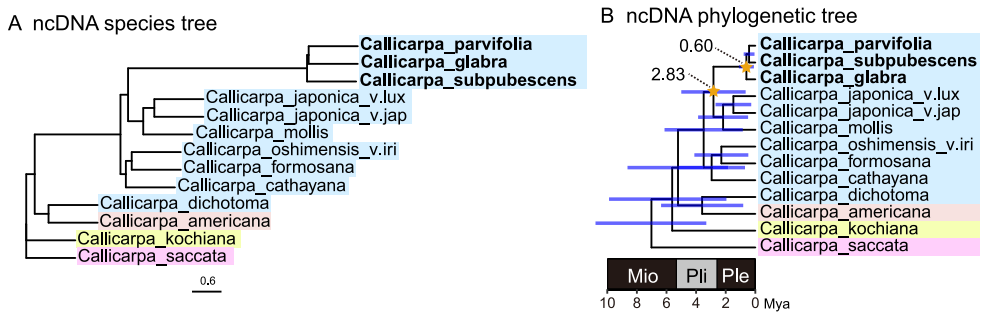

Fig. 3. (A) Chronogram of Callicarpa and outgroups inferred from BEAST2 using 30 nuclear coding loci. Blue bars above each node indicate the 95% highest posterior densities. The posterior probability exceeded 0.95 for all nodes. The yellow star represents the divergence point between the three endemic species of Ogasawara and other species. (B) Species tree using 11,538 loci inferred from ASTRAL analysis. All nodes had a support value of 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Estimated divergence time of (A) the node between three Ogasawara Islands endemic species and the most recently related species and (B) the crown node of the three species.

	Data set	Age (Mya)	95 % HPD
	Whole chloroplast genome	2.93	1.52–4.60
A	86 Single-copy nuclear gene	2.03	0.99–3.24
	Whole nuclear genome	2.83	0.95–5.30
	Whole chloroplast genome	0.41	0.07–0.89
B	86 Single-copy nuclear gene	0.49	0.17–0.90
	Whole nuclear genome	0.60	0.18–1.19

Abbreviation: HPD; highest posterior density.

Ogasawara Islands originated mainly in three geographic regions: the tropical or subtropical regions of Southeast and East Asia, the temperate regions of the Japanese Archipelago, and the South Pacific Islands (Ono, 1991). Phylogenetic analysis based on the chloroplast gene fragments and the whole chloroplast genome placed the three Ogasawara islands endemic species at the base of a clade that included multiple species distributed through East Asia (Group F, Fig. 2, S1). The results of the analysis using nuclear whole genome showed that it formed a sister group with the temperate deciduous species *C. japonica*, which is distributed in the temperate regions of mainland China, the Korean Peninsula, Japan, and Taiwan, and with *C. mollis* that is found in Japan and South Korea within the East Asian clade (Fig. 3A, B). Results of this study based on the chloroplast two loci (Fig. 1F) and previous molecular phylogenetic studies based on two nuclear loci and eight chloroplast loci has shown consistently that *C. candicans*, which is distributed in Micronesia (https://naturalhistory2.si.edu/botany/micronesia/), is a separate lineage located further outside the East Asian clade (Cai et al., 2023; Liu et al., 2023). Unfortunately, our sampling of taxa used in wCp, scNr and Nr analysis did not use any of the taxa distributed in the South Pacific Islands, including *C. candicans*. Considering these results, however, it is likely that the ancestors of these three species originated in East Asia.

4.2. Divergence time estimation

Based on the whole nuclear and chloroplast genome data, the three Ogasawara Island endemic species diverged from their most closely related continental species approximately 3 Mya (Table 1). This consistency across different datasets indicates the robustness of the results. Our results support the hypothesis that the Ogasawara Islands appeared above sea level before the middle Pleistocene (Imaizumi, 1984; Kaizuka, 1977). The exact age at which the Ogasawara Islands emerged above sea level is still unknown. However, based on studies using molecular clocks for plants and animals (e.g., land plants (Noda et al., 2022), land snails (Chiba, 1999), freshwater snails (Miura et al., 2008) and freshwater fish (Mukai et al., 2005)), including our results, the Ogasawara Islands may have already had environmental conditions that allowed these plants and animals to establish at least approximately three million years ago. Many species of East Asian *Callicarpa*, including the three species endemic to the Ogasawara Islands, produce bright purple berries, suggesting that they mainly depend on birds for seed dispersal (Liu et al., 2023). Therefore, it can be assumed that the ancestral species migrated to the Ogasawara Islands through long-distance dispersal(s) from East Asia or nearby continental islands during late Pliocene to the early Pleistocene. However, whether the migration time of the ancestral species is consistent with the divergence time of the most closely related species is controversial. In the present study, phylogenetic analysis based on the chloroplast genome could not identify a single nearest-related species, and the support values of the nodes within the sister group were low. Furthermore, the branches between the sister group and three Ogasawara-endemic species were considerably longer in the nuclear genome trees. It is possible that more phylogenetically related species were not included in this analysis or were already extinct. In such cases, the true migration time may have been over-estimated.

Three *Callicarpa* species, *C. glabra*, *C. parvifolia* and *C. subpubescens*, which are endemic to the Ogasawara Islands, showed monophyly in both the chloroplast and nuclear genome trees, regardless of the analysis method. This result is consistent with the fact that, although these three species are ecologically differentiated, they are dioecious (Kawakubo, 1990b). The mean crown age of these species was estimated to be approximately 0.5 Mya (Table 1). Previous studies based on coalescent simulations have proposed that the adaptive radiation of *Callicarpa* genus in the Ogasawara islands began about 0.17 Mya (Setsuko et al., 2024a). The estimated age in this study was 2–3 times older than that, but considering the width of the confidence interval, the uncertainties of the generation time, and the mutation rate assumed in the simulation analysis, there appear to be no significant inconsistencies. These results suggest that after a single ancestor migrated to the islands during the late Pliocene, it acquired dioecy before the three species diverged. Subsequently, during the late Pleistocene, the *Callicarpa* clade progressively diverged, forming *C. subpubescens* at relatively mesic forest edges, *C. parvifolia* in dry rocky areas, and *C. glabra* in intermediate shrublands (Setsuko et al., 2024a; Sugai et al., 2019). The genetic comparisons of the three dioecious species of the islands with the continental hermaphrodite relative species revealed in the present study may provide new insight into the genomic evolution that led to the acquisition of dioecy.

4.3. Causes of phylogenetic discrepancies between datasets and analysis methods

In our analysis of chloroplast whole genomes and nuclear genes, we found inconsistent tree topologies and low support values that hindered the resolution of interspecific relationships within the East Asian clade

(Fig. 1F, 2, S1). The genus *Callicarpa* appeared to have diversified in East Asia more recently than approximately 4 Mya (Fig. 2). Rapid diversification prevents the generation of sufficient phylogenetic signals between species, particularly between chloroplasts and single-copy genes with low mutation rates (Moore et al., 2010; Small et al., 1998). Therefore, our tree estimates might have suffered from incomplete lineage sorting (ILS) (Maddison, 1997; Suh et al., 2015). Another possible reason for this is the effect of interspecific hybridizations. The reproductive isolation of *Callicarpa* species appears weak, with rich examples of natural or artificial hybrids [e.g., *C. dichotoma* × *C. bodinieri*, (Lin et al., 2013), *C. japonica* × *C. mollis*, *C. shikokiana* × *C. japonica* var. *luxurians* (Nihara, 2016) and *C. kochiana* × *C. japonica* (Yamanaka, 1988)]. It has been suggested that introgressive hybridization occurs between *C. japonica* and *C. mollis* throughout their distribution area (Tsukaya et al., 2003). The widespread occurrence of past gene introgression and chloroplast capture through hybridization in this genus may complicate its phylogenetic relationships. Among the species examined in the present study, *C. oshimensis* var. *iriomotensis* was placed in disparate clades in the wCp and Nr phylogenetic trees, implying that it may have undergone introgressive hybridization in the past. Region-specific introgression may also explain why *C. kochiana*, *C. cathayana* and *C. formosana* are not monophyletic within the same species (Fig. S1). There are some inconsistencies between the phylogenetic tree and divergence times obtained in this study and those of previous studies (Cai et al., 2023; Liu et al., 2023). For example, the estimated crown ages of major clades within *Callicarpa* genus were approximately three times more recent in this study. For example, the crown age of the entire clade of *Callicarpa* genus, including *C. arborea*, which is located at the base of the genus in this study, was approximately ten Mya in this study, but 30 Mya in the previous two studies (Cai et al., 2023; Liu et al., 2023). One possible factor is the difference between datasets. The analyses conducted in previous studies Cai et al. (2023) and Liu et al. (2023) both using a dataset combining two nuclear loci (ITS & ETS) and eight chloroplast fragment loci (matK, rpl32-trnl, trnD-trnT, trnH-psbA, psbJ-petA, trnQ-5′rps16, 3′trnV-ndhC, trnS-trnG), whereas in the present study, we used over ten thousand nuclear loci and the entire chloroplast genomes. Performing analyses on larger number of loci can help to mitigate the effects of ILS on phylogenetic estimates and narrow the confidence intervals for divergence time estimates. Differences in the speciation models and the number of calibration points may also be factors. The previous studies used the Yule model (Yule, 1925) for the tree prior of BEAST2 analysis, whereas birth–death model, which is a more generalized speciation model, was used in this study. The previous studies also used two fossil records recognized in Lamiaceae, whereas the present study used one fossil record for Lamiaceae and one for Bignoniaceae and loose constraints on the crown age of Lamiales as a secondary calibration point. Furthermore, in taxa such as *Callicarpa* where introgressive hybridization has been reported, the tree topologies of nuclear and plastid genomes, such as chloroplasts may be different. Therefore, phylogenetic analyses combining these sequences may lead to incorrect phylogenetic relationships. However, because the number of species examined our study was smaller than that in the previous studies, the complete phylogeny within *Callicarpa* genus is still unknown. To further elucidate the phylogenetic relationships within the genus, it is necessary to obtain a large number of genome-wide phylogenetic markers from more species and perform separate analyses of the nuclear and chloroplast genomes.

5. Conclusion

By analyzing whole chloroplast and nuclear genome sequences, we reconstructed some of the phylogenetic relationships within the *Callicarpa* genus. Our phylogenetic analysis of over ten thousand nuclear genes showed that the endemics of the three Ogasawara Islands are closely related to the East Asian species *C. japonica* and *C. mollis*. However, we were unable to obtain strong evidence to determine the closest relatives of the three species because the discrepancy between the tree topologies of the nuclear and chloroplast genomes. Phylogenetic relationships within the genus may be complicated by past introgressive hybridization and ILS due to relatively recent diversification in some clades. To elucidate the complex interspecific relationships of the genus, further research using more markers and species for each nuclear and chloroplast genome is required. One significant discovery from this study is the strong evidence indicating that the three *Callicarpa* species evolved from a common ancestor. This aligns with the observation that these species frequently exhibit a recently evolved sex-determining system, known as dioecy. Considering the estimated crown age of the insular species, the evolution of the dioecy system is inferred to have occurred once between the Late Pliocene and Late Pleistocene. The phylogenetic results obtained in this study offer crucial background information regarding the evolutionary shift in the sex expression system within the insular *Callicarpa*.

CRediT authorship contribution statement

Kazutoshi Masuda: Writing – review & editing, Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Hiroaki Setoguchi:** Supervision, Funding acquisition. **Koki Nagasawa:** Writing – review & editing, Investigation. **Suzuki Setsuko:** Writing – review & editing. **Shosei Kubota:** Writing – review & editing, Investigation. **Shin S. Satoh:** Writing – review & editing, Investigation. **Shota Sakaguchi:** Writing – review & editing, Project administration, Investigation, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108234.

Data availability

Data will be made available on request.

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