Local environmental adaptation contributes to the maintenance of ecotypes of *Callicarpa subpubescens* (Lamiaceae), in spite of frequent hybridization and low pre- and post-mating barriers

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Abstract

Callicarpa subpubescens is endemic to the oceanic Ogasawara Islands, and multiple ecotypes have been suggested to exist within southern part of this group, the Hahajima Islands, each associated with unique localized habitats. We determined the habitat characteristics of each ecotype, the presence or absence of pre- and post-mating isolation, the amount of gene flow among ecotypes in adult trees and naturally pollinated seeds using EST-SSR markers, and discussed how ecotypes are maintained in this species. There were four ecotypes in the Hahajima Islands, one of which presumed to be derived from hybridization of the remaining two ecotypes. The spatial distribution and habitat of each ecotype showed distribution which have depended on the suitable environment for each ecotype, i.e. local adaptation. The leaf morphology and size distribution of each ecotype also indicated the appropriate forms for each habitat. Flowering times more or less overlap among the ecotypes, indicating that pre-mating isolation is not perfect. Artificial cross-pollination showed that no post-mating isolation exists between ecotypes. Hybridization rates in adult trees and naturally pollinated seeds were 37.2% and 26.4%, respectively, and most of the hybrids were backcrosses and few F1. The hybridization rates of each ecotype and paternal correlation indicated that the flowering synchrony and spatial distribution of ecotypes contributed to hybridization among ecotypes. The reversion to the original ecotype which adapted to the environment through backcrossing would contribute to the maintenance of the ecotypes.

Title; Local environmental adaptation contributes to the maintenance of ecotypes of *Callicarpa subpubescens* (Lamiaceae), in spite of frequent hybridization and low pre- and post-mating barriers

Running title; Ecotype diversification in endemic *Callicarpa*

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ABSTRACT

Callicarpa subpubescens is endemic to the oceanic Ogasawara Islands, and multiple ecotypes have been suggested to exist within southern part of this group, the Hahajima Islands, each associated with unique localized habitats. We determined the habitat characteristics of each ecotype, the presence or absence of pre- and post-mating isolation, the amount of gene flow among ecotypes in adult trees and naturally pollinated seeds using EST-SSR markers, and discussed how ecotypes are maintained in this species. There were four ecotypes in the Hahajima Islands, one of which presumed to be derived from hybridization of the remaining two ecotypes. The spatial distribution and habitat of each ecotype showed distribution which have depended on the suitable environment for each ecotype, i.e. local adaptation. The leaf morphology and size distribution of each ecotype also indicated the appropriate forms for each habitat. Flowering times more or less overlap among the ecotypes, indicating that pre-mating isolation is not perfect. Artificial cross-pollination showed that no post-mating isolation exists between ecotypes. Hybridization rates in adult trees and naturally pollinated seeds were 37.2% and 26.4%, respectively, and most of the hybrids were backcrosses and few F_1. The hybridization rates of each ecotype and paternal correlation indicated that the flowering synchrony and spatial distribution of ecotypes contributed to hybridization among ecotypes. The reversion to the original ecotype which adapted to the environment through backcrossing would contribute to the maintenance of the ecotypes.

KEYWORDS

adaptive introgression, adaptive radiation, cryptic species, hybrid zone, ongoing speciation, the Bonin Islands

INTRODUCTION

Adaptive radiations are recognized as a pattern of ecological speciation in which a single species, over a relatively short period of time, differentiates into multiple closely related sympatric species with morphological and physiological differentiation resulting from adaptation to contrasting environments or ecological niches (Gillespie et al., 2001; Givnish, 1997; Schluter, 2000). Adaptive radiations can involve ecological speciation whereby adaptation to different environments or ecological niches leads to the evolution of isolation barriers and the development of reproductive isolation (Rundle & Nosil, 2005; Schluter, 2001). Reproductive isolation can be broadly distinguished into two classes: pre- and post-mating barriers. The former prevents gene flow between different species or populations by changing flower color (Bradshaw & Schemske, 2003; Hoballah et al., 2007), morphology (Yang et al., 2007), odor (Okamoto et al., 2015) and flowering phenology (Martin et al., 2007) etc., and the latter prevent fertilization or the production of viable or fertile hybrid offspring after pollination (Case & Willis, 2008; Sandstedt et al., 2021). Adaptive radiations are well suited for studying environmental adaptation in ecological speciation because they are characterized by the rapid occurrence of many species that exhibit diverse environmental adaptations. Adaptive radiations have been shown to occur in a variety of plants and animals, especially on oceanic islands (Baldwin, 1997; Chiba & Cowie, 2016; Grant & Grant, 1996), probably due to the small number of species available to occupy their diverse ecological niches. Many papers report that adaptive radiation is occurring, however, few studies have examined how species and ecotypes are maintained after diversification by directly measuring the degree of pre- and post-mating isolation (Christie & Strauss, 2019), and quantifying gene flow from naturally pollinated seeds (Goulson & Jerrim, 1997). In order to understand how biodiversity is maintained on oceanic islands, it is very important to clarify the adaptive evolutionary processes of island organisms.

In recent years, it has become clear that hybridization between different evolutionary lineages or taxa has caused rapid diversification of ecological traits and promoted adaptive radiations (Meier et al., 2017). It has also been shown that introgression, the transfer of genes from one taxon to another taxon through hybridization and recurrent backcrossing, is potentially advantageous to new niches because it can add novel genes which is advantageous to survival to the recipient taxon (Chhatre et al., 2018; Suarez-Gonzalez et al., 2016). Thus, introgression allows the rapid colonization of recipient taxon to a new niche that the donor taxon has inhabited (Arnold & Kunte, 2017). Hybrid zones are areas in which genetically distinct taxa come into contact and form hybrids that exhibit traits intermediate to those of the parent species (Barton & Hewitt, 1985). Studies of hybrid zones suggest that most hybrids are less fit than their parents in their parents' niches, but more fit in novel niches (Arnold & Hodges, 1995; Barton, 2001; Burke & Arnold, 2001; Lexer et al., 2003). Ecological conditions that facilitate the establishment of hybrid zones would also likely to promote adaptive radiation since they both require new and previously unused niches (Seehausen, 2004).

The Ogasawara Islands are oceanic islands which are volcanic origin, located in the northwest Pacific Ocean in Japan, approximately 1,000 km south from Tokyo. They comprise four island groups: the Mukojima, Chichijima, Hahajima (collectively called the Bonin Islands) and the Volcano Islands. Their total land area is small comprising approximately 80 km^2, however, their endemic species rates are as high as 40 % for the vascular flora (Ono et al., 1986) and more than 90 % in land snails (Tomiyama & Kurozumi, 1991). The elevation of the Hahajima Islands is the highest among the Bonin Islands, and topography of the islands are also more varied compared with the other islands. Especially, cloud cover and fog frequently occurs at high elevations allowing the area to develop mesic scrub as tall as 1-2 m which is unique to the islands (Shimizu, 1992).

The genus *Callicarpa* (Lamiaceae) in the Ogasawara Islands consists of three recognized endemic species, *Callicarpa parvifolia* , *C. glabra* and *C. subpubescens* , which are considered to represent adaptive radiation (Ono, 1991). They are dioecious while most species of*Callicarpa* are hermaphrodite (Kawakubo, 1990). *Callicarpa parvifolia* and *C. glabra* are distributed only in the Chichijima Islands, while *C. subpubescens* is distributed widely across the Ogasawara Islands including the isolated Volcano Islands. Pollen are mainly dispersed by insects, such as endemic small bees, introduced honey bees (Abe, 2006) and endemic *Xylocopa* bees (Setsuko S., personal observation). The sizes of fruits and seeds are approximately 3 and 2mm, respectively, and small birds, such as brown-eared bulbul (*Hypsipetes amaurotis*) disperse their seeds (Sugai et al. 2019). Sugai et al. (2019) investigated the population genetic structure and phylogenetic analyses of these three *Callicarpa* throughout the Ogasawara Islands, and found that three species in the Chichijima Islands were clearly genetically distinct, while *C. subpubescens*in the Hahajima Islands was differentiated into three genetic groups, and the spatial distribution of the genetic groups appeared to be related to habitat differences rather than geographic gradients. Kawakubo (1986) also showed that variation in leaf morphology of*C. subpubescens* in the Hahajima Islands were much higher than that of *C. subpubescens* in the Chichijima Islands. These facts suggested these genetically distinct groups of *C. subpubescens* in the Hahajima Islands may form distinct ecotypes that could represent ongoing adaptive radiation. Sugai et al. (2019) also uncovered significant admixture of genetic groups in some populations, suggesting the presence of gene flow and hybridization among genetic groups. Information on the frequency of hybridization, which genetic group pairs have a higher hybridization rate, and whether different life history stages (e.g., adult trees vs. seeds) have different hybridization rates, is necessary to understand the maintenance mechanism of each distinct genetic groups.

In this study, we conducted a detailed genetic analysis of *C. subpubescens* throughout the Hahajima and satellite Imoutojima Islands, which is located 5.6 km south-southeast of the southern tip of the Hahajima Island, including isolated trees, using 14 expressed sequence tag (EST)–based microsatellite (SSR) markers to answer following questions. 1) Are there associations between genetic groups and spatial distribution, habitats, leaf morphology and size structure of each genetic group, i.e., can genetic groups be called ecotypes? If so, 2) Is there pre- and post-mating reproductive isolation mechanism between ecotypes? 3) What are the hybridization rates of each ecotype in adult trees and naturally pollinated seeds? 4) How are ecotypes maintained?

MATERIALS AND METHODS

Sample collection

We comprehensively sampled leaves of 581 and 114 trees from the whole Hahajima and Imoutojima Islands (collectively referred to as the Hahajima Islands), respectively (Fig. 1). We named these data as adult trees, with regard to the data of natural pollinated seeds explained below. Adult trees include nine populations in the Hahajima Islands sampled by Sugai et al. (2019) (SHHA, SHHB, SHHC, SHHD, SHHE, SHHF, SHHG, SHIA and SHIB). The locations of the adult trees were recorded using a GPS receiver (Garmin GPSmap 60CSx). Leaf samples were desiccated with silica gel for DNA extraction.

In the Hahajima Islands, four ecotypes named "G: Glabrescent", "T: Tall", "D: Dwarf" and "M: Middle" were identified both morphologically and genetically (Table 1, see details in results). To investigate the current gene flow among ecotypes at the seed stage, naturally pollinated seeds were sampled from 76 maternal trees from five sites (12–19 trees per site) dominated by each rcotype in 2013 (Fig. 1, Table S1). For the ecotype G, we sampled seeds from two sites, named as Gn and Gs. Seeds of *C. subpubescens* are small as 2 mm and difficult to extract DNA directory from seeds, thus we extract DNA from germinated seedlings. Seeds were preserved at 4 °C wrapped with wet moss for about six month (prechilling) and then, germinated at room temperature. A total of 1,260 seedlings from five sites (201–304 seeds per site) were obtained (Table S1), and preserved at -20°C until DNA extraction.

DNA extraction and genotyping

Genomic DNA was extracted from the sampled leaves and germinated seedlings using a modified CTAB method. Genotypes of each trees were characterized at 17 EST-SSR markers listed in table S2, developed for *C. subpubescens* (Setsuko et al., 2018). PCR was carried out in 6 µl reaction mixtures containing ca. 1 ng genomic DNA, 2.5 µl Type-it Multiplex PCR Master Mix (Qiagen, Hilden, Germany), and 0.2 µM of each primer under the following conditions: 95 °C for 5 min, then 35 or 38 cycles of 94 °C for 30 s, 55 °C or 60 °C for 90 s, 72 °C for 90 s, followed by final extension at 60 °C for 30 min. PCR fragments were separated using a 3130 Genetic Analyzer (Applied Biosystems, CA, USA) and genotyped using GeneMarker software (SoftGenetics, PA, USA).

Characteristics of EST-SSR markers

To check whether each EST-SSR locus met the requirements for population genetic analysis, we used BayeScan 2.1 (1,000,000 simulations) (Foll, 2012) to identify outlier loci, which had excessively high or low F_{ST} compared to neutral expectations. The existence of null alleles were checked using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004) and linkage disequilibrium between loci in each population was tested using GENEPOP 4.7 (Raymond & Rousset, 1995; Rousset, 2008). For these analyses, seven populations in the Hahajima Islands which did not indicate admixed pattern in Sugai et al. (2019), listed in table S2, were used because adult trees we sampled were not always aggregated as a population (Fig. 1) and admixed population might bias these results.

Genetic analysis

We used the Bayesian clustering program STRUCTURE 2.3.4 (Falush et al., 2007; Pritchard et al., 2000) to identify genetic groups of adult *C. subpubescens* trees in the Hahajima Islands, and checked whether the genetic groups correspond to morphological ecotypes. This program assigns individuals into K subpopulations (clusters) based on an admixture model and a correlated allele frequencies model. We used runs involving 100,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in period of 50,000 iterations. The analysis was run 30 times with each K (ranging from 1 to 10). The optimal value of K was selected by assessing the likelihood distribution (mean Ln P(K)) and ΔK values (Evanno et al., 2005). Results were graphically summarized and checked the existence of minor clusters using the online version of CLUMPAK (Kopelman et al., 2015). In this analysis, we defined that the genetic cluster with the largest Q value as the ecotype of the adult trees, and trees assigned Q [?] 0.9 for each cluster as pure adult trees, and $Q<$ 0.9 as hybrid adult trees (Kato et al., 2014; Li et al., 2021). The hybridization rate for each ecotype was calculated as the percentage of hybridized trees relative to the total number of trees exhibiting the ecotype. For hybrid adult trees, largest and second largest Q values within the tree were used to estimate which ecotypes interbreed and formed the hybrid. Hybrid adult trees with 0.4 [?] largest $Q < 0.6$ was defined as first or second filial generation hybrids (F_1 or F_2), and hybrids with 0.6 [?] largest $Q < 0.9$ as backcross hybrids (Li et al., 2021).

The results of STRUCTURE analysis suggested ecotype M has resulted from hybridization between ecotypes G and T (see details in results), we also conducted principal coordinate analysis (PCoA) and HIest (Fitzpatrick, 2012) analysis using pure adults of ecotype G, T and M to test the possibility of a hybridization origin of ecotype M. PCoA was conducted using GenAlEx 6.501 (Peakall & Smouse, 2012). HIest is the program estimating the ancestry (S) and interclass heterozygosity (H $_{\mathrm{I}}$). Both S and H $_{\mathrm{I}}$range from 0 to 1. S is a similar to Q value estimated by STRUCURE and was defined as ancestry of ecotype T (i.e., 0 and 1 means pure ecotypes G and T, respectively). Expected values of$H$ $_{\mathrm{I}}$ for pure ecotypes, first filial generation hybrids (F_1), second filial generation hybrids (F_2) and first-generation backcross hybrids (BC_1) are 0, 1, 0.5 and 0.5, respectively. By plotting$S$ and H $_{\mathrm{I}}$ on a triangular plot, we can see the hybrid status of the two ecotypes. Reference allele frequencies of ecotypes G and T were calculated using pure adult ecotypes G and T. When calculating allele frequencies, for the alleles detected in only ecotype M, such alleles were assumed to be detected only once in both ecotypes G and T. Parameters were estimated by the maximum likelihood method with a simulated annealing (SANN) algorithm of 10,000 iterations, surf options and start-grid value of 100. To evaluate the power of genetic markers used, we simulated 1,000 individuals each of pure ecotype G, pure ecotype T, F_1, F_2, $\mathrm{BC}_1$to G ($\mathrm{BC}_{1\mathrm{G}}$) and BC_1 to T ($\mathrm{BC}_{1\mathrm{T}}$) using a sample function of R assuming an infinite population size.

Current hybridization rates among ecotypes at seed level was estimated using naturally pollinated seeds sampled from five sites (Fig. 1). We assessed the Q values of each seed using the USEPOPINFO option in the STRUCTURE. This analysis performed using pure adult trees (POPFLAG = 1) and seeds sampled from pure adult trees (POPFLAG = 0). Allele frequencies were updated using only the reference data with POPFLAG = 1.K was fixed at four and run 30 times with 100,000 MCMC iterations after a burn-in period of 50,000 iterations. STRUCTURE analysis for adult trees revealed that 27 out of 76 maternal trees were hybrids, thus we eliminate seeds of hybrid trees from this analysis, and used total 847 seeds (85–272 seeds per site) from 49 pure maternal trees (6–15 trees per site) (Table S1). We defined seeds assigned Q [?] 0.9 for each cluster as pure seeds, and $Q < 0.9$ as hybrid seeds. Hybridization rates for naturally pollinated seeds were calculated as the percentage of the number of hybridized seeds relative to the total number of seeds. For hybrid seeds, the ecotypes showing the largest Q values other than that corresponding to the ecotype of the maternal tree was defined as the ecotype of the pollen parent.

Paternal correlation was estimated between all seed-parent pairs from the obtained genotypes of maternal trees and seeds by using the POLDISP software package (Robledo-Arnuncio et al., 2007). Then, we compared the paternal correlation within site and between sites. In this analysis, we used all genotyped 1,260 seeds from all 76 maternal trees, including seeds from hybrid trees (Table S1), since paternal correlation is the probability of seeds sired by the same paternal tree in different maternal trees, thus paternal correlation is not affected by whether the maternal trees are pure or hybrid.

Characteristics of ecotypes

To investigate the habitat of each ecotype, we extracted forest type of the adult trees which were categorized as pure ecotype from a vegetation map (Fig. 1) sourced from the Biodiversity center of Japan (1999-), and elevation and slope of the trees using ArcGIS Desktop 10.8.2 (ESRI Japan, Tokyo, Japan). Elevation and slope values were extracted from 10-m mesh digital elevation model provided by the Geospatial Information Authority of Japan. We used medium and fine categories of the vegetation map, which reflecting dominant species, physiognomy and geographical conditions. As for the extracted vegetation categories of *C. subpubescens* on Hahajima Island, mesic scrub (forest height, 1–2 m), dry scrub (1–6 m) and mesic forest (4–20 m) accounted for 69 % of the total categories, 19 % was plantation forest, and the remaining 10 % was *Freycinetia formosana* scrub and alien grassland of*Kalanchoe pinnata* , etc. We classified them into three major forest types: mesic forest, mesic scrub and dry scrub (Shimizu, 1992). Specifically, alien grasslands were classified as dry scrub, and*F. formosana* scrubs were included in the mesic scrub from the ecological characteristics and habitats of each species. Plantation forests were excluded from the analysis because the original forest type was not able to be determined.

To characterize the leaf morphology of each ecotype, we sampled two to five intact leaves from a total 28 pure adult trees (seven trees per ecotype), and examined the following 11 leaf traits: entire length, blade length, width of a leaf blade, hair density on the upper surface and on the lower surface of a leaf (number of hairs per 4 mm^2), number of serrations per 30 mm, thickness of leaf blade, ratio of blade length to entire length of a leaf, ratio of width to length of a leaf blade, which were examined in Kawakubo (1986), leaf area (LA) and leaf mass per area (LMA). These characteristics were subjected to principal component analysis (PCA) to test the morphological aggregation of leaves of each ecotype.

To characterize size distribution of adult trees of each ecotype, we measured the maximum stem length, the maximum diameter at breast height (DBH) and counted the number of stems per tree for total 81 trees (13–24 trees per ecotype) which were categorized as pure ecotype.

Pre- and post-mating reproductive barrier

To evaluate the existence of pre-mating isolation among ecotypes, flowering phenology of total 57 trees (9–18 trees per ecotype) which were categorized as pure ecotype were investigated. The number of flowing cymes were counted for each tree once a month for 10 months (May in 2014 to January in 2015).

To evaluate the existence of post-mating isolation among ecotypes, we conducted artificial inter-cross, pollination between different ecotypes, and intra-cross, pollination between same ecotypes, experiments using plants derived from cutting seedling raised in the greenhouse. For inter-cross pollination, total 16 cymes of five maternal plants of ecotype D were crossed with two paternal plants of ecotype G (G×D), and for intra-cross pollination, total 18 cymes of five maternal plants of ecotype D were crossed with eight paternal plants of ecotype D (D×D, Table S3). Fruit set rates were calculated for each cyme from the equation [(number of fruits / number of flowers in the cyme) × 100]. Total 240 seeds from 10 pairs of inter-crosses and 384 seeds from 16 pairs of intra-crosses were sowed and monitored the germination for six months (Table S3). Germination rate for each crossing pair were calculated from the equation [(number of germinated seedlings / number of sown seeds) × 100]. Ninety-six germinated seedlings for each cross type were transferred to pots and tracked the mortality for one year in a same laboratory environment with LED lights and regular watering (Table S3). Mortality rate for each crossing pair were calculate from the equation [(number of dead seedlings / number of seedlings transferred to pots) × 100].

In the course of mortality tracking, we compared the differences in soil moisture requirements between ecotypes. In addition to the seedlings derived from artificial crossing (G×D and D×D), natural pollinated seeds of site Gn were sown and grown under the same conditions. EST-SSR analysis was performed for seedlings from site Gn using the same method as for the adult trees, and only pure seedlings of ecotype G were used in the experiment. Three months after transferred to pots, watering was temporarily stopped and soil moisture content when the seedlings start wilting was measured using a soil moisture sensor (SM300, Delta-T Devices Ltd). The volumetric soil water content (ϑ % vol.) was calculated from the following equation: $\vartheta = -27.8V^5 + 30.3V^4 - 0.7V^3 - 9.0V^2 + 3.8V$, where V is measured voltage value. This equation was obtained from the relationship between V and volume water content of soil used for cultivation of seedlings.

RESULTS

Characteristics of microsatellite markers

We conducted an outlier test using Bayescan, and outliers were not detected in any of the 17 EST-SSR markers at false discovery rate 0.05 (Table S2). Three (Cal_0219, Cal_0351 and Cal_1632) out of 17 markers might have null alleles since estimated null allele frequency was significant in more than two out of seven populations (Table S2). No significant linkage disequilibrium was observed between loci in any population for the 17 markers. Thus, we used 14 markers for further analyses.

Ecotypes of *Callicarpa subpubescens* in the Hahajima Islands

The STRUCTURE analysis showed that the log-likelihood converged and reached a plateau at $K = 6$ (Fig. S1a). At $K = 2$, [?]$K$was highest, with smaller peaks at $K = 4$ and 6 (Fig. S1b). As we explained below, four phenotypical ecotypes of *C. subpubescens*were recognized in the Hahajima Islands, and $K = 2$ could not differentiate the ecotypes (Fig. S1c). $K = 4$ corresponded to the phenotypical ecotypes. $K = 6$ differentiate the ecotype G in three, northern and southern parts of Hahajima, and Imoutojima Islands, probably caused by isolation by distance. Thus, we judged $K = 4$ could represent the phenotypical ecotypes of *C. subpubescens* in the Hahajima Islands.

At $K = 3$ in the STRUCTURE analysis, genetic cluster correspond to ecotype M when $K = 4$ showed admixed pattern of ecotypes G and T (Fig. 2a), and most trees had higher Q values of ecotype T than those of ecotype G. In PCoA analysis using pure adults of ecotype G, T and M, axes 1 and 2, which explain 27.6 % of the variation, clearly separate three ecotypes (Fig. S2a). The axes 1 and 3, which explain 21.8 % of the variation, ecotypes G and M were overlapped, while ecotype T was separated (Fig. S2b). PCoA suggested ecotype M was closer to ecotype G than T. In HIest analysis, triangular plots between S andH_{I} for the observed data showed most points of ecotypes G and T were close to each pure ecotype, although points of ecotype G were more scattered than those of ecotype T (Fig. S3a). Points of ecotype M were located in the left half area and most points were located on the base and left-side lines of the triangle. These observed distribution of points of ecotype M were rather similar to simulated one of first-generation backcross hybrids to ecotype G ($\text{BC}_{1\text{G}}$) (Fig. S3b).

Spatial distribution, habitat and morphological traits of ecotypes

Pure ecotype D had the narrowest distribution range, and allopatrically distributed with other ecotypes (Fig. 1), and 78 % inhabited in the dry scrub on steep cliffs at elevations of 186.4 ± 37.6 m (Fig S4). On the other hands, pure ecotype G had the widest distribution range with entire Hahajima Island from low to high elevations (el. 158.5 ± 105.1 m), and 93 % inhabited in the mesic forests. Both pure ecotypes T and M were distributed on the ridges of the central mountain, but pure ecotype T was distributed at lower elevations than pure ecotype M (el. 302.2 ± 58.3 m) and 88 % inhabited in the mesic forests, while pure ecotype M was more concentrated in higher elevation areas (el. 394.0 ± 24.3 m) and inhabited in the mesic scrub (31 %) as well as mesic forests (69 %).

Principal component analysis was conducted using eleven leaf morphological traits of pure adult trees of each ecotype, and first and second principal components explained 71.3 % and 23.5 % of the variation, respectively, accounted for 94.8 % of the total variation (Fig. 3). Distribution of each plot was not clearly separated, but different ecotypes did not overlap each other. Ecotype M was located between ecotypes G and T. Leaf morphology of ecotype G was characterized by large leaf area, few numbers of hairs of both sides and few numbers of serrations (Fig. S5). That of ecotype D had small, rounded and thick leaf with many hairs. Ecotype T was characterized by short petiole and many hairs. Ecotype M had moderate size and number of hairs compared with other ecotypes.

Maximum stem length of pure adult trees was significantly different among ecotypes except between ecotypes G and M ($p < 0.05$, Fig. 4a), and DBH was significantly different among ecotypes except between ecotypes G and D, and G and M ($p < 0.05$, Fig. 4b). The largest in maximum stem length and DBH was ecotype T (average 7.1 m, 9.8 cm, respectively), followed by M (average 3.5 m, 5.5 cm, respectively), G (average 3.0 m, 2.7 cm, respectively) and D (average 1.5 m, 0.7 cm, respectively) in this order. The number of stems within individual tree was significantly larger in ecotype D (average 7.7) than other ecotypes (average 1.1–1.5) ($p < 0.05$, Fig. 4c).

Pre- and post-mating reproductive isolation mechanism

Main flowering times of pure ecotypes G and M were almost the same from June to July having same peak in July. Flowering time of pure ecotype T was from July to December with the peak in October (Fig. 5). Flowering time of pure ecotype D was long, from July to January except September, with two peaks August and November, and overlapped with most trees of other ecotypes. Flowering patterns were similar to those reported by Sugai et al. (2019), except ecotype D had a higher number of flowering trees in June and August. Although there were differences in flowering peak in all ecotype pairs except the pair G-M, the flowering periods more or less overlapped among ecotypes. This suggest all ecotypes have the potential to hybridize with different ecotypes and thus, pre-mating isolation among ecotypes is not perfect.

There was no significant difference in fruit set rate between inter-cross G×D and intra-cross D×D ($p = 0.63$, Fig. S6a). In germination rate, seeds from inter-cross G×D was significantly higher than intra-cross D×D ($p < 0.05$, Fig. S6b), and in mortality rate of seedlings was not significantly different between inter- and intra-cross ($p = 0.68$, Fig. S6c). The genetic distance between ecotypes G and D is rather large (Fig. 2a). Although we could not conduct artificial crossing for all ecotype pairs, no mechanism of post-mating isolation between any of the pairs were suggested. Volumetric soil water content when the seedlings start wilting was significantly lower in intra-cross D×D than in pure ecotype G, while inter-cross G×D showed intermediate values and not significantly different from pure ecotype G and intra-cross D×D (Fig. S6d).

Hybridization rate and paternal correlation

We identified 365 pure adult trees on Hahajima Island, of which 154, 67, 53 and 91 were ecotypes G, T, D and M, respectively. In contrast, 216 adult trees on Hahajima Island were hybrids, of which 184 were backcrosses and 32 were F_1 or F_2. The total hybridization rate of adult trees on Hahajima Island was 37.2 %. Hybridization rate of adult trees at each ecotype were about 20 % for ecotypes T and M, and about 40 % for ecotypes G and M (Fig. 2a, Table 2a). Among 216 hybrid adult trees, ecotype pair G-M accounted for the highest percentage at 52.3 %, while the ecotype pair T-D and D-M both accounted for the lowest percentage at 6.0 % (Table S4). The total hybridization rate of naturally pollinated seeds was 26.4 %. Hybridization rate of naturally pollinated seeds at each ecotype were lowest for site T with 7.7 % and highest for site Gs with 48.2 % (Fig. 2b, Table 2b). Among 224 hybrid seeds, ecotype pair G-M accounted for the highest percentage at 27.2 %, while the ecotype pair T-D accounted for the lowest percentage at 8.0 % (Table S4). The ecotype of the pollen parents of the hybrid seeds varied by site, with ecotype M being the most common in Gn, while ecotype T was the most common in Gs (Table 2). In site M, ecotype T was the most common pollen parent of hybrid seeds.

Paternal correlations were always positive among maternal trees of the same sites and were particularly high in site T and D (Fig. 6). On the other hands, paternal correlations among maternal trees of the different sites were negative except site pairs Gn-Gs and Gn-M, and especially low in site pairs Gn-T and Gs-T.

DISCUSSION

Ecotypes of *Callicarpa subpubescens* in the Hahajima Islands

Phenotypic and genetic clustering analysis revealed that there are four ecotypes of *C. subpubescens* in the Hahajima Islands. Sugai et al. (2019) reported the existence of three genetic groups in the Hahajima Islands, and the population SHHE which showed admixed pattern was identified as ecotype M in this study. A genetic cluster with small number of samples can be difficult to detect in STRUCTURE analysis (Meirmans, 2019). Thus, the greater number of adult trees collected in this study likely allowed us to clearly identify ecotype M as a separate genetic cluster. The results of STRUCTURE, PCoA and HIest suggest that ecotype M have resulted from hybridization between ecotypes G and T. Ecotype M showed intermediate distribution between ecotypes G and T in PCA analysis of leaf morphology. Maximum stem length and DBH of ecotype M was also intermediate between ecotypes G and T, although there was no significant difference between ecotypes G and M. Flowering time of ecotype M was similar to ecotype G. The phenomenon that speciation accelerated through hybridization has been found in many plants and animals (Abbott et al., 2010; Mallet, 2007). The presence of four ecotypes within a single tree species in small islands with only 20 km^2 would be attributed not only to adaptive radiation, but also to hybridization among ecotypes through secondary contact.

Local adaptation of ecotypes

Ecotype D is mainly distributed in the dry scrub on steep cliffs, while the other ecotypes are distributed in mesic forests and mesic scrub. Seedlings of intra-cross D×D wilted at a lower soil moisture content than those of pure ecotype G. Maximum stem length and DBH of ecotype D were low, and had smaller thick leaf with many hairs. It has been known there are negative correlation between drought tolerance and tree height (McGregor et al., 2021), and small, thick, trichome-rich leaves are also known to be an adaptation to dry areas (Ilyas et al., 2021; Tsujii et al., 2016). These facts suggest ecotype D is adapted to lower soil moisture, and considered to be separately inhabited with other ecotypes in terms of soil moisture. In the Bonin Islands, several endemic tree species are known to exhibit different genetic groups within a species that are distributed in mesic forests and dry scrub (Sugai et al., 2022; Tsuneki et al., 2014). This suggests genetic differentiation by soil moisture conditions is probably a common pattern of differentiation in the Bonin Islands.

Ecotype G, which is distributed in the understory of mesic forests, has almost no leaf hairs, while ecotypes T, D and M, which constitute forest canopy, have hairs on their leaves. Leaf hairs are known to reduce the photoinhibition caused by strong sunlight (Ripley et al., 1999). Growing ecotype G in a sunny location causes leaf burn and atrophy, while no such phenomenon occurs in other three hairy ecotypes (Setsuko S., personal observation), suggesting ecotype G is clearly not adapted to full sun exposure. Thus, ecotype G and other hairy ecotypes are considered to have undergone adaptation to the contrasting light intensity regimes characterizing the canopy and understory environments of mesic forests.

Ecotypes M and T mostly occur in separate habitats, although a small number of ecotype T is distributed within the distribution area of ecotype M. Ecotype M was predominantly distributed on the high-elevation of mountain ridges in mesic scrub, which is characterized by lower forest height, or at the edge of mesic forests. Ecotype M constitutes forest canopy of mesic scrub or mesic forests with low tree height, at most 4–5 m, while, ecotype T constitutes forest canopy of mesic forests as high as 10 m or more. Unfortunately, no forest height data were available for each adult tree in this study, and vegetation maps were used as an alternative, but future surveys will need to be conducted.

Since ecotype M is derived from the hybridization of ecotypes G and T, the distribution of ecotype M can therefore be considered as a hybrid zone. The hybrid zone has likely formed because ecotype M might be adaptive in a new niche, mesic scrub, that other ecotypes had not previously dominated, while ecotype M is not adapted to habitat of parents ecotypes G and T. Mesic scrub is distributed in areas with frequent cloud cover in areas above 350 m elevation on Hahajima Island (Shimizu, 2001). Frequent cloud cover tends to reduce the amount of sunlight (Loope & Giambelluca, 1998). Ecotype M might be able to dominate in mesic scrub because ecotype M is more shade tolerant than ecotype T due to its parentage from ecotype G. If this is the case, ecotype M might be an example of adaptive introgression (Suarez-Gonzalez et al., 2018). On the other hand, ecotype M is not distributed around seed sampling site T and the west of ecotype M distribution. Forests heights of these areas are higher than those in the area which ecotype M is distributed (Shimizu, 2008). The reason ecotype M cannot survive in those areas might be because ecotype M, with ecotype G as its parent, cannot grow as tall as ecotype T. To test these possibilities, future studies are needed to determine the differences in shade tolerance and growth rates between ecotypes T and M by common garden experiments, and what genes ecotype M has acquired from its parental ecotypes.

Hybridization rates among ecotypes and contributing factors

Paternal correlations were generally positive within the same site and negative between different sites, and especially large negative values were observed for site pairs Gn-T and Gs-T, which differ in flowering peaks, and exceptionally positive correlations were observed for site pairs Gn-Gs and Gn-M, which had synchronized flowering peaks. This is consistent with the results that the most common hybrid mating ecotype pair was G-M in both adult trees and naturally pollinated seeds. These results indicate that the degree of overlap in flowering time contributes to the degree of hybridization between ecotypes, as shown by other studies (Campbell et al., 2016; Field et al., 2011). On the other hand, ecotype D had the longest flowering time from summer to early winter and had potential to hybridize with any other ecotypes. However, hybridization rates of adult trees and natural pollinated seeds of ecotype D were not very high, and paternal correlations were negative for pairs D and all other sites. This was probably because ecotype D is locally distributed on dry scrub on steep cliffs. Pollen dispersal is limited by distance (Adams, 1992), and hybridization rates are therefore expected to lower in locally isolated ecotypes (Lagache et al., 2013).

Ecotype maintenance mechanism

The total hybridization rates were about 30–40 % at both adult tree and natural pollinated seed stages, suggest ecotypes of *C. subpubescens* on Hahajima Island is maintained despite the relatively high gene flow between ecotypes. This could be explained as follows: The result of STRUCTURE analysis of adults ($K = 4$) suggested most of the hybrid individuals are backcrosses. Even when F_1 are produced by hybridization between ecotypes, backcrossing pollination would be more frequently occur than F_2 because F_1 is rarer than pure trees (Tochigi et al., 2021). As backcrosses are repeated, the genetic composition of the offspring derived from hybridization would reverts to the genetic composition of the pure ecotypes.

There would be a reason for the small number of F_1 or F_2. Soil moisture when the seedlings start wilting was lowest in intra-cross D×D, intermediate in inter-cross G×D and highest in pure ecotype G. This suggests that fitness of hybrids in the dry habitat is inferior to pure ecotype D. In endemic tree taxa in the Bonin Islands, *Machilus* , hybrids with high genetic composition of mesic ecotype inhabit on dry habitat are removed from dry habitat as well as mesic ecotype individuals as they grow up (Tsuneki, 2012). This result indicates fitness of F_1 or F_2 hybrids of mesic and dry ecotypes in the dry habitat is lower than backcrosses to dry ecotypes, result in lower number of F_1 or F_2. In fact, in this study, F_1 or F_2 were much less than backcrosses. These facts would suggest that selection against hybrids, especially F_1 or F_2, is occurring. Lower fitness of hybrids would also contribute the maintenance of parental ecotypes (Lepais & Gerber, 2011; Twyford et al., 2015), and the reversion to the original ecotype which adapted to the environment through backcrossing would contribute to the maintenance of the ecotypes. However, in the present study, we only compared the fitness of ecotypes of G and D and F_1hybrids between them, so it is necessary to ascertain the relationship between adaptive traits and survival rates among other ecotype pairs and their hybrids.

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DATA ACCESSIBILITY

Genotype data of EST-SSRs used for this study are available from supporting information.

AUTHOR CONTRIBUTIONS

S.S., K.S., K.H. and H.K. designed the research. S.S., K.S., K.H. and H.K. sampled materials. S.S. performed all the laboratory work. S.S., K.S. and I.T. performed analysis. All co-authors discussed the results. S.S and I.T. wrote the paper.

Table 1 Name, id and characteristics of each ecotype

Ecotype	Ecotype id	Forest type and Habitat	Elevation	Slope
			(range, average (m))	(range, average (°))
Glabrescent	G	Understory of mesic forests	(13–424, 158.5)	(3–30, 17.8)
Tall	T	Forming crown of mesic forests	(215–427, 302.2)	(3–41, 17.3)
Dwarf	D	Forming crown of dry scrub	(101–279, 186.4)	(4–37, 25.1)
Middle	M	Forming crown of mesic forests and mesic scrub	(321–456, 394.0)	(4–41, 19.4)

Table 2 Purity and hybridization rates (%) for adult trees for each ecotypes (a) and naturally pollinated seeds for each seed sampling site (b)

a)

Ecotype	Purity rate (%)	Pairwise hybridization rate (%) (2nd largest Q)	Pairwise hybridization rate (%) (2nd largest Q)	Pairwise hybridization rate (%) (2nd largest Q)	Pairwise hybridization rate (%) (2nd largest Q)	Total hybridization rate (%)
(Largest Q)		G	T	D	M	
G	58.3	-	8.7	4.9	28	41.7

Ecotype	Purity rate (%)	Pairwise hybridization rate (%) (2nd largest Q)	Pairwise hybridization rate (%) (2nd largest Q)	Pairwise hybridization rate (%) (2nd largest Q)	Pairwise hybridization rate (%) (2nd largest Q)	Total hybridization rate (%)
T	79.8	4.8	-	4.8	10.7	20.2
D	77.9	4.4	13.2	-	4.4	22.1
M	55.2	23.6	15.2	6.1	-	44.8

b)

Seed sampling site	Purity rate (%)	Pairwise hybridization rate (%) (Pollen parent)	Pairwise hybridization rate (%) (Pollen parent)	Pairwise hybridization rate (%) (Pollen parent)	Pairwise hybridization rate (%) (Pollen parent)	Total hybridization rate (%)
		G	T	D	M	
Gn	67.9	-	5.7	7.9	18.6	32.1
Gs	51.8	-	27.1	12.9	8.2	48.2
T	92.3	4.8	-	2.6	0.4	7.7
D	72.7	10.6	5.6	-	11.1	27.3
M	58.6	16.4	24.3	0.7	-	41.4

Figure legends

Fig. 1 Spatial distribution of adult trees of pure ecotypes and hybrids on topographic (left) and pure ecotypes on vegetation map (right) of Hahajima Island. Enlarged distribution maps in the dashed square show five seed collection sites and spatial distribution of maternal trees. Letters indicated by arrows and letters in parentheses to the right of the seed collection site name are population names and location used in Sugai *et al.* (2019).

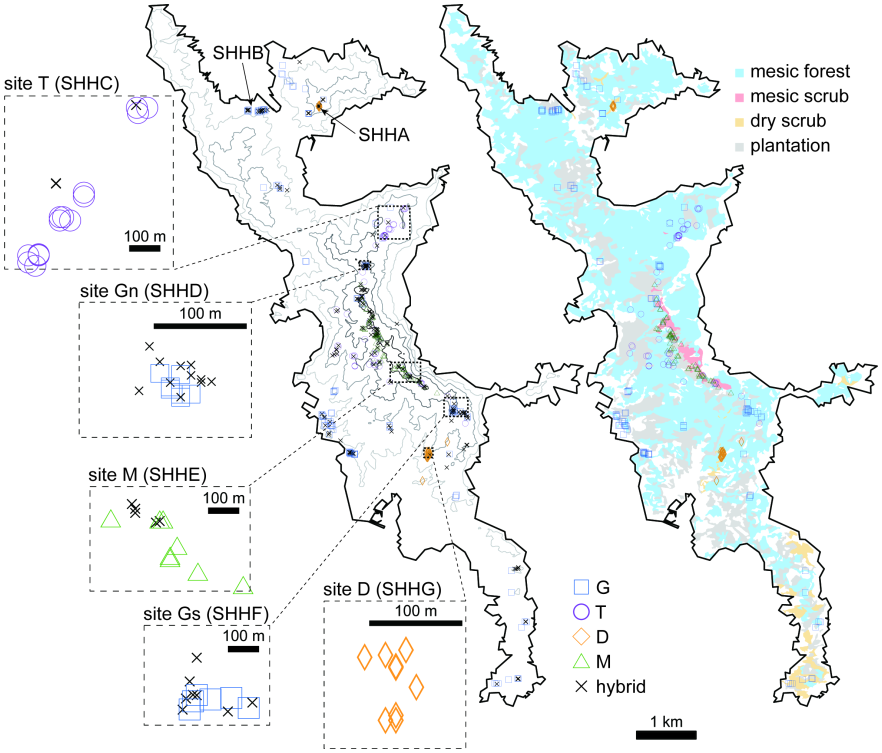
Fig. 2 Results of STRUCTURE analysis. (a) Bar plots of adult trees of each ecotype on Hahajima Island at K =3 and 4. Neighbor-joining trees to the right of the bar plot show the relationships of each cluster, and the numbers beside the circles indicate theF_{ST} values of each cluster. (b) Bar plots of naturally pollinated seeds for each site at K =4. Vertical columns represent individuals and heights of bars are proportional to the posterior means of the estimated admixture proportions. Individuals were ordered by Q value at K =4 for each ecotype. Horizontal red bars above the bar plot indicate individuals with $Q < 0.9$ (hybrids), and the numbers above indicate the hybridization rate for each category.

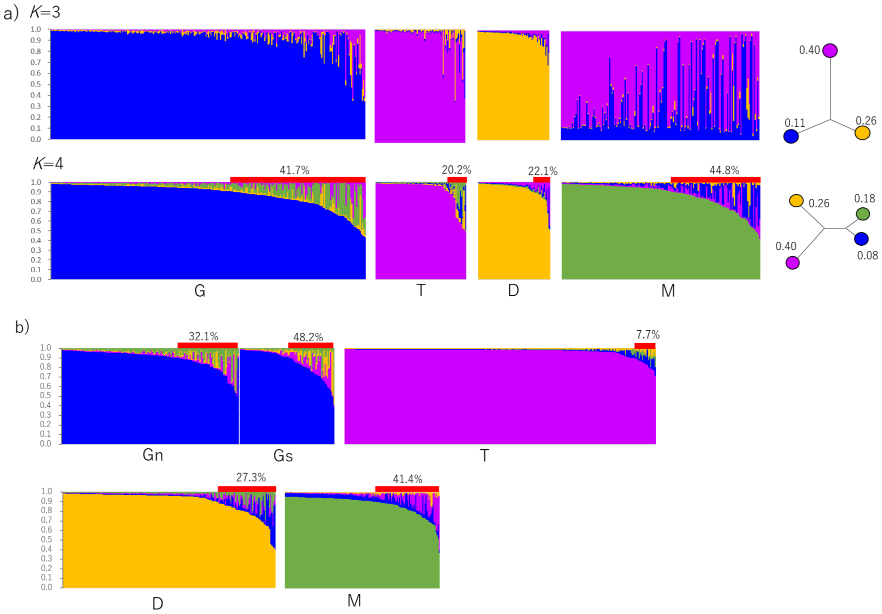
Fig. 3 Distributions of the first and second principal components (PC1 and PC2) for the 11 leaf morphological traits. Hair_U; leaf hair density on the upper surface, Hair_L; leaf hair density on the lower surface, Serration; number of serrations, LA; leaf area, LMA; leaf mass per area.

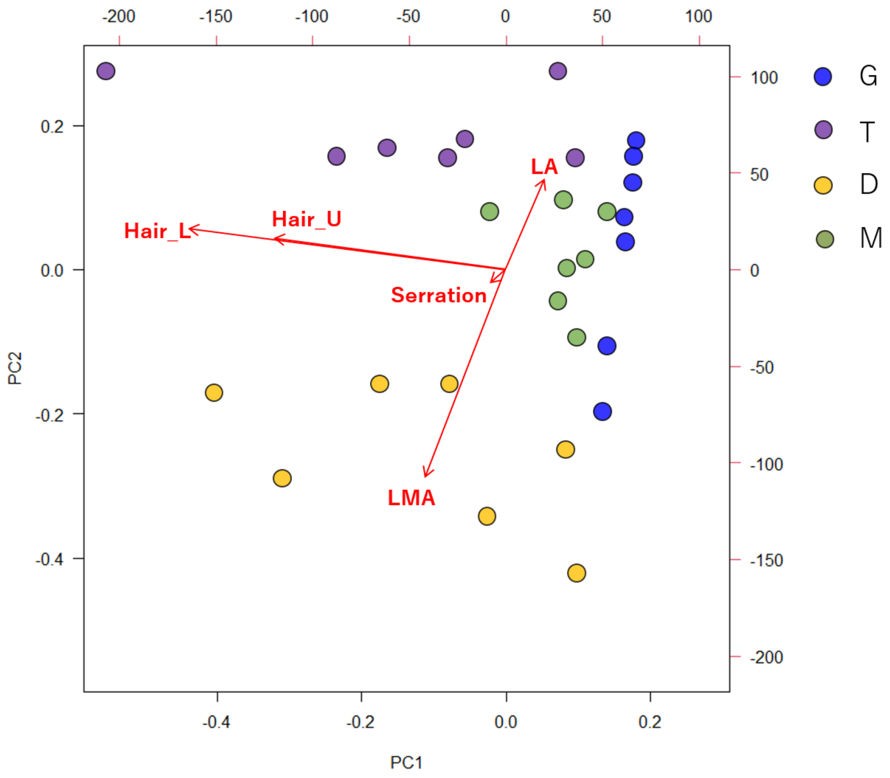
Fig. 4 Size distribution of pure adult trees for each ecotype. Different letters indicate significant difference among ecotypes (p< 0.05, pairwise t-test with Bonferroni correction).

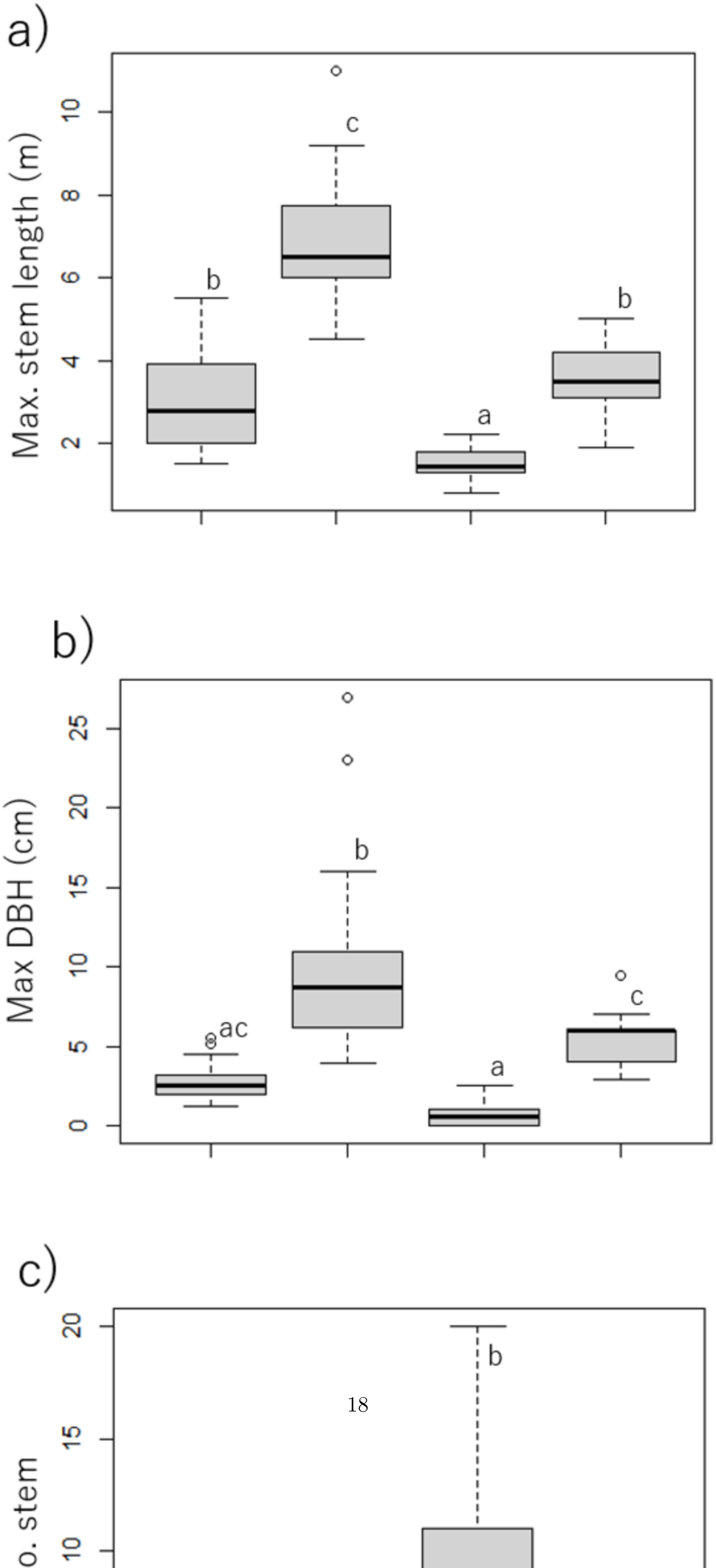
Fig. 5 Temporal changes of number of flowering cymes for each pure adult tree. Each horizontal line indicates an individual. Darker color indicates a greater number of cymes flowering.

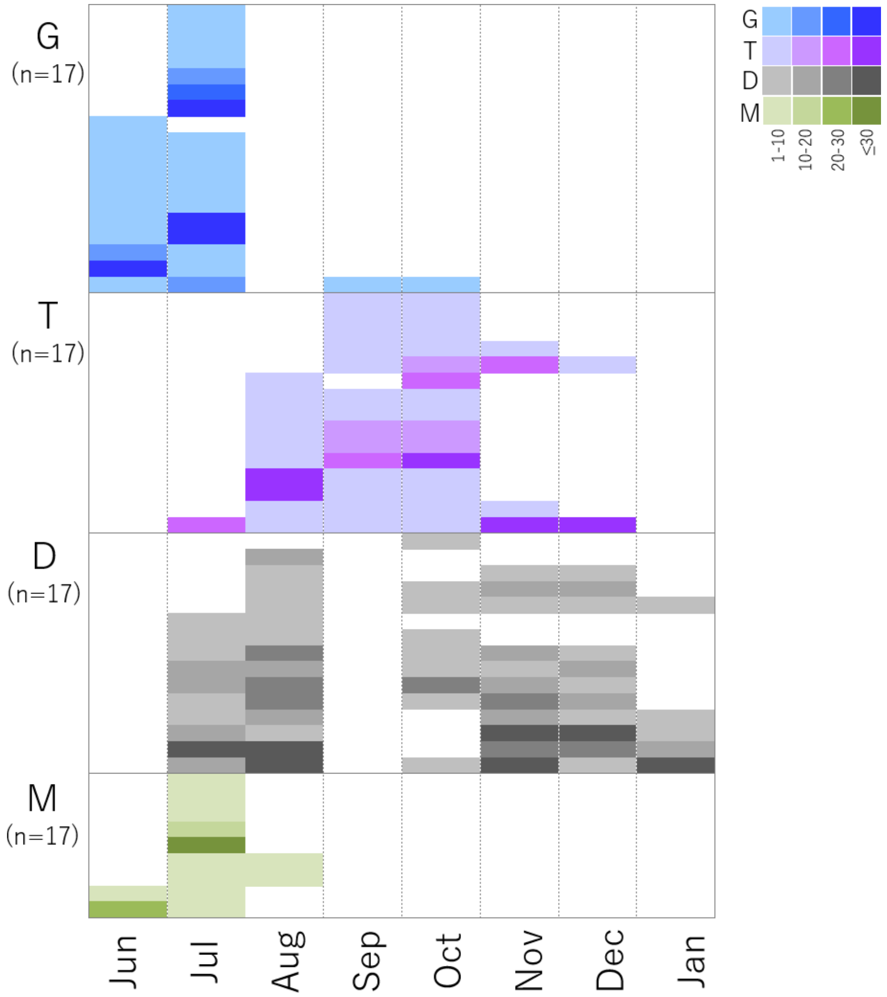
Fig. 6 Paternal correlation within (black bars) and between sites (gray bars).

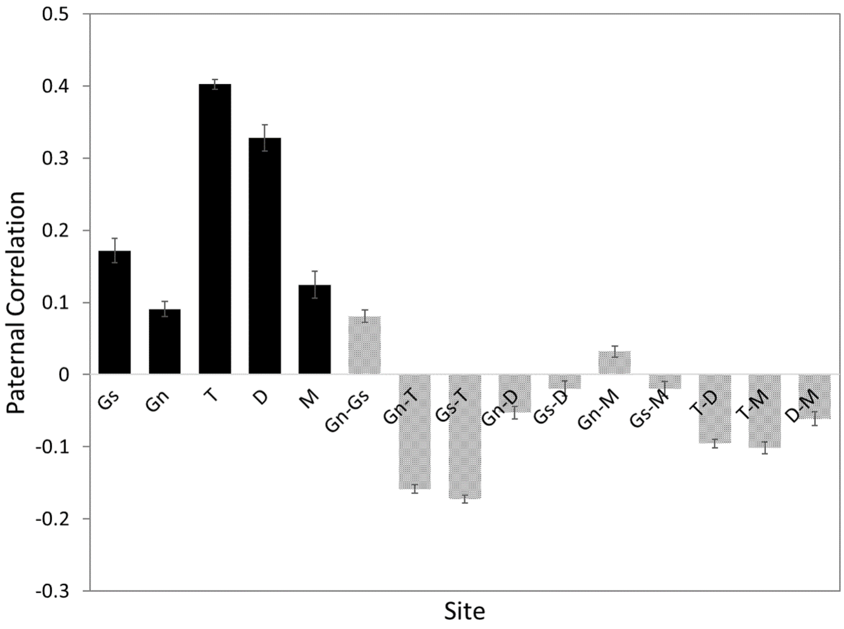

SHHB
SHHA
site T (SHHC)
100 m
site Gn (SHHD)
100 m
site M (SHHE)
100 m
site Gs (SHHF)
100 m
site D (SHHG)
100 m
mesic forest
mesic scrub
dry scrub
plantation
G
T
D
M
hybrid
1 km


a) K=3
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.40
0.11
0.26
K=4
41.7%
20.2%
22.1%
44.8%
0.26
0.18
0.40
0.08
G
T
D
M
b)
32.1%
48.2%
7.7%
Gn
Gs
T
27.3%
41.4%
D
M


-200
-150
-100
-50
0
50
100
0.2
0.0
-0.2
-0.4
100
50
0
-50
-100
-150
-200
-0.4
-0.2
0.0
0.2
PC1
PC2
LA
Hair_U
Hair_L
Serration
LMA
G
T
D
M


a)
Max. stem length (m)
2
4
6
8
10
b
c
a
b
b)
Max DBH (cm)
0
5
10
15
20
25
ac
b
a
c
c)
o. stem
10
15
20
b


G
(n=17)
T
(n=17)
D
(n=17)
M
(n=17)
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan
G
T
D
M
1-10
10-20
20-30
≤30



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`SupplementaryTables&Figs.docx` available at https://authorea.com/users/580224/articles/621375-local-environmental-adaptation-contributes-to-the-maintenance-of-ecotypes-of-callicarpa-subpubescens-lamiaceae-in-spite-of-frequent-hybridization-and-low-pre-and-post-mating-barriers