

# Adaptive radiation of the *Callicarpa* genus in the Bonin Islands revealed through double-digest restriction site-associated DNA sequencing analysis

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## Abstract

The Bonin Islands, comprising of the Mukojima, Chichijima, and Hahajima Islands, are known for their isolated and distinctive habitats, hosting a diverse array of endemic flora and fauna. In these islands, adaptive radiation has played a remarkable role in speciation, particularly evident in the *Callicarpa* genus that is represented by three species: *Callicarpa parvifolia* and *Callicarpa glabra* exclusive to the Chichijima Islands, and *Callicarpa subpubescens*, distributed across the entire Bonin Islands. Notably, *C. subpubescens* exhibits multiple ecotypes, differing in leaf hair density, flowering time, and tree size. In this study, we used double-digest restriction site-associated DNA sequencing to analyze species, ecotypes and geographical variations within *Callicarpa* in the Bonin Islands. We aimed to determine detailed phylogenetic relationships, investigate species and ecotype diversification patterns, estimate divergence times, and explore cryptic species using genetic and phenotypic data. Genetic analysis revealed that *C. parvifolia* and *C. glabra* formed a single, distinct genetic groups. Conversely, *C. subpubescens* showed seven genetic groups corresponding to different ecotypes and regions, with one ecotype derived from the hybridization of two others. Phylogenetic and population demography analyses, focusing on six Chichijima and Hahajima Islands-based species/ecotypes, indicated the divergence of an ecotype adapted to tall mesic forests approximately 170 kya, whereas the other five species/ecotypes diverged nearly simultaneously around 73–77 kya. Environmental changes during the glacial period likely contributed to this process of adaptive radiation. Moreover, leaf morphology, flowering time, and genetic analyses suggested the presence of two cryptic species within *C. subpubescens*.

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## ABSTRACT

The Bonin Islands, comprising of the Mukojima, Chichijima, and Hahajima Islands, are known for their isolated and distinctive habitats, hosting a diverse array of endemic flora and fauna. In these islands, adaptive radiation has played a remarkable role in speciation, particularly evident in the *Callicarpa* genus that is represented by three species: *Callicarpa parvifolia* and *Callicarpa glabra* exclusive to the Chichijima Islands, and *Callicarpa subpubescens*, distributed across the entire Bonin Islands. Notably, *C. subpubescens* exhibits multiple ecotypes, differing in leaf hair density, flowering time, and tree size. In this study, we used double-digest restriction site-associated DNA sequencing to analyze species, ecotypes and geographical variations within *Callicarpa* in the Bonin Islands. We aimed to determine detailed phylogenetic relationships, investigate species and ecotype diversification patterns, estimate divergence times, and explore cryptic species using genetic and phenotypic data. Genetic analysis revealed that *C. parvifolia* and *C. glabra* formed a single, distinct genetic groups. Conversely, *C. subpubescens* showed seven genetic groups corresponding to different ecotypes and regions, with one ecotype derived from the hybridization of two others. Phylogenetic and population demography analyses, focusing on six Chichijima and Hahajima Islands-based species/ecotypes, indicated the divergence of an ecotype adapted to tall mesic forests approximately 170 kya, whereas the other five species/ecotypes diverged nearly simultaneously around 73–77 kya. Environmental changes during the glacial period likely contributed to this process of adaptive radiation. Moreover, leaf morphology, flowering time, and genetic analyses suggested the presence of two cryptic species within *C. subpubescens*.

Key words: ddRAD-Seq, Lamiaceae, Oceanic islands, Quaternary, Ogasawara Islands

## INTRODUCTION

Oceanic islands are geographically isolated—never been connected to large landmasses. Owing to their distinctive characteristics and limited colonization opportunities, the biotas of oceanic islands exhibit a greater degree of endemic flora and fauna, often resulting from frequent ecological speciation and adaptive radiation (Gillespie et al., 2001; Givnish, 1997). Adaptive radiation refers to the rapid diversification of lineages into multiple taxa, each adapted to distinct ecological niches (Rundle & Nosil, 2005; Schluter, 2000). Some examples include Darwin’s finches in the Galapagos Islands (Grant, 1986; Grant, 1998; Grant & Grant, 2007) and silverswords and Hawaiian lobeliads in the Hawaiian Islands (Carlquist et al., 2003; Givnish et al., 2009).

The Bonin Islands, comprising of the Mukojima, Chichijima, and Hahajima Islands, are oceanic islands located approximately 1,000 km south of Tokyo, Japan (Fig. S1). Despite their smaller land area (70 km<sup>2</sup>) and lower altitude (463 m) compared with other oceanic islands, such as the Canary Islands (area,

7,490 km<sup>2</sup>; elevation, 3,718 m) and the Galapagos Islands (area, 7,870 km<sup>2</sup>; 1707 m), the Bonin Islands exhibit a high proportion of endemic vascular flora, with ~43% of species being endemic (Ono et al., 1986), exceeding 26% and 43% endemism in the Canary Islands (Aedo et al., 2013) and the Galapagos Islands (Porter, 1978), respectively. Diverse habitats, including forests, grasslands, and coastal areas, providing varied microclimates and supporting multifarious plant species, coupled with the islands' isolation, have facilitated the evolution of endemic species adapted to specific ecological conditions in the Bonin Islands. The role of adaptive radiation in driving the notable endemism rate of the Bonin Islands is underscored by various genera, such as *Crepidiastrum* (Ito & Ono, 1990), *Symplocos* (Soejima et al., 1994), and *Pittosporum* (Ito et al., 1997), which have experienced diversification yielding three to four species each.

In the Bonin Islands, the genus *Callicarpa* is another example of adaptive radiation (Ono, 1991; Shimizu & Tabata, 1991). Comprising three endemic species, *Callicarpa parvifolia*, *Callicarpa glabra*, and *Callicarpa subpubescens*. *Callicarpa parvifolia* and *C. glabra* are restricted to the Chichijima Islands, whereas *C. subpubescens* exhibits a wider distribution across the Mukojima and Hahajima Islands (Fig. S1). Using microsatellite markers, Sugai et al. (2019) demonstrated genetic differentiation among the three *Callicarpa* species in the Chichijima Islands, as well as multiple genetic groups within *C. subpubescens* in the Mukojima and Hahajima Islands. Furthermore, Setsuko et al. (2023) identified four ecotypes in the Hahajima Islands, one derived from hybridization of two other ecotypes, with these ecotypes found to be locally adapted to soil moisture and light conditions. However, detailed phylogenetic relationships, as well as species/ecotype diversification timing and order remain unclear. By examining differentiation patterns and divergence time, it may be possible to infer how plants in the oceanic islands diversified with geohistorical and/or environmental changes (Kadereit & Abbott, 2021).

In this study, we aimed to elucidate detailed phylogenetic relationships within the *Callicarpa* genus in the Bonin Islands, determine species and ecotype diversification patterns, estimate divergence times, and investigate cryptic species. To achieve these objectives, we employed double-digest restriction site-associated DNA sequencing (ddRAD-Seq), a genomic approach for detecting a large number of single nucleotide polymorphisms (SNPs) throughout the genome. Known for high resolution, ddRAD-Seq is suitable for investigating intricate phylogenetic relationships within closely related taxa (Wagner et al., 2013). The results of this study will offer fundamental insights into the evolutionary history of *Callicarpa* in the Bonin Islands, contribute essential knowledge on factors influencing species diversification in island ecosystems, and enhance our understanding of speciation processes. Moreover, the identification of potential cryptic species highlights the importance of comprehensive genetic analyses in biodiversity studies.

## MATERIALS AND METHODS

### Study species and sampling

*Callicarpa parvifolia* grows in sunny dry dwarf scrub on rocky ground in the Chichijima Islands (Toyoda, 2003), with a flowering peak in July (Table 1), whereas *C. glabra* grows in the understory of dry scrub in the Chichijima Islands (Toyoda, 2014) and has its flowering peak in August. These species are classified as “endangered” and “critically endangered” in the Red List of Threatened Species of Japan (Ministry of the Environment Government of Japan, 2020), respectively. In contrast, *C. subpubescens* is not listed as a threatened species and is widely distributed in the Bonin and Volcano Islands. *Callicarpa subpubescens* exhibits different ecotypes, each with distinct habitats and some with different flowering peaks. For example, the Chichijima Islands' ecotype (S) inhabits the forest edge of mesic forests, with peak flowering in June. The Hahajima Islands have four ecotypes: the glabrescent ecotype (SG), the tall ecotype (ST), the dwarf ecotype (SD), and the hybrid ecotype (SH; previously called the intermediate ecotype M; Setsuko et al. 2023). The ecotype SG inhabits the understory of mesic forests, with a flowering peak in July. The ecotype ST forms the canopy of tall mesic forests, with a flowering peak in October. The ecotype SD forms the canopy of dry scrub, with two flowering peaks in August and November. The ecotype SH forms the canopy of mesic scrub (cloud forests) or inhabits the forest edge of mesic forests, with a flowering peak in July (Setsuko et al., 2023; Sugai et al., 2019). The Mukojima Islands have two ecotypes (STm and Sm), which are genetically close to the ecotype ST in the Hahajima Islands and the ecotype S in the Chichijima Islands

(Sugai et al. 2019; also refer to the Results section). However, substantial forest areas have been lost due to feral goats (Shimizu, 2003), making it challenging to determine their original habitats. Flowering surveys on the Mukojima Islands were conducted only once in July 2010, as the islands are currently uninhabited, with no ocean liner services, and distant from inhabited Chichijima Island.

To cover species and ecotypes of each island in the Bonin Islands, leaf samples were collected from 94 individuals across 14 populations for DNA extraction (Table 1; Fig. S1). These samples included two populations in the Mukojima Islands, six populations from the Chichijima Islands, and six populations from the Hahajima Islands. As outgroups, one individual each of *Callicarpa japonica* and *Callicarpa mollis*, which grow in Kyoto Prefecture, Japan, was also collected (Fig. S1). Silica gel was used to immediately dry leaf samples used for DNA extraction.

Leaves were also sampled for phenotypic measurements from the same individuals used for DNA extraction. As leaf morphology varies within individuals depending on sunlight exposure, leaves were collected from the sunlit upper canopy. However, owing to time constraints, sunlit leaves could not be collected from the SGI population.

### Reference genome development

To obtain the reference genome of *C. subpubescens*, the individual with the highest homozygosity (based on previous studies using SSR markers, Setsuko et al. 2023) was selected from 51 individuals cultivated in a greenhouse at the Forestry and Forest Products Research Institute. DNA was extracted from fresh leaves using the Genomic-tip (Qiagen, Germany). Library construction, using the SMRTbell Template Prep Kit (PacBio, USA), was performed according to the manufacturer’s instructions. The DNA library was further fractionated using BluePippin (Sage Science, USA) to eliminate fragments < 15 kb in size and sequenced using four single-molecule real-time cells on the Sequel system (PacBio, USA). DNA extraction, library preparation, and sequencing were conducted by the Kazusa DNA Research Institute (Chiba, Japan).

*De novo* genome assembly for *C. subpubescens* involved preprocessing to split chimera sequences using yacrdr (Marijon et al., 2020). The assembly, conducted using wtdbg2 v. 2.5 (Ruan & Li, 2020), resulted in a genome size of approximately 450 Mb (Masuda et al., unpublished). The original dataset was approximately  $81 \times$  the size of the *C. subpubescens* genome. The dataset comprised a total of 482,624,924 bases and 6,011 reads, with read lengths of 1,129–5,172,107 bp (mean: 80,290 bp). The N50 sequence length was 623,636 bp. The quality of the assembly was assessed using the web tool gVolante (Nishimura et al., 2017). Using BUSCO (Simão et al., 2015) implemented in gVolante, approximately 86.1% of the complete core plant genes (1,440 in total) were detected in the assembly.

### ddRAD genotyping and SNP filtering

DNA was extracted using a modified CTAB method (Milligan, 1992). The DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, MA, USA) and adjusted to 12.6 ng/μl through dilution with TE buffer. Sequencing libraries were prepared following a modified version of Peterson’s protocol for ddRAD-seq (Peterson et al., 2012). For detailed library preparation methods, refer to appendix 1. The libraries were sequenced using a HiSeq2000 platform (Illumina, CA, USA) with 51 bp single-end reads at BGI Japan (Kobe, Japan).

SNPs were detected using dDocent (Puritz et al., 2014) and Stacks (Catchen et al., 2013; Catchen et al., 2011), resulting in three datasets: denovo, referenced, and demography datasets. The detection conditions and number of SNPs used in each analysis are summarized in Table S1. In all data sets, we excluded five individuals with low individual-level genotyping rates from SNP detection. In the referenced and denovo datasets, SNPs were detected using dDocent, following its tutorial. When using the denovo dataset, the reference genome of *C. subpubescens* was not used as a reference sequence and the two outgroup individuals were not included. In contrast, when using the referenced dataset, the reference genome and two outgroup individuals were used. Total raw SNPs generated via dDocent were filtered using vcftools -0.1.14 to meet the conditions outlined in Table S1. For the demography dataset, SNPs were re-extracted from the

.bam files created for the referenced dataset using dDocent. First, gstacks from Stacks version 2.60 was used to generate catalogs of variable sites (Rochette et al., 2019). Subsequently, populations from Stacks were employed to extract SNPs with the following options: `-r 0.8 -p X -min-mac 1 -max-obs-het 0.5 -vcf` (where X represents the number of species/ecotypes in each dataset). The pairwise two-dimensional minor allele site frequency spectrum (2D-mSFS) was calculated from the .vcf file using the R script 2D-msfs-R (<https://github.com/garageit46/2D-msfs-R>). Missing data were addressed through bootstrapping within the same ecotype.

### Population genetic structure analysis

For individual-based genetic structure analysis, the denovo dataset and ADMIXTURE program (Alexander et al., 2009) were used, with  $K$  values 1–15, as well as 30 iterations per  $K$  value, employed for analysis. The results were visualized using CLUMPAK (Kopelman et al., 2015), and the  $K$  value with the lowest cross-validation (CV) error was considered the optimal  $K$ .

Neighbor-net network analysis on the referenced dataset was conducted using SplitsTree4 (Huson & Bryant, 2006). Two analyses were performed: one including the ecotype SH suggested to originate from hybridization, and one excluding SH.

To reveal phylogenetic relationships, the two individuals with the highest number of SNPs from each population in the referenced dataset were selected. A maximum likelihood phylogenetic tree was constructed using MrBayes ver. 3.2.7 (Ronquist et al., 2012). Model selection was performed using MrModelTest ver 2.4 (Nylander, 2002) with PAUP\* (Swofford, 2002), employing a general model of DNA substitution (the GTR) with gamma-distributed rate variation across sites. MrBayes analysis conditions were as follows: `mcmc ngen = 400000; savebrlens = yes; printfreq = 100; samplefreq = 100; nchains = 4`. Similar to SplitsTree analysis, MrBayes analysis was conducted both with and without the inclusion of the ecotype SH.

### Population demography inference

To identify divergence patterns among species/ecotypes and their timing, a coalescent-based maximum likelihood method was employed, estimating parameters of the population demographic model with fastsimcoal2 version 2705 (Excoffier et al., 2021). Divergence models were sequentially applied for three to six ecotypes in the Chichijima and Hahajima Islands (Fig. S2a–e) and from three to five ecotypes in the Chichijima, Hahajima, and Mukojima Islands (Fig. S2f and g). Comparative divergence patterns for a model involving more than four-ecotype divergences were designed based on the results from lower ecotype divergence modeling, admixture, phylogenetic tree, and neighbor-net network analyses. As the ecotype SH on Hahajima Island originated from hybridization between ecotypes SG and ST (Setsuko et al. 2023; also refer to the Results section), we did not include it in the present analysis to reduce complexity. Migration between species/ecotypes was only considered within the same island groups, as each island group was isolated by the sea, even during the Last Glacial Maximum (Setsuko et al., 2017). Only recent migration was assumed based on preliminary analyses; although ancient migration was also considered, such models exhibited much lower log-likelihood values (data not shown). Details of each model are presented in Figs. S3–9.

The likelihood of each model was maximized from 50 random starting values, 40 expectation-conditional-maximization (ECM) optimization cycles, and 100,000 coalescent simulations. The mutation rate was set to  $1.74 \times 10^{-8}$ , estimated in a woody species, *Populus tremula* (Gossmann et al., 2012). We considered the model with the lowest Akaike’s information criterion value as the best model. The goodness of fitness of the best model was checked by visually comparing observed and simulated 2D-mSFSs. The confidence interval of the best model was calculated via parametric bootstrapping. We simulated the model using fastsimcoal2 with maximum likelihood estimate parameter values 100 times and obtained its 2D-mSFSs. Using the simulated 2D-mSFSs as input data, parameters of the best model were recalculated with the observed parameter values as a starting value, 15 ECM cycles, and 100,000 coalescent simulations. Finally, the 95% confidence interval (CI) was calculated from the obtained parameter values. Considering the ecological traits of our study species, five years per generation were used to convert an event time from generations ago to years ago.

## Leaf morphology

All individuals from the Bonin Islands were subjected to leaf phenotypic analysis, except population SGI, which lacked collected sunlit leaves. For each individual, 1–5 leaves were measured for the same 11 traits outlined by Setsuko et al. (2023): total length, blade length, blade width, hair density on the upper and lower surfaces of the leaf (i.e., number of hairs per 4 mm<sup>2</sup>), number of serrations per 30 mm, thickness of the leaf blade, leaf area, leaf mass per area, ratio of blade length to total leaf length, and ratio of leaf blade width to length. Principal component analysis (PCA) was performed on the measured traits. The two individuals from the Mukojima Islands that exhibited an admixed pattern were excluded from PCA (Figs. 1 and 2).

## RESULTS

### Phylogenetic relationships

In ADMIXTURE analysis, the minimum CV error occurred at  $K = 8$  (Fig. 1a), revealing that the 14 populations in the Bonin Islands can be divided into eight genetic clusters: species P, species G, and ecotype S in the Chichijima Islands; ecotypes ST, SG, and SD in the Hahajima Islands; and ecotypes STm and Sm in the Mukojima Islands (Fig. 1b). Ecotype SH on Hahajima Island exhibited a mixture of ecotypes ST and SG on these islands. Populations of the same ecotype from different islands within the same island groups shared the same genetic cluster, whereas populations of the same ecotype from different island groups exhibited different genetic clusters.

Neighbor-net network analysis using SplitsTree revealed that the outgroups were positioned at the tips of exceptionally long branches (Fig. S10). Fourteen populations in the Bonin Islands clustered into nine genetic groups (Fig. 2). The additional group relative to the ADMIXTURE analysis was due to ecotype SH forming an independent genetic cluster. Ecotype SH was positioned between ecotypes SG and ST in the Hahajima Islands, forming a reticulate structure. Conversely, in the network diagram without ecotype SH, ecotype SG was located between ecotype S and species P but not between ecotypes S and ST, suggesting that ecotypes SG and ST were not genetically similar (Fig. 2b). Akin to the results from ADMIXTURE analysis, populations of the same ecotype from different islands within the same island groups shared the same genetic groups, whereas populations of the same ecotype from different island groups exhibited different genetic groups.

The phylogenetic tree generated using Mr. Bayes showed that, with ecotype SH, the 14 populations in the Bonin Islands were divided into clade 1 (species P and G) and clade 2 (ecotypes S, SG, SD, SH, and ST) (Fig. 3a). Clade 1 was further divided into two subclades, with species P and G, as monophyletic clades. Clade 2 was divided into subclade 2-1 (ecotypes S and Sm) and subclade 2-2 (ecotypes SG, SD, SH, ST, and STm).

The phylogenetic tree excluding ecotype SH exhibited a different arrangement of species and ecotype positions, splitting into clade 1 (species P and G, and ecotypes S, Sm, and SG) and clade 2 (ecotypes SD, ST, and STm) (Fig. 3b). In this tree, the positions of ecotypes S and SG differed compared with the tree including ecotype SH. Clade 1 was divided into two clades: subclade 1-1 (species P and G) and subclade 1-2 (ecotypes S, Sm, and SG). Clade 2 was divided into two clades: subclade 2-1 (ecotype SD) and subclade 2-2 (ecotypes ST and STm).

### Inference of population demography

Regarding divergence patterns among the six species/ecotypes (species P, species G, and ecotype S in the Chichijima Islands; and ecotypes ST, SG, and SD in the Hahajima Islands), at step (a), among the three species divergence models in the Chichijima Islands, model a2 was the best model (Fig. S2a; Table S2). At step (b), among the four-ecotype divergence models in the Chichijima and Hahajima Islands, model b1 was the best model (Fig. S2b; Table S3). Similarly, at steps (c) and (d), among the three- and four-ecotype divergence models in the Hahajima Islands and in the Chichijima and Hahajima Islands, models c2 and d2 were the best models (Fig. S2c and d; Tables S4 and S5). Among six-ecotype divergence models in the Chichijima and Hahajima Islands, built considering steps (a)–(d), model e1 was the best model (Fig. S2e; Table S6). In this model, most ecotypes, except for S and SD, exhibited significant recent population

expansion at 70.2 kya (95% CI could not be estimated; refer to Table S7), whereas S and SD did not show significant population size change (Fig. 4; Tables S7 and S8). Divergence times  $T_2$ – $T_5$  showed a narrow range of 72.9–76.9 kya. However, the divergence time between ST and the other ecotypes was 170.7 (95% CI: 162.2–183.4) kya (more than twofold that of  $T_2$ – $T_5$ ).

Regarding the divergence patterns of the five ecotypes in the Chichijima, Hahajima, and Mukojima Islands, at step (f), among the three-ecotype divergence models in these islands, model f1 was the best model (Fig. S2f; Table S9). At step (g), among the five-ecotype divergence models in the Chichijima, Hahajima, and Mukojima Islands, model g2 was the best model (Figure S2g; Table S10). In this model, ecotype S and the two ecotypes in Mukojima Islands (Sm and STm) showed significant recent population reduction at 37.1 (95% CI: 33.9–40.5) kya, whereas the two ecotypes in the Hahajima Islands (SG and ST) exhibited significant recent population expansion (Fig. 4b; Tables S8 and S11). Divergence times  $T_2$  and  $T_3$  were 81.4 (72.7–86.4) and 82.3 (75.0–88.2) kya, respectively, and were very close. However, the divergence time between the two ancestral lineages was 168.8 (95% CI: 160.6–179.1) kya (more twofold that of  $T_2$  and  $T_3$ ).

Migrant numbers per generation estimated in the best models e1 and g2 were 0.020–0.511 and all significantly lower than 1.0 (Tables S7 and S11). These two best models indicated that, although the timings of recent population size change and the onset of migration differed between models, the timings of divergence between ecotypes were very similar (approximately 80 and 170 kya; Fig. 4; Tables S7 and S11), i.e., most ecotypes underwent recent divergence (approximately 73–77 kya), whereas ST experienced ancient divergence (around 170 kya).

### Leaf morphology

In PCA of leaf morphologies, species P, species G, and ecotype SD exhibited distinct distributions (Fig. 5a). Most *C. subpubescens* ecotypes did not show independent distributions, whereas ecotype SH showed a relatively narrow distribution but was included in the ecotype ST distribution. The leaves of species P were characterized as small, round, remarkably hairy, and thick. Species G possessed small, elongated, and moderately thick leaves lacking hair (Fig. S11). Ecotype SD showed small, round leaves, similar to those of species P, but with a significantly lower hair mass and smaller leaf thickness.

## DISCUSSION

### Phylogenetic relationships and divergence patterns

We investigated the phylogenetic relationships of various genetic groups, considering the presence and absence of the hybrid ecotype of *C. subpubescens* (SH; Figs. 2 and 3). When a hybrid ecotype arises between two distantly related parent ecotypes, the parent ecotype is expected to be located closer to the hybrid ecotype compared with its original position on the phylogenetic tree. In the Central American genus *Aphelandra*, the inclusion of hybrids between distantly related species in phylogenetic analyses caused a breakdown in cladistic structure and significant topological changes (McDade, 1992). Thus, we concluded that the phylogenetic tree lacking hybrid ecotype SH (Fig. 3b) represents the true phylogenetic relationships, and ecotype SG is considered to have originated from ecotype S in the Chichijima Islands, subsequently colonizing the Hahajima Islands as ecotype SG.

Based on the phylogenetic tree lacking the hybrid ecotype (Fig. 3b) and the population demography model (Model e1 in Fig. S2e), we determined that the ancestral ecotype ST in the Hahajima Islands is the most ancient. Subsequently, ecotype SD in the Hahajima Islands underwent divergence. This was followed by species P and G in the Chichijima Islands, as well as ecotype S. Finally, ecotype SG in the Hahajima Islands emerged from ecotype S in the Chichijima Islands. Population demography analysis revealed that ecotype ST diverged from the ancestral species approximately 170 kya, whereas the other species and ecotypes underwent nearly simultaneous divergence around 73–77 kya (Table S6). Given that 73–77 kya, corresponds to the time when Marine Isotope Stage (MIS) 5 (interglacial period) changed to MIS 4 (glacial period) (Martinson et al., 1987), estimated to have been a period of rapid cooling, cold weather may have triggered the simultaneous divergence of this taxon. Kadereit and Abbott (2021) reviewed studies examining divergence times from

phylogenetic trees from all continents and major climatic zones, finding that many plant speciation events occurred in the Quaternary and suggesting that climate change during this period was the cause.

However, it should be noted that estimated divergence times are highly dependent on the generation time used, as these times are calculated by multiplying generation time by approximate generation time. Cultivation experiments involving ecotypes SD and SG confirmed that flowering occurred within 1–2 years of sowing (Setsuko S., personal observation). We consider a 5-year generation time to be suitable for estimating divergence time; however, to validate our findings, it is essential that comparative analyses are conducted using alternative methods, such as BEAST (Drummond A, 2007), which employs fossil data for a more precise age calibration.

In this study, species and ecotypes adapted to a dry environment (species P and ecotype SD) and forest understory and forest edge environments (species G and ecotypes S and SG) diverged at the same time from ecotype ST that constitutes the canopy of tall mesic forests. Although speciation timing may lack precision due to methodological challenges regarding glacial or interglacial periods, it is evident that simultaneous diversification occurred concurrently. Considering the characteristics of the divergent species/ecotypes, the timing of speciation likely aligns with the onset of aridification on the islands. Initially, species/ecotypes adapted to the forest understory and forest edge environment may seem unrelated to aridification. However, species G inhabits forests that are not tall (dry scrub) (Toyoda, 2014), ecotype S inhabits the edges of mesic forests, and ecotype SG does not flower in overly dark forests (Setsuko S., personal observation). Therefore, they are all considered maladaptive to taller forests where ecotype ST grows. This suggests that the environment has changed from a forest with high tree height to a forest with reduced tree height and increased forest edges (Olson et al., 2018), potentially caused by the aridification of the island. Examples of organisms rapidly altering their phenotypes upon aridification have been reported in animals, such as Darwin’s finches (Grant & Grant, 2006), and in plants, such as *Mimulus* and *Brassica* (Dickman et al., 2019; Johnson et al., 2022). Selection has also been observed on the *HMG2* gene, causing beak size variation during drought in Darwin’s finches (Lamichhaney et al., 2016), and multiple genes associated with drought response traits evolving during drought in *Brassica* (Franks et al., 2016; Johnson et al., 2023). In the *Callicarpa* genus in the Bonin Islands, rapid adaptation to aridification may have led to speciation. Therefore, future research will involve identifying genes associated with drought adaptation.

Surprisingly, we revealed that the differentiation timing of the five species/ecotypes, occurring 73–77 kya, and the migration of ecotypes Sm and STm in the Mukojima Islands from ecotypes S in the Chichijima Islands and ST in the Hahajima Islands, respectively, took place during approximately the same period, around 81–82 kya (Fig. 4; Table S6 and S10). Aridification usually hinders the successful fruit reproduction of previously abundant plants (Abobatta, 2021), leading to food shortages across the entire island; therefore, it is plausible that avian seed dispersers may have moved to new islands in search of food (Boyle & Conway, 2007).

Population size changes, exhibiting large increases of more than two orders, were observed for species/ecotypes P, G, and SG in model e1 (Fig. 4a; Tables S6 and S8). Dry scrub, the habitat of species P and G, may have increased due to aridification during the glacial period. Ecotype SG currently inhabits the understory of mesic forests throughout the Hahajima Islands and has the largest population of any ecotype in the Hahajima Islands (Setsuko et al. 2023). Compared with the Chichijima Islands, the area of mesic forests is larger in the Hahajima Islands (Shimizu, 1999), and the substantial increase in population size of ecotype SG may be due to successful adaptation to the mesic forest environment through migration from the Chichijima Islands to the Hahajima Islands. However, large decreases of more than two orders were observed in ecotype Sm and STm in model g2 (Fig. 4b; Tables S8 and S10). This may be due to the limited number of individuals that migrated from the original island populations to the Mukojima Islands (i.e., the founder effect).

### Cryptic species

Phenotypic and genetic differences, as well as diversification patterns, were used to assess classifications as

distinct species. First, the leaf phenotypes of species P (*C. parvifolia*) and G (*C. glabra*), as well as ecotype SD (*C. subpubescens*), exhibited distinct and distinguishable distributions in the PCA of leaf morphology (Fig. 5). However, the ecotypes of *C. subpubescens*, excluding ecotype SD, displayed PCA plots with less clarity in terms of separation.

*Callicarpa parvifolia*, endemic to the Chichijima Islands grows in very dry and sunny rocky areas and is notably distinguishable from other *Callicarpa* species and ecotypes owing to its small, rounded, remarkably hairy, and thick leaves (Fig. S11). Similarly, *C. glabra*, also endemic to the Chichijima Islands grows in the dry scrub understory and is characterized by its small, slender, hairless, and rather thick leaves. Phylogenetically, these two species form a sister clade, each maintaining monophyly (Fig. 3b). Accordingly, the current taxonomy of the two species appears reasonable.

Ecotype SD, found only in the Hahajima Islands, forms a canopy in dry scrubs, with leaves that are small, round, hairy, and thick, similar to *C. parvifolia* (Fig. S11). However, it can be distinguished from *C. parvifolia* by significantly lower leaf hair density and thickness. Phylogenetically, ecotype SD and *C. parvifolia* were located different clades (Fig. 3b). A parallel evolutionary process of adaptation to dry environments likely resulted in a morphology closely resembling that of *C. parvifolia*. Additionally, compared with ecotype S from the Chichijima Islands, treated as *C. subpubescens*, ecotype SD differs both in its leaf morphology and flowering phenology, with prolonged flowering from summer to winter (Table 1), warranting recognition as a new species.

Although ecotype ST in the Hahajima Islands could not be clearly distinguished from other ecotypes of *C. subpubescens* except ecotype SD in leaf morphology (Fig. 5), it can be distinguished from other ecotypes using flowering phenology (the flowering season of ecotype ST is autumn, whereas those of ecotypes S, SG, and SH are summer; Table 1). Phylogenetically, we consider ecotype ST to be a cryptic species, as it diverged from the ancestral species at an age more than twofold greater than that of other genetic groups based on population demography analysis (Fig. 4).

Both ecotype S in the Chichijima Islands and ecotype SG in the Hahajima Islands flower in summer (Table 1). Ecotype S is identical to the registered type specimen of *C. subpubescens* (specimen no. K000674714), exhibiting fine, soft hairs on its leaves, whereas ecotype SG has almost no hairs on its leaves. However, the number of hairs did not differ compared with ecotype SG (Fig. S11), and the plot distributions of ecotypes S and SG overlapped in PCA (Fig. 5). Despite belonging to different genetic groups, no obvious phenotypic differences were observed. Several examples exist in the Bonin Islands of the same species differentiated genetically due to gene flow restriction caused by the different island groups they inhabit (Setsuko et al., 2017; Setsuko et al., 2022; Setsuko et al., 2020; Sugai et al., 2013). Therefore, considering ecotype SG as homologous to ecotype S is reasonable.

Ecotype SH, thought to be derived from a hybrid of ecotypes ST and SG, is found only on Hahajima Island (Figs. 1 and 2a). Ecotypes SH and ST possessed similar leaves, with ecotype SH occupying part of the broader distribution of ecotype ST in the PCA plot (Fig. 5). However, the plot distributions of ecotypes SH and SG in PCA did not overlap. As the flowering season of ecotypes SG and SH is summer and that of ecotype ST is autumn (Table 1), ecotypes SH and ST are distinguished by their flowering periods, whereas ecotypes SH and SG are roughly distinguished by their leaf morphologies. In naturally distributed individuals, habitat information can aid taxonomic classification of ecotypes SG and SH, as ecotype SH inhabits high-elevation cloud forests and forms the forest canopy layer, whereas ecotype SG inhabits the understory of mesic forests, except in the high-elevation areas of the Hahajima Island (Setsuko et al., 2023). Based on these findings, we propose that *C. subpubescens* can be divided into three species in addition to one hybrid-derived taxon, rather than one species.

Concerning ecotypes in the Mukojima Islands, the flowering of STm and Sm was investigated only once in July, with these ecotypes found to be in the early and late stages of flowering, respectively. July marked the beginning of flowering for ecotype ST in the Hahajima Islands, and the end of flowering for ecotype S in the Chichijima Islands (Table 1). Combining flowering information with the phylogenetic tree results

from this study, Sm was considered the same ecotype as S, whereas STm was considered the same ecotype as ST. Determining whether ecotypes STm and Sm in the Mukojima Islands can be considered identical to ecotypes ST and S, respectively, necessitates a more thorough investigation into the flowering periods of ecotypes STm and Sm. Furthermore, although only a minimal number of hybrid individuals (two) were found in the Mukojima Islands (Figs. 1 and 2), the extent to which they form hybrid zones, akin to ecotype SH on Hahajima Island (Setsuko et al., 2023), remains unclear. However, given the coexistence of lineages from different origins on the same island, there is a possibility that this codistribution contributes to hybrid speciation (Kagawa & Takimoto, 2018). Further research should include more comprehensive investigations into hybridization in the Mukojima Islands.

### Long seed dispersal between the island groups

In phylogenetic tree analysis, ecotypes S in the Chichijima Islands, SG in the Hahajima Islands, and Sm in the Mukojima Islands together with subclade 1–2, whereas ecotypes ST in the Hahajima Islands and STm in the Mukojima Islands formed another subclade (Fig. 3b), indicating potential interisland group migration. The primary seed disperser of *Callicarpa* is the brown-eared bulbul (*Hypsipetes amauroti* s). Furthermore, metabarcoding from the feces of the Japanese wood pigeon (*Columba janthina nitens*), endemic to the Bonin Islands, occasionally showed the presence of *Callicarpa* seeds (Ando et al., 2016). Although pigeons are not considered efficient seed dispersers due to seed crushing in their gizzards, seeds up to 3 mm in size remain intact in their feces (Shibazaki & Hoshi, 2006). Additionally, until 1920, the jungle crow (*Corvus macrorhynchos*) was present in the Bonin Islands (Higuchi, 1984). Estimated seed dispersal distances for these bird species, based on body size (Dunning Jr, 2007), and calculated using a phylogenetic generalized least squares model for seed retention time (Yoshikawa et al., 2019) and flight speed (Tennekes, 2009), are as follows: brown-eared bulbul, 39.7 km; Japanese wood pigeon, 88.8 km; and jungle crow, 110 km. The current distances between the Mukojima and Chichijima, Chichijima and Hahajima, and Mukojima and Hahajima Islands are 32, 35, and 110 km, respectively. The presence of the same ecotypes in different island groups is likely a result of long-distance seed dispersal by these birds. Particularly, ecotype ST, inhabiting the mesic forests of the Hahajima Islands, occurs in the Mukojima Islands but not in the Chichijima Islands, despite similar mesic forests in the latter. It is speculated that ecotype ST migrated between the Hahajima and Mukojima Islands through birds with high flight ability, bypassing the Chichijima Islands. However, due to genetic differentiation even among the same ecotypes in different island groups, the occurrence of long-distance seed dispersal between islands is expected to be extremely rare.

### Conclusion

Our study on the *Callicarpa* genus in the Bonin Islands provides crucial insights into phylogenetic relationships, divergence patterns, and speciation drivers. The ancestral ecotype ST of *C. subpubescens* was over twofold older than the other species/ecotypes, followed by ecotype SD, species P and G, ecotype S, and finally ecotype SG, all diverging simultaneously. The concurrent diversification of species/ecotypes adapted to different environments suggests a connection to island aridification. Shifts from tall forests to lower forests with increased forest edges and an increase in dry environments likely triggered rapid phenotypic and genetic changes for adaptation, as observed in other organisms. Future research will focus on identifying genes associated with drought adaptation. Cryptic species were found within *C. subpubescens*, with ecotypes SD and ST considered distinct species based on leaf morphology, flowering phenology, and genetic divergence patterns. Although genetically distinct, ecotypes S and SG show no significant phenotypic differences and can be treated as the same species. Ecotype SH, presumed to be a hybrid between ecotypes ST and SG and found only on Hahajima Island, exhibited different characteristics from its parent ecotypes. Long-distance seed dispersal events likely contributed to the presence of the same ecotypes in different island groups. Certain birds, such as the brown-eared bulbul, Japanese wood pigeon, and jungle crow, are potential seed dispersers, with estimated dispersal distances aligning with the longer distances between islands. However, genetic differentiation among the same ecotypes in different island groups suggests rare long-distance dispersal. Our study provides a comprehensive understanding of phylogenetic relationships, divergence patterns, and speciation processes in *Callicarpa* in the Bonin Islands. Moreover, it emphasizes the importance of

hybridization, adaptive radiation in response to changing environments, and long-distance seed dispersal in shaping the evolutionary history of plants in oceanic islands.

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#### DATA ACCESSIBILITY AND BENEFIT-SHARING

Genotype data has been deposited at FigShare: <https://figshare.com/account/articles/24356665>. The PacBio Sequel raw reads are available at NCBI Sequence Reads Archive (DRA017215), and the reference genome sequence of *C. subpubescens* has been deposited at DDBJ/EMBL/GenBank under the accessions BTTA01000001–BTTA0106011.

#### AUTHOR CONTRIBUTIONS

SS, SN, IY and HK designed the research. SS, SN, KS and HK sampled materials. SN and AJN performed the laboratory work. TUI performed de novo genome assembly. SS and IT performed data analysis. All co-authors discussed the results. SS and IT wrote the paper.

Table 1 Population characteristics of the three *Callicarpas* species in the Bonin Islands examined in this study.

Species	Island group	Island	Population ID	Species/ Ecotype <sup>+</sup>	No. individuals	Leaf hairs (mean d
<i>C. parvifolia</i>	Chichijima	Anijima	Pa	P	7	195.4, 558.0
		Chichijima	Pc	P	7	57.8, 556.9
<i>C. glabra</i>	Chichijima	Anijima	Ga	G	7	1.1, 2.8
		Chichijima	Gc	G	7	3.4, 2.6
<i>C. subpubescens</i>	Mukojima	Mukojima	STm	STm	7	17.5, 37.9
		Mukojima	Sm	Sm	7	7.1, 14.2
	Chichijima	Anijima	Sa	S	5	4.4, 19.0
		Chichijima	Sc	S	7	0.7, 15.6
	Hahajima	Hahajima	SGh	SG	7	0.9, 0.1
		Imoutojima	SGi	SG	6	0.7, 7.7
		Hahajima	SDh	SD	7	21.8, 64.0
		Imoutojima	SDi	SD	6	19.2, 38.5
		Hahajima	STh	ST	7	22.3, 91.1
		Hahajima	SHh	SH	7	17.5, 37.9

<sup>+</sup>First letter is the initial letter of the species name, second letter is the ecotype abbreviation of the Hahajima and Mukojima Islands for *C. subpubescens*, and last lowercase letter is the initial letter of the island's name; <sup>++</sup>Sugai et al. (2019); <sup>SS</sup>Setsuko et al. (2023).

#### Figure legends

Fig. 1 Results of ADMIXTURE analysis performed on 89 individuals from 14 populations of the Bonin Islands using denovo dataset. The cross-validation (CV) error values for each run (a) and bar plots depicting the genetic admixture proportions for  $K = 2-8$ . Vertical columns represent individuals; heights of bar plots are proportional to the posterior means of the estimated admixture proportions.

Fig. 2 Neighbor-net network tree reconstructed using 89 individuals from 14 populations (a) and 82 individuals from 13 populations (excluding ecotype SH, b) of the Bonin Islands and two outgroup individuals using the referenced dataset. Phylogenetic clade colors correspond to those used in Figs. 1 and 3.

Fig. 3 Phylogenetic tree of analysis inferred from Mr. Bayes using 30 individuals from 14 populations (a) and 28 individuals from 13 populations (excluding ecotype SH, b) of the Bonin Islands and two outgroup individuals using the referenced dataset. Phylogenetic clade colors correspond to those used in Figs. 1 and 2.

Fig. 4 Best (a) six- and (b) five-ecotype divergence models. These correspond to models e1 and g2 in Fig. S2, respectively. Period shown in dashed lines assumes migration between ecotypes. Direction of migration shows the movements of individuals (i.e., forward-in-time). Ecotype colors correspond to those used in Figs. 1–3.

Fig. 5 Distributions of the first and third principal components (pc1 and pc3) for eleven leaf morphological traits of seven species/ecotypes (a), and those of the second and third principal components (pc2 and pc3) for ecotypes of eleven leaf morphological traits of five species/ecotypes (ecotypes P and SD are masked) (b).

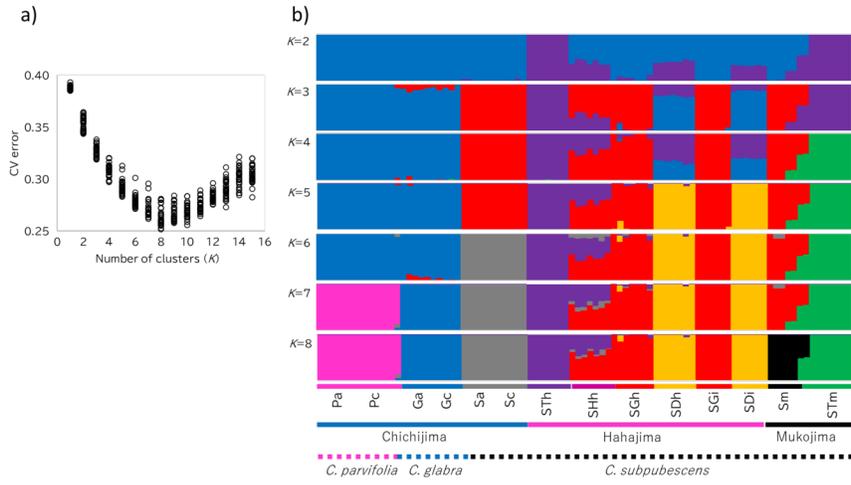


Fig. 1

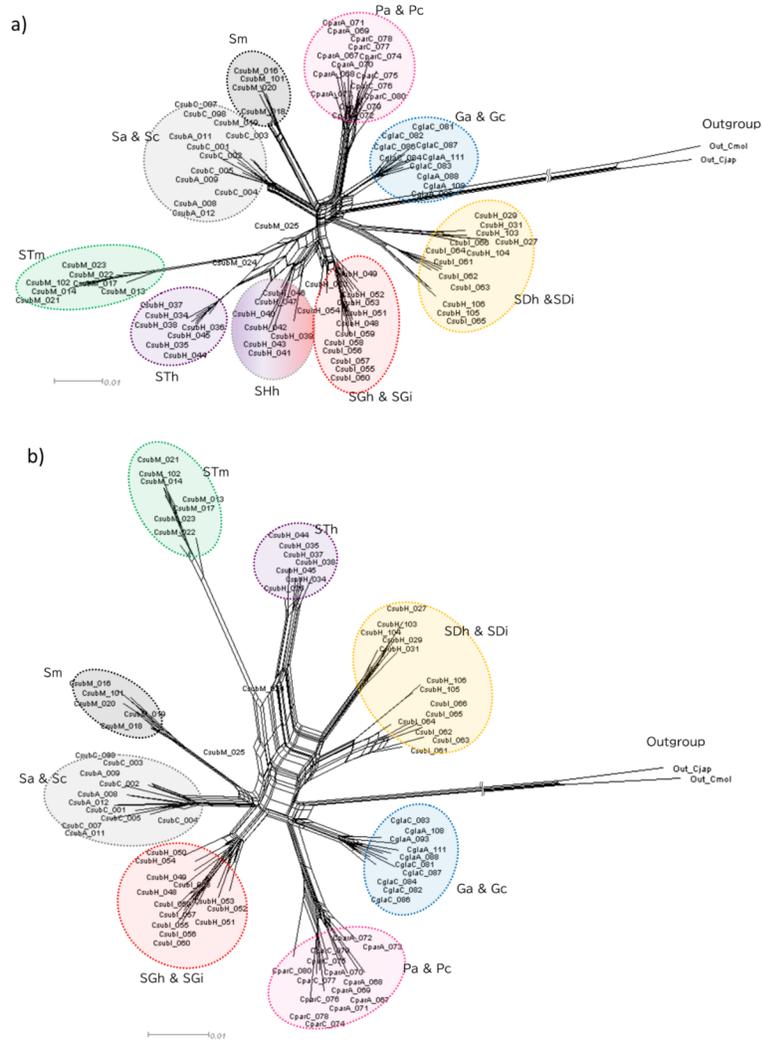


Fig. 2

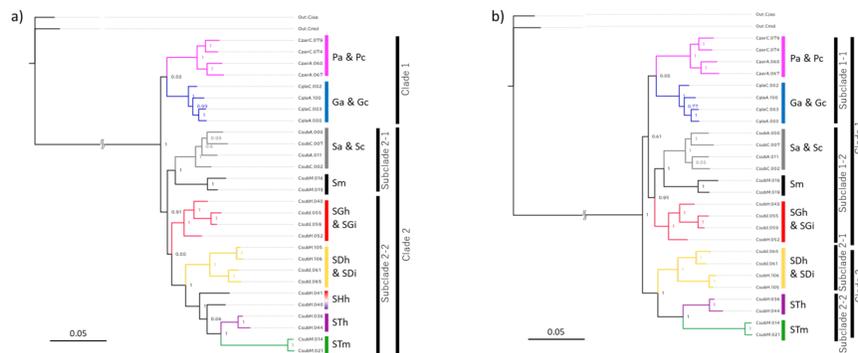


Fig. 3

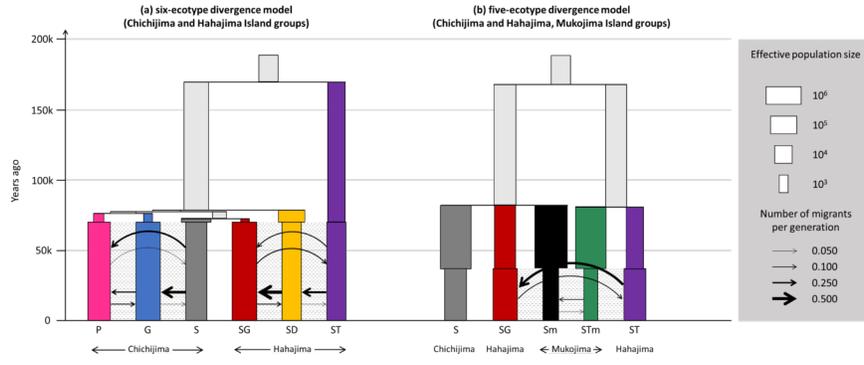


Fig. 4

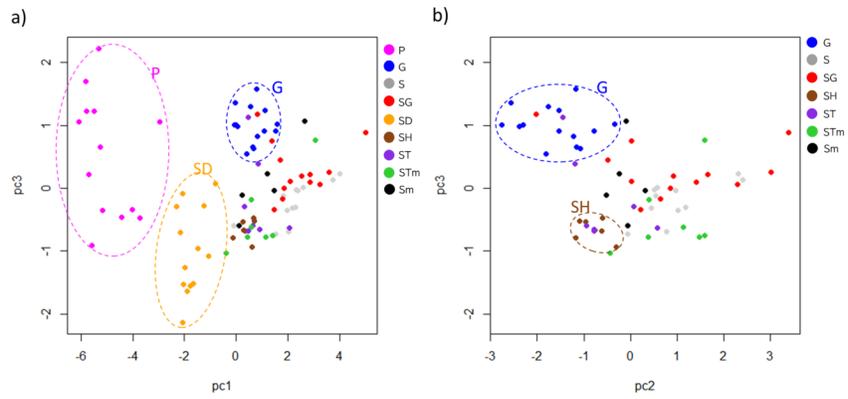


Fig. 5