Ecotype maintenance in *Callicarpa subpubescens* via local environmental adaptation amidst frequent hybridization and low pre- and post-mating barriers

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Article

Keywords: adaptive introgression, adaptive radiation, Callicarpa subpubescens, cryptic species, hybrid zone, ongoing speciation, the Bonin Islands

Posted Date: June 28th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3085244/v1

Additional Declarations: There is no duality of interest

Version of Record: A version of this preprint was published at Heredity on May 7th, 2024. See the published version at https://doi.org/10.1038/s41437-024-00684-3.

Abstract

Adaptive radiations frequently occur on oceanic islands, but the mechanisms behind the maintenance of species and ecotypes after such radiations remain poorly understood, especially regarding potential gene flow among sympatric congeners. *Callicarpa subpubescens* is endemic to the oceanic Ogasawara Islands, and it has been suggested that multiple ecotypes exist within the southern part of this group, the Hahajima Islands, each associated with a unique localized habitat. Here, we determined the habitat characteristics of each ecotype and the degree of pre- and post-mating isolation by investigating flowering time overlap cross compatibility, and gene flow among ecotypes using an EST-SSR marker analysis of adult trees and naturally pollinated seeds. Our study confirmed the presence of four distinct ecotypes on the Hahajima Islands, with one likely arising from hybridization. Each ecotype was confined to specific island habitats, and distinct leaf morphologies and plant heights suggested local adaptation. However, overlapping flowering times and successful artificial cross pollination indicated little pre- and post-mating isolation. Hybridization rates in adult trees and naturally pollinated seeds were 37.2% and 26.4%, respectively, and most hybrids were backcrosses with few first- or second-generation hybrids. The hybridization rates of each ecotype and paternal correlation indicated that the flowering synchrony and close spatial distribution of ecotypes contributed to hybridization among ecotypes. Overall, *C. subpubescens* likely maintains four distinct ecotypes in spite of frequent hybridization probably due to selection pressures that result in reversion to the original ecotype via backcrossing, since this is well-adapted to the local environment.

Introduction

Adaptive radiations are a pattern of ecological speciation in which a single species, over a relatively short period of time, differentiates into multiple closely related sympatric species showing morphological and physiological differentiation resulting from adaptation to contrasting environments or ecological niches (Gillespieet al. 2001; Givnish 1997; Schluter 2000). Adaptive radiations can involve ecological speciation, in which adaptation to different environments or ecological niches leads to the development of isolation barriers and reproductive isolation (Rundle and Nosil 2005; Schluter 2001). Reproductive isolation involves two broad types of isolation mechanism: pre- and post-mating barriers. The former prevents gene flow between different species or populations, for example by changing flower color (Bradshaw and Schemske 2003; Hoballahet al. 2007), morphology (Yanget al. 2007), odor (Okamoto et al. 2015), and/or flowering phenology (Martin et al. 2007) etc. In contrast, the latter prevent fertilization or the production of viable or fertile hybrid offspring after pollination (Case and Willis 2008; Sandstedt et al. 2021). Adaptive radiations are well suited for studying environmental adaptation during ecological speciation because they are characterized by the rapid emergence of many species that exhibit diverse environmental adaptations. Adaptive radiations have been shown to occur in many plants and animals, especially on oceanic islands (Baldwin 1997; Chiba and Cowie 2016; Grant and Grant 1996), probably due to the small number of species available to occupy diverse ecological niches. Many papers have reported when adaptive radiations occur, 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 and Strauss 2019), or by quantifying gene flow in naturally pollinated seeds (Goulson and Jerrim 1997). It is therefore important to clarify the adaptive evolutionary processes of island organisms to understand how biodiversity is maintained on oceanic islands.

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

The Ogasawara Islands are oceanic islands located in the northwest Pacific Ocean off the coast of Japan, approximately 1,000 km south of Tokyo. This island chain comprises four distinct groups: the Mukojima, Chichijima, and Hahajima Islands (collectively called the Bonin Islands) and the Volcano Islands. Their total land area is small (~80 km^2), but their endemic species rates are as high as 40% for vascular flora (Onoet al. 1986) and more than 90% for land snails (Tomiyama and Kurozumi 1991). The elevation of the Hahajima Islands is the highest among the Bonin Islands, and their topography is also more varied relative to the others. Furthermore, cloud cover and fog frequently occur at high elevations, which allow the area to develop endemic mesic scrub that reach 1–2 m in height (Shimizu 1992).

The genus *Callicarpa* (Lamiaceae) in the Ogasawara Islands includes three recognized endemic species, *Callicarpa parvifolia*, *C. glabra*, and *C. subpubescens*, which are considered to represent an adaptive radiation (Ono 1991). All three are dioecious, despite most *Callicarpa* species being hermaphroditic (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 on the isolated Volcano Islands. Pollen dispersal occurs via insects, such as endemic small bees, introduced honey bees (Abe 2006), and endemic *Xylocopa* bees (SS personal observation). The sizes of fruits and seeds are approximately 3 mm and 2 mm, respectively, and small birds, such as the brown-eared bulbul (*Hypsipetes amaurotis*) are known to disperse their seeds (Sugai et al. 2019). Sugai et al. (2019) investigated the population genetic structure and phylogenetic analyses of these three *Callicarpa* species throughout the Ogasawara Islands using 14 microsatellite (SSR) markers. They found that the three species were clearly genetically distinct in the Chichijima Islands, while in the Hahajima Islands *C. subpubescens* had differentiated into three genetic groups, the spatial distribution of which appeared to be related to habitat differences rather than geographic gradients. Kawakubo (1986) also showed that variation in the leaf morphology of *C. subpubescens*in the Hahajima Islands was much higher than in the Chichijima Islands. These facts suggest that genetically distinct groups of *C. subpubescens* in the Hahajima Islands may form distinct ecotypes that represent ongoing adaptive radiation. Sugai et al. (2019) also uncovered significant admixture of genetic groups in some populations, which suggests that gene flow and hybridization occurred among genetic groups. Information on the frequency of hybridization, which genetic group pairs had higher hybridization rates, and whether different life history stages (e.g., adult trees vs. seeds) have different hybridization rates, is necessary to understand the mechanisms maintaining the distinctness of genetic groups.

In this study, we conducted a detailed genetic analysis of *C. subpubescens* throughout the Hahajima and satellite Imoutojima Islands, which is located approximately 5.6 km south-southeast of the southern tip of Hahajima Island (Fig. S1). To do so, we analyzed samples from populations and isolated trees using 14 expressed sequence tag (EST)–based SSR markers. We aimed to answer the following questions: 1) Are there associations between genetic group and spatial distribution, habitat, leaf morphology, and the size structure of each genetic group. That is, are these genetic groups really ecotypes? If so, 2) Are there pre- and post-mating reproductive isolation mechanisms 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 Hahajima and Imoutojima Islands (hereafter collectively referred to as the Hahajima Islands), respectively (Fig. 1, Fig. S1). These leaf samples were used to provide data for adult trees. Adult trees included nine populations taken from the Hahajima Islands that were sampled by Sugai et al. (2019) (i.e., SHHA, SHHB, SHHC, SHHD, SHHE, SHHF, SHHG, SHIA, and SHIB). Tree locations were recorded using a GPS receiver (Garmin GPSmap 60CSx). Upon harvest, leaf samples were desiccated using silica gel for DNA extraction.

During this study, we identified four ecotypes in the Hahajima Islands by their distinctive morphological and genetic features. These ecotypes were named “G: Glabrescent,” “T: Tall,” “D: Dwarf,” and “M: Middle” (Table 1). To investigate the current gene flow among ecotypes at the seed stage, in 2013 naturally pollinated seeds were sampled from 76 maternal trees from five sites (i.e., 12–19 trees per site) dominated by each ecotype (Fig. 1, Table S1). For ecotype G, we sampled seeds from two sites, Gn and Gs. Seeds of *C. subpubescens* are small (i.e., ~2 mm) and it is therefore difficult to extract DNA directly from seeds; we therefore extracted DNA from germinated seedlings. Cold stratification for seeds were performed at 4°C in wet moss for six months and were then germinated at room temperature. We obtained a total of 1,260 seedlings from five sites (201–304 seeds per site; Table S1). These were preserved at −20°C until DNA extraction.

DNA extraction and genotyping

Genomic DNA was extracted from sampled leaves and seedlings using a modified CTAB method. Genotypes of each sample were characterized by the 17 EST-SSR markers listed in Table S2, which were developed for *C. subpubescens* (Setsukoet 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. PCR conditions were as follows: 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 then 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 analyses, we used BayeScan 2.1 (1,000,000 simulations) (Foll 2012) to identify outlier loci, which we defined as those with excessively high or low F_{ST} compared to neutral expectations. The existence of null alleles was checked using Micro-Checker version 2.2.3 (Van Oosterhoutet al. 2004) and linkage disequilibrium between loci in each population was tested using GENEPOP version 4.7 (Raymond and Rousset 1995; Rousset 2008). For these analyses, seven populations in the Hahajima Islands that did not have a pattern of admixture in Sugai et al. (2019), listed in Table S2, were used because the adult trees we sampled did not always aggregate as a population (Fig. 1) and population admixture might bias these results.

Genetic analysis

We used the Bayesian clustering program STRUCTURE version 2.3.4 (Falushet al. 2007; Pritchard et al. 2000) to identify genetic groups of adult *C. subpubescens* trees in the Hahajima Islands, then checked whether these genetic groups corresponded to morphological ecotypes. This program assigns individuals to *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 repeated 30 times for each value of *K* from 1 to 10. The optimal value of *K* was selected by assessing the likelihood distribution (mean Ln P(*K*)) and Δ*K* values (Evannoet al. 2005). Next, we checked for the existence of minor clusters using the online version of CLUMPAK (Kopelmanet al. 2015). For this analysis, we defined the genetic cluster with the largest *Q* value as the ecotype of adult trees. Trees assigned at $Q \geq 0.9$ to each cluster were then considered to be pure adult trees, and trees with $Q < 0.9$ were considered to be hybrid adult trees (Kato et al. 2014; Li et al. 2021). The hybridization rate of each ecotype was calculated as the percentage of hybridized trees relative to the total number of trees of that ecotype. For hybrid adult trees, largest and second largest *Q* values for each type of tree were used to estimate which ecotypes interbreed and formed the hybrid. Hybrid adult trees with $0.4 \leq$ largest $Q < 0.6$ was defined as first or second filial generation hybrids (i.e., F_1 or F_2), and hybrids with $0.6 \leq$ largest $Q < 0.9$ were considered to be backcross hybrids (Liet al. 2021).

The results of our STRUCTURE analysis suggested that ecotype M resulted from hybridization between ecotypes G and T (see results). Therefore, we also conducted a principal coordinate analysis (PCoA) and a HIest (Fitzpatrick 2012) analysis using pure adults of ecotypes G, T, and M to test hybridization origin possibilities for ecotype M. PCoA was conducted using GenAlEx version 6.501 (Peakall and Smouse 2012). HIest is a program that estimates ancestry (*S*) and interclass heterozygosity (H_I), where both *S* and H_I range from 0 to 1. *S* is similar to the *Q* value estimated by STRUCURE and was defined in terms of ancestry of ecotype T—i.e., values of 0 and 1 indicate that a putative hybrid is actually a pure ecotype G or T, respectively. Expected values of H_I for pure ecotypes, first filial generation hybrids (F_1), second filial generation hybrids (F_2), and first-generation backcross hybrids (BC_1) were 0, 1, 0.5, and 0.5, respectively. By plotting *S* and H_I on a triangular plot, we can see the hybrid status of the two ecotypes. The 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 only in ecotype M, these alleles were assumed to be detected only once in both ecotypes G and T. Parameters were estimated using the maximum likelihood method with a simulated annealing algorithm of 10,000 iterations, surf options, and a start-grid value of 100. To evaluate the power of the genetic markers used, we simulated 1,000 individuals each of pure ecotype G, pure ecotype T, F_1, F_2, BC_1 to G (BC_{1G}), and BC_1 to T (BC_{1T}) using a sample function of R and assuming an infinite population size.

Current hybridization rates among ecotypes on the seed level were estimated using naturally pollinated seeds sampled at five sites (Fig. 1). We assessed the *Q* values of each seed using the USEPOPINFO option in STRUCTURE. This analysis was 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 we ran models 30 times with 100,000 MCMC iterations after a burn-in period of 50,000 iterations. STRUCTURE analysis of adult trees revealed that 27 of 76 maternal trees were hybrids (Table S1), and therefore we eliminated seeds of hybrid trees from this analysis. We therefore used a total of 847 seeds (i.e., 85–272 seeds per site) from 49 pure maternal trees (i.e., 6–15 trees per site). We defined seeds with an assigned $Q \geq 0.9$ for each cluster as pure seeds, and those with $Q < 0.9$ 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 ecotype showing the largest *Q* value other than that of the ecotype of the maternal tree was defined as the ecotype of the pollen parent.

Paternal correlation was estimated among all seed-parent pairs from the obtained genotypes of maternal trees and seeds using POLDISP (Robledo-Arnuncioet al, 2007). Then, we compared the paternal correlation within sites and between sites. For this analysis, we used all 1,260 seeds genotyped 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 unaffected by whether the maternal tree is pure or hybrid.

Ecotype characteristics

To investigate the habitat of each ecotype, we extracted the forest type of adult trees that were categorized as pure ecotypes from a vegetation map (Fig. 1) sourced from the Biodiversity Center of Japan (1999-), and obtained elevation and slope data for all pure adult trees using ArcGIS Desktop version 10.8.2 (ESRI Japan, Tokyo, Japan). Elevation and slope values were extracted from a 10 m mesh digital elevation model provided by the Geospatial Information Authority of Japan. We used the medium and fine categories of the vegetation map, which indicated the dominant species, physiognomy, and geographical conditions. For the extracted vegetation categories of *C. subpubescens* on Hahajima Island, mesic scrub (i.e., a forest height of 1–2 m), dry scrub (1–6 m), and mesic forest (4–20 m) accounted for 69% of all total categories. The rest included 19% that was plantation forest and a remaining 10% that was *Freycinetia formosana* scrub and alien grassland of *Kalanchoe pinnata* and other species. We classified these into three major forest types: mesic forest, mesic scrub, and dry scrub (Shimizu, 1992). In this scheme alien grasslands were classified as dry scrub and *F. formosana* scrub was included in the mesic scrub category based on the ecological characteristics and habitats of each species. Plantation forests were excluded from our analyses since the original forest

type was unknown. In addition, we also investigated the forest height, the presence or absence of overstory trees of each adult *C. subpubescens* pure ecotype, and the species of overstory tree, if any. The relative photosynthetic photon flux density (rPPFD) was calculated from the following equation by simultaneously measuring the photosynthetic photon flux density (PPFD) above the canopy of each adult *C. subpubescens* pure ecotypeand at a nearby open site: rPPFD = (PPFD above the canopy / PPFD at open site) × 100.

To characterize the leaf morphology of each ecotype, we sampled two to five intact leaves from a total of 28 pure adult trees (i.e., seven trees per ecotype). Moreover, we also examined the following 11 leaf traits: total length, blade length, width of leaf blade, hair density on the upper and lower surface of the leaf (i.e., number of hairs per 4 mm^2), number of serrations per 30 mm, thickness of leaf blade, leaf area (LA), leaf mass per area (LMA), ratio of blade length to total leaf length, and the ratio of leaf blade width to length; where applicable, these measurements were taken as described by Kawakubo (1986). 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. Our samples included a total of 81 trees (i.e., 13–24 trees per ecotype) that were categorized as pure ecotypes.

Pre- and post-mating reproductive barriers

To determine whether pre-mating isolation exists among ecotypes, the flowering phenology of 57 trees (i.e., 9–18 trees per ecotype) that were categorized as a pure ecotype were investigated. The number of flowing cymes was counted for each tree once a month for eight months (i.e., May 2014 to January 2015).

To determine whether post-mating isolation exists among ecotypes, we conducted artificial inter-crossings via pollination between different ecotypes, and intra-crossing via pollination within the same ecotypes. These experiments used plants derived from cutting seedlings raised in a greenhouse. For inter-cross pollination, a total 16 cymes from five maternal plants of ecotype D were crossed with two paternal plants of ecotype G (G × D). For intra-cross pollination, a total of 18 cymes from 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 using the following equation: (number of fruits / number of flowers in the cyme) × 100. Next a total of 240 seeds from 10 pairs of inter-crosses and 384 seeds from 16 pairs of intra-crosses were sown. Their germination was monitored for six months (Table S3). The germination rate for each crossing pair was calculated using the following equation: number of germinated seedlings / number of sown seeds) × 100. Ninety-six germinated seedlings from each cross type were then transferred to pots and their mortality was tracked for one year in a laboratory environment with LED lighting and regular watering (Table S3). The mortality rate for each crossing pair was calculated using the following equation: (number of dead seedlings / number of seedlings transferred to pots) × 100.

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

Results

Characteristics of microsatellite markers

We conducted an outlier test using BayeScan, and no outliers were detected for any of the 17 EST-SSR markers at a false discovery rate of 0.05 (Table S2). Three (Cal_0219, Cal_0351 and Cal_1632) of 17 markers may have null alleles since estimated their null allele frequency was significant in more than two out of the seven populations (Table S2). No significant linkage disequilibrium was observed between loci in any population for the 17 markers. Thus, we excluded three loci that might have null alleles and used 14 markers for further analyses.

Ecotypes of *Callicarpa subpubescens* in the Hahajima Islands

The STRUCTURE analysis showed that the log-likelihoods converged and reached a plateau at $K = 6$ (Fig. S2a). At $K = 2$, ΔK was highest, with smaller peaks at $K = 4$ and 6 (Fig. S2b). As we explain below, four phenotypical ecotypes of *C. subpubescens* were recognized in the Hahajima Islands; $K = 2$ could not differentiate these ecotypes (Fig. S2c), while $K = 4$ corresponded to the phenotypical ecotypes. $K = 6$ differentiated between three subtypes of ecotype G, which were found in the northern and southern parts of the Hahajima Islands and in the Imoutojima

Islands, and this was therefore probably caused by isolation by distance. Thus, we determined that $K = 4$ could represent the phenotypical ecotypes of *C. subpubescens* in the Hahajima Islands.

In a STRUCTURE analysis at $K = 3$, we observed a genetic cluster that corresponded to ecotype M at $K = 4$ that showed an admixed pattern of ecotypes G and T (Fig. 2a). In this group, most trees had higher Q values of ecotype T than those of ecotype G. A PCoA analysis using pure adults from ecotypes G, T, and M revealed two axes (1 and 2) that explained 27.6% of the variation and clearly separated the three ecotypes (Fig. S3a). Moreover, axes 1 and 3, which collectively explain 21.8% of the variation, showed an overlap between ecotypes G and M, while ecotype T was separated (Fig. S3b). The PCoA suggested ecotype M was closer to ecotype G than T. In the HIest analysis, triangular plots between S and H_I for the observed data showed that most points of ecotypes G and T were close to each pure ecotype, although the ecotype G datapoints were more scattered than the ecotype T datapoints (Fig. S4a). Specifically, we observed that datapoints for ecotype M were located in the left half of the plot area with located on the base and left-side lines of the triangle. The observed distribution of datapoints of ecotype M was therefore rather similar to a simulation of first-generation backcross hybrids to ecotype G (BC_{1G}) (Fig. S4b).

Spatial distribution, habitat, and morphological traits of ecotypes

Pure ecotype D had the narrowest distribution range and was allopatrically distributed with other ecotypes (Fig. 1). We found that 78% inhabited the dry scrub with a mean height of 2.1 m on steep cliffs at elevations of 186.4 ± 37.6 m (Fig S5). No overstory trees existed in 11% of the locations of pure ecotype D, while 82% had alien *Leucaena leucocephala* trees in the overstory; regardless, the mean rPPFD was as high as 88%. On the other hand, pure ecotype G showed the widest distribution range, being present over all of Hahajima Island from low to high elevation (i.e., elevation: 158.5 ± 105.1 m). Moreover, 93% of the trees of this ecotype inhabited mesic forests that had a mean height of 7.8 m. All pure ecotype G individuals examined were present in the understory of mesic forests, with a mean rPPFD of 22%. Next, pure ecotypes T and M were both distributed on the ridges of the central mountain, but pure ecotype T (el. 302.2 ± 58.3 m) was distributed at a lower elevation than pure ecotype M (el. 394.0 ± 24.3 m). 88% of ecotype T trees inhabited mesic forests with a mean height of 7.9 m, while pure ecotype M was more concentrated in higher elevation areas and inhabited both mesic scrub (31%) and mesic forests (69%) with a mean forest height 3.9 m. Pure ecotype T had no overstory trees in 69% of the locations and constituted the forest canopy, while 30% had native trees in the overstory. On the other hand, 71% of pure ecotype M trees also had no overstory trees and therefore constituted the forest canopy, while 29% had alien *Bischofia javanica* in the overstory and showed a forest height of more than 5 m. The mean rPPFD was therefore as high as 88% for ecotype T and 86% for ecotype M.

Next, PCA was conducted using eleven leaf morphological traits of pure adult trees for each ecotype. This analysis revealed that the first and second principal components explained 71.3% and 23.5% of the variation, respectively, and accounted for 94.8% of the total variation (Fig. 3). The distribution of each plot was not clearly separated, but different ecotypes did not overlap with each other. Ecotype M was located between ecotypes G and T. The leaf morphology of ecotype G was characterized by large leaf area, few hairs on either side of the leaf, and few leaf serrations (Fig. S6). The leaf morphology of ecotype D featured small, rounded, and thick leaves with many hairs. Ecotype T was characterized by short petioles and many hairs. Finally, ecotype M had moderate-sized leaves and a moderate number of hairs compared to the other ecotypes.

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

Pre- and post-mating reproductive isolation mechanisms

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

We found no significant difference in fruit set rate between inter-cross G × D and intra-cross D × D ($p = 0.63$, Fig. S7a). Moreover, with respect to germination rate, we found that seeds from inter-cross G × D were significantly more likely to germinate than intra-cross D × D ($p < 0.05$, Fig. S7b), and that the seedling mortality rate was not significantly different between inter- and intra-cross seedlings ($p = 0.68$, Fig. S7c). We note that the genetic distance between ecotypes G and D is rather large (Fig. 2a). Although we could not conduct artificial crossings for all ecotype

pairs, we found no evidence of mechanisms of post-mating isolation among ecotypes. Volumetric soil water content when seedlings began to wilt was found to be significantly lower in intra-cross D × D seedlings than in pure ecotype G, while inter-cross G × D seedlings showed intermediate values that did not significantly different from those of pure ecotype G and intra-cross D × D (Fig. S7d).

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, we also found 216 adult trees on Hahajima Island that 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 therefore 37.2%. The hybridization rate of adult trees of each ecotype was ~20% for ecotypes T and M and ~40% for ecotypes G and M (Fig. 2a, Table 2a). Among the 216 hybrid adult trees, the ecotype pair G-M accounted for the highest percentage at 52.3%, while the ecotype pairs T-D and D-M both accounted for the lowest percentage at 6.0% (Table S4). Next, we found that the total hybridization rate of naturally pollinated seeds was 26.4%. The hybridization rate of naturally pollinated seeds for each ecotype was lowest at site T (7.7%) and highest at site Gs (48.2%; Fig. 2b, Table 2b). Among 224 hybrid seeds identified, the G-M ecotype pair was the most abundant at 27.2%, while the T-D ecotype pair was the least abundant 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 at Gn, while ecotype T was the most common at Gs (Table 2). At site M, ecotype T was the most common pollen parent of hybrid seeds.

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

Discussion

Ecotypes of *Callicarpa subpubescens* in the Hahajima Islands

Phenotypic and genetic clustering analysis revealed the existence of 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 in that study showed an admixture pattern, was identified here as ecotype M. A genetic cluster with relatively few samples can be difficult to detect by 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 analyses suggest that ecotype M may have resulted from a hybridization event between ecotypes G and T. An admixture analysis using more than 2,000 SNPs obtained by restriction site associated DNA sequencing (RAD-Seq) also showed that ecotype M is an admixture of ecotypes T and G (Setsuko et al. unpublished). A PCA analysis of leaf morphology revealed that ecotype M showed a distribution intermediate between ecotypes G and T. Moreover, the maximum stem length and DBH of ecotype M was also intermediate between ecotypes G and T, although we found no significant differences between ecotypes G and M. The flowering time of ecotype M was similar to that of ecotype G. In many plants and animals, speciation has been observed to accelerate via hybridization (Abbottet al. 2010; Mallet 2007). The presence of four ecotypes within a single tree species on small islands of approximately 20 km^2 in size may be attributed not only to adaptive radiation, but also to hybridization among ecotypes via secondary contact.

Local adaptation of ecotypes

Ecotype D is mainly distributed in dry scrub locations on steep cliffs, while the other ecotypes are distributed in mesic forests and mesic scrub. Seedlings of intra-cross D × D wilted at lower soil moisture content than pure ecotype G seedlings. The maximum stem length and DBH of ecotype D were low and it had smaller, thicker leaves that contained many hairs. Previous studies have identified a 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). Taken together, these facts suggest that ecotype D is adapted to lower soil moisture and this may be why it inhabits soil types that the other ecotypes do not. In the Bonin Islands, several endemic tree species are known to exhibit different genetic groups within a single species that are distributed in mesic forest and dry scrub (Sugaiet al. 2022; Tsuneki et al. 2014). This suggests that genetic differentiation by soil moisture conditions is probably a common pattern of differentiation in the Bonin Islands.

Ecotype G, which is distributed throughout the understory of the mesic forests, has almost no leaf hairs, whereas ecotypes T and M, which grow in bright areas and constitute the forest canopy, have hairs on their leaves. Ecotype D, that also have hairs on their leaves, may have originally constituted the canopy of dry scrub, although at present the upper layer is covered by alien trees. Leaf hairs are known to reduce photoinhibition caused by strong sunlight (Ripleyet al. 1999). Growing ecotype G in a sunny location causes leaf burn and atrophy, while no such phenomenon occurs in the other three hairy ecotypes (SS personal observation). Taken together, these findings suggest that 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 that characterize the canopy and understory environments of mesic forests.

Ecotypes M and T mostly occur in separate habitats, although a few ecotype T plants are distributed within the distribution area of ecotype M. Ecotype M was predominantly distributed along high-elevation mountain ridges in mesic scrub, which is characterized by lower forest height, or at the edge of mesic forests. Ecotype M constitutes the forest canopy of mesic scrub or mesic forests with low tree height (i.e., at most 4 m excluding alien trees), while ecotype T constitutes the forest canopy of mesic forests (which can be as high as 8 m or more).

Since ecotype M is assumed to be derived from the hybridization of ecotypes G and T, the distribution of ecotype M can therefore be considered as a hybrid zone. This hybrid zone likely formed because ecotype M is adaptive in a new niche, mesic scrub, in which the other ecotypes had not previously dominated. Conversely, ecotype M is not adapted to the habitats of its parents (i.e., 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), and frequent cloud cover tends to reduce the amount of sunlight (Loope and Giambelluca 1998). Ecotype M may therefore be able to dominate in the mesic scrub because ecotype M is more shade-tolerant than ecotype T due to its parentage from ecotype G, which is distributed in the mesic forest understory. If this is the case, ecotype M may be an example of adaptive introgression (Suarez-Gonzalezet al. 2018). On the other hand, ecotype M inhabited mesic scrub and mesic forests with low forest height, and did not inhabit mesic forests with high forest height, where ecotype T inhabited. The reason why ecotype M cannot survive in those areas may be because ecotype M, which has ecotype G as a parent, cannot grow as tall as ecotype T. To test these possibilities, future studies are needed. Specifically, common garden experiments are needed to determine whether differences in shade tolerance and growth rates are between ecotypes T and M, as well as which 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. Moreover, we observed substantial negative values for site pairs Gn-T and Gs-T, which differ in flowering peaks, and exceptionally positive correlations for site pairs Gn-Gs and Gn-M, which showed synchronized flowering peaks. This is consistent with the results that the most common hybrid mating ecotype pair was G-M, both in adult trees and in naturally pollinated seeds. These results indicate that the degree of overlap in flowering time contributes to the degree of hybridization between ecotypes, as has been shown by other studies (Campbellet al. 2016; Field et al. 2011). On the other hand, ecotype D had the longest flowering time (i.e., from summer to early winter) and therefore had potential to hybridize with all other ecotypes. Interestingly, the hybridization rates of adult trees and natural pollinated seeds of ecotype D were not very high, and paternal correlations were negative for pairs that included D and any of the other sites. This was probably because ecotype D is locally distributed on dry scrub on steep cliffs. Pollen dispersal is therefore limited by distance (Adams 1992), and hybridization rates are therefore expected to lower for locally isolated ecotypes (Lagache et al. 2013).

Ecotype maintenance mechanism

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

Next, we note that there may be a reason for the small number of F_1 or F_2. Soil moisture when the seedlings began to wilt was lowest in intra-cross D × D, intermediate in inter-cross G × D, and was the highest for pure ecotype G. This suggests that fitness of hybrids in the dry habitat is inferior to pure ecotype D. In *Machilus*, a tree species endemic to the Bonin Islands, hybrids with high genetic composition of mesic ecotypes that inhabit dry habitats are removed from dry habitat alongside pure mesic ecotype individuals as they mature (Tsuneki 2012). This result indicates that the fitness of F_1 or F_2 hybrids of mesic and dry ecotypes in dry habitats is lower than in backcrosses to dry ecotypes, which results in a lower number of F_1 or F_2 trees. In fact, in this study, we found very few F_1 or F_2 relative to backcrosses. These facts suggest that selection against hybrids, especially F_1 or F_2, is occurring. The lower fitness of hybrids would also contribute the maintenance of parental ecotypes (Lepais and Gerber 2011; Twyfordet al. 2015), and reversion to the original ecotype, which is adapted to a specific environment, through backcrossing would contribute to ecotype maintenance. However, in the present study, we compared only the fitness of ecotypes of G, D, and F_1 hybrids, so it remains necessary to elucidate the relationship between adaptive traits and survival rates among other ecotype pairs and hybrids between them.

Declarations

Acknowledgements

The authors are grateful to Y. Nakamura for providing the location of the adult trees; Dr. C. Migita, A. Hisamatsu, M. Yokoya and Y. Yoshii for their experimental support; Drs. T. Nagamitsu, N. Nakanishi, and J.R.P. Worth for their valuable advice. We also thank Metropolis of Tokyo, the Ministry of the Environmental Government of Japan, and Forestry Agency of Japan for allowing this study. This research was conducted using the Ogasawara Field Research Station of Tokyo Metropolitan University. This work was funded by Grants-in-Aid for Science Research from the Japanese Society for Promotion of Science (JP26290073, JP15K07203, JP21K05694), the Environment Research and Technology Development Fund of the Ministry of the Environment, Japan (4-1402).

Author Contributions

SS, KS, KH, and HK designed the research. SS, KS, KH, and HK sampled materials. SS performed all the laboratory work. SS, KS, and IT performed data analysis. All co-authors discussed the results. SS and IT wrote the paper.

Conflict of Interest

The authors declare that they have no competing interests.

Data Archiving

Genotype data of EST-SSRs used for this study are available from 10.6084/m9.figshare.23542611.

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Tables

Table 1. Name, ID, and characteristics of each ecotype

Ecotype	Ecotype ID	Forest type and habitat	Elevation	Slope	Forest height	rPPFD	Leaf hair density	Flowering time	Tree size	Growth form
			(Range, average (m))	(Range, average (°))	(Average (m))	(Average (%))	(Average density of the upper and lower surfaces)	(Range, peak)	(Average max. stem length (m), Average max. stem DBH (cm))	(Average no. stems)
Glabrescent	G	Understory of mesic forest	(13–424, 158.5)	(3–30, 17.8)	High	Dark	Glabrescent	Summer	Middle	Tree
					(7.8)	(21.7)	(0.5, 0.4)	(Jun.–Jul., Jul.)	(3.0 m, 2.72 cm)	(1.3)
Tall	T	Crown of mesic forest	(215–427, 302.2)	(3–41, 17.3)	High	Bright	Many	Autumn	Tall	Tree
					(7.9)	(88.0)	(50.8, 52.6)	(Jul.–Dec., Oct.)	(7.1 m, 9.97 cm)	(1.1)
Dwarf	D	Crown of dry scrub	(101–279, 186.4)	(4–37, 25.1)	Low	Bright	Many	Summer-Winter	Dwarf	Bush
					(2.1)	(88.1)	(32.3, 42.1)	(Jul.–Jan., Aug & Nov.)	(1.5 m, 0.68 cm)	(7.7)
Middle	M	Crown of mesic forest and mesic scrub	(321–456, 394.0)	(4–41, 19.4)	Low	Bright	Middle	Summer	Middle	Tree
					(3.9)	(85.7)	(16.9, 13.1)	(Jun.–Jul., Jul)	(3.5 m, 5.52 cm)	(1.5)

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

a)

Ecotype	Purity rate (%)	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
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)				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

Figures

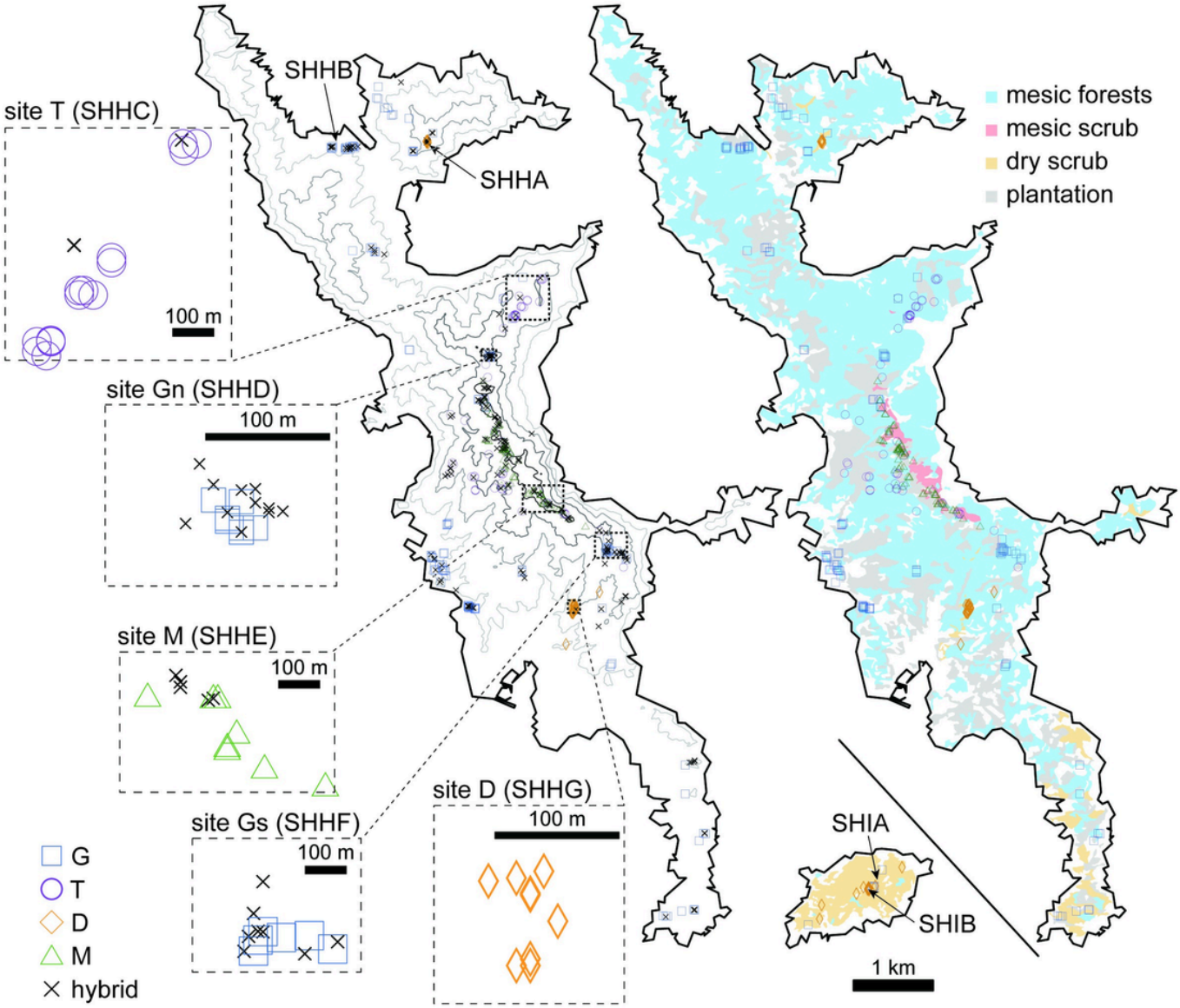

Figure 1

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

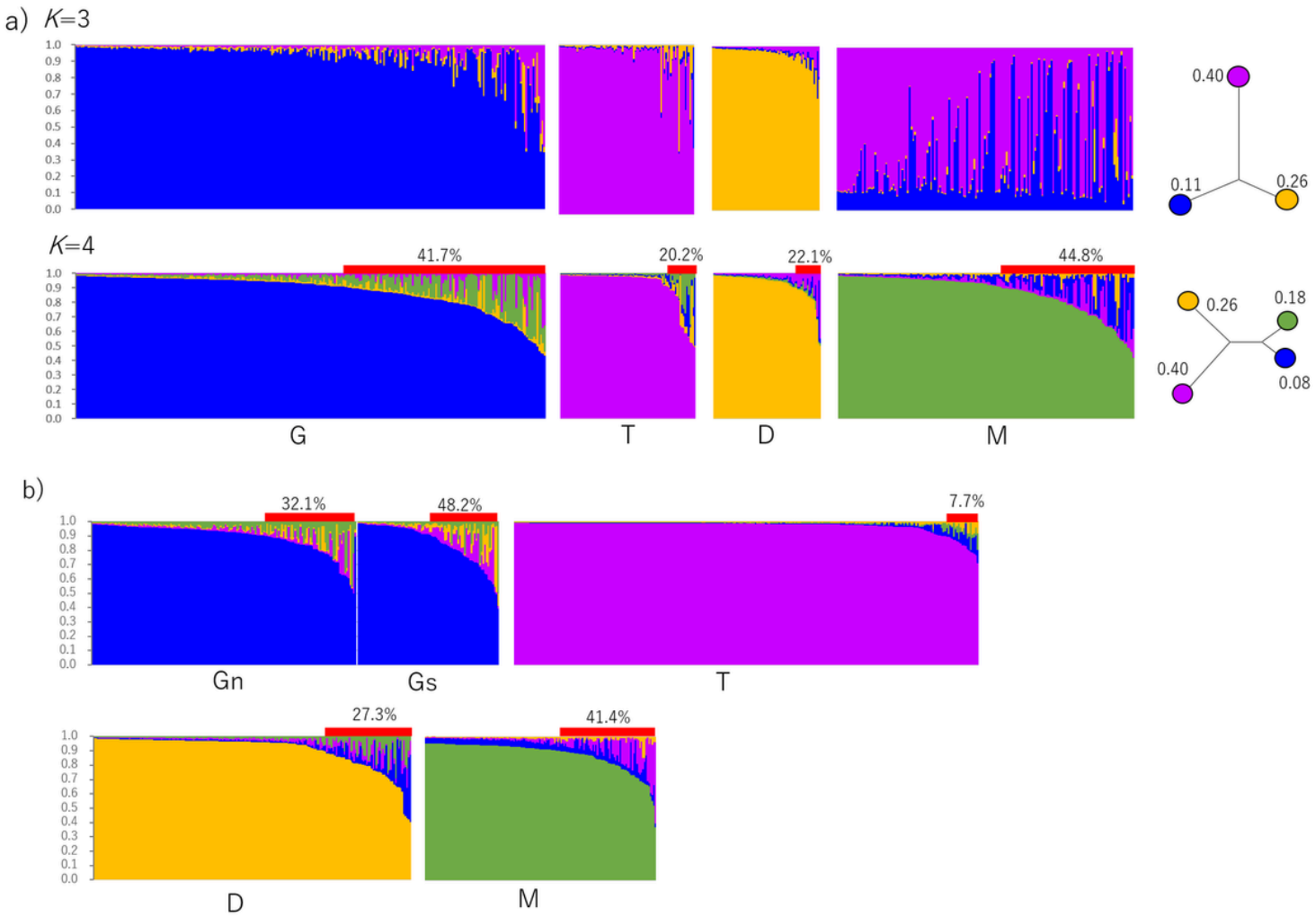

Figure 2

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

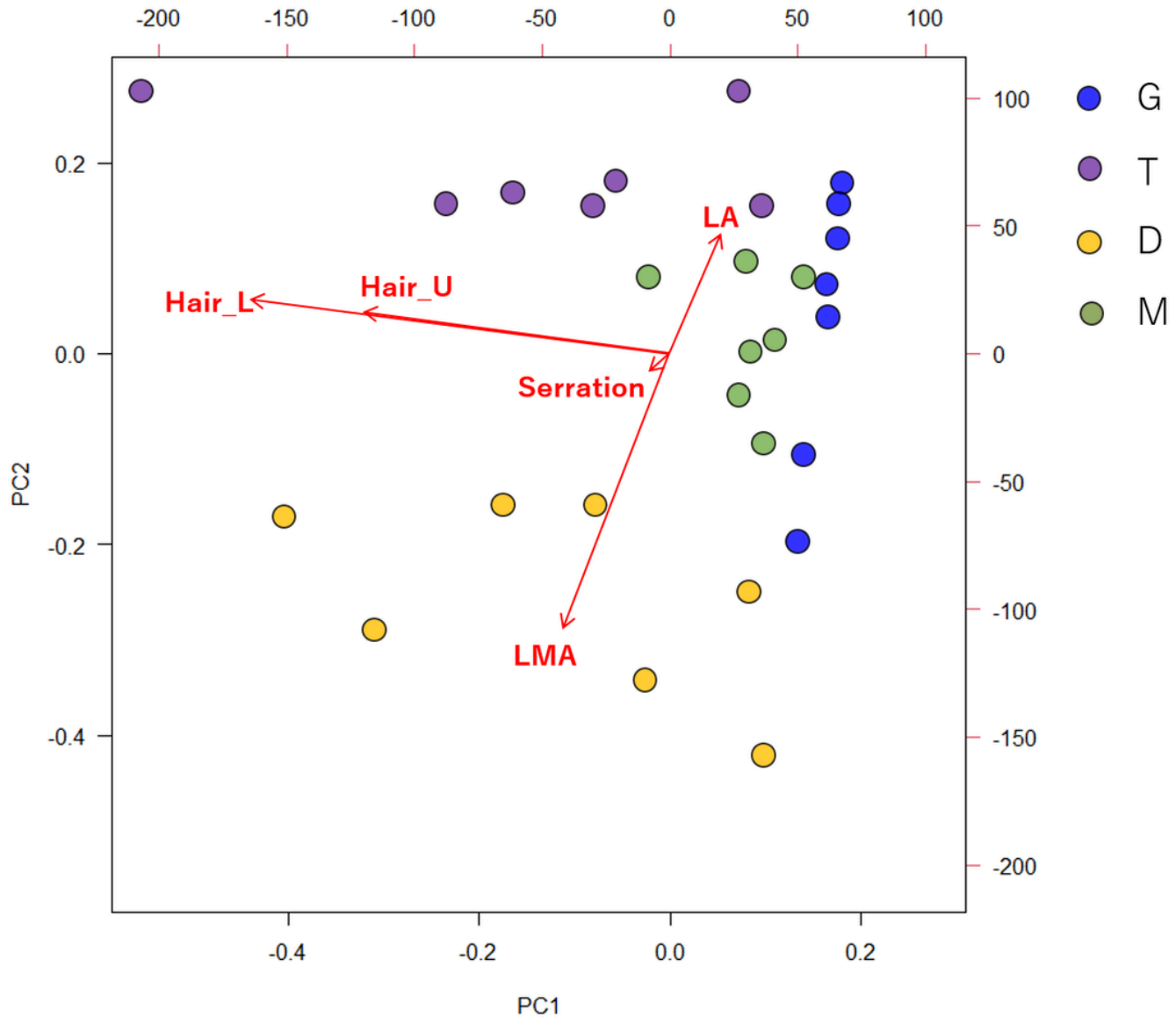

Figure 3

Distributions of the first and second principal components (i.e., PC1 and PC2) for eleven 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.

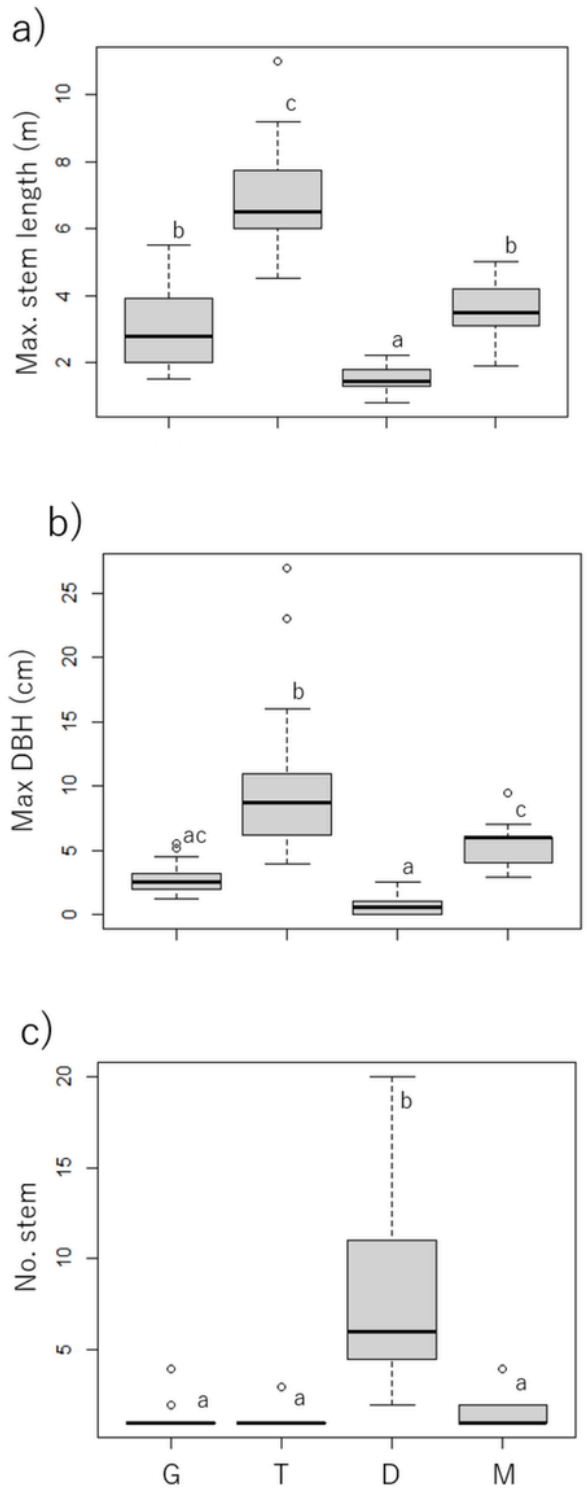

Figure 4

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

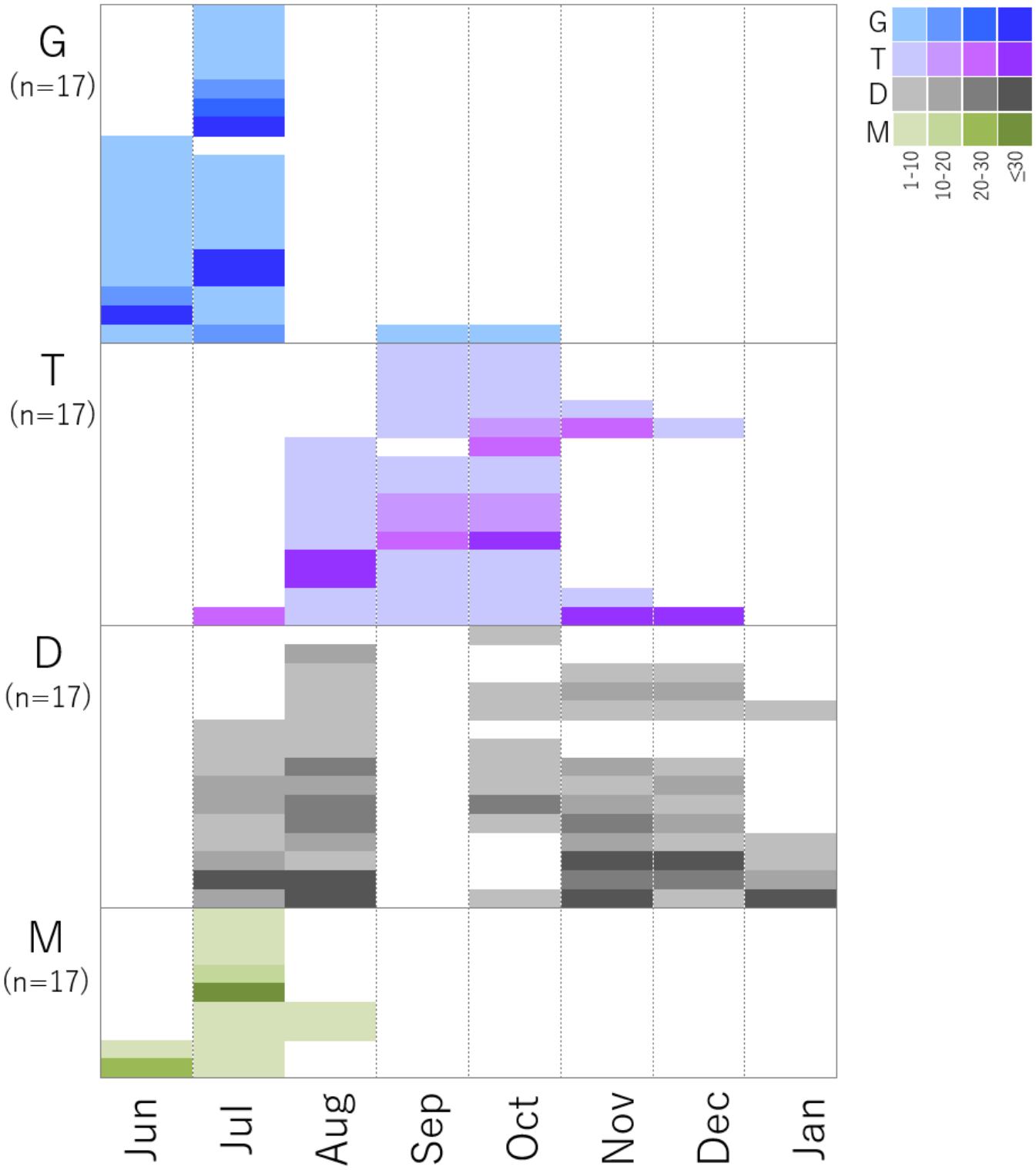

Figure 5

Temporal changes in the number of flowering cymes of each pure adult tree. Each horizontal line indicates an individual. Darker colors indicate a greater number of flowering cymes.

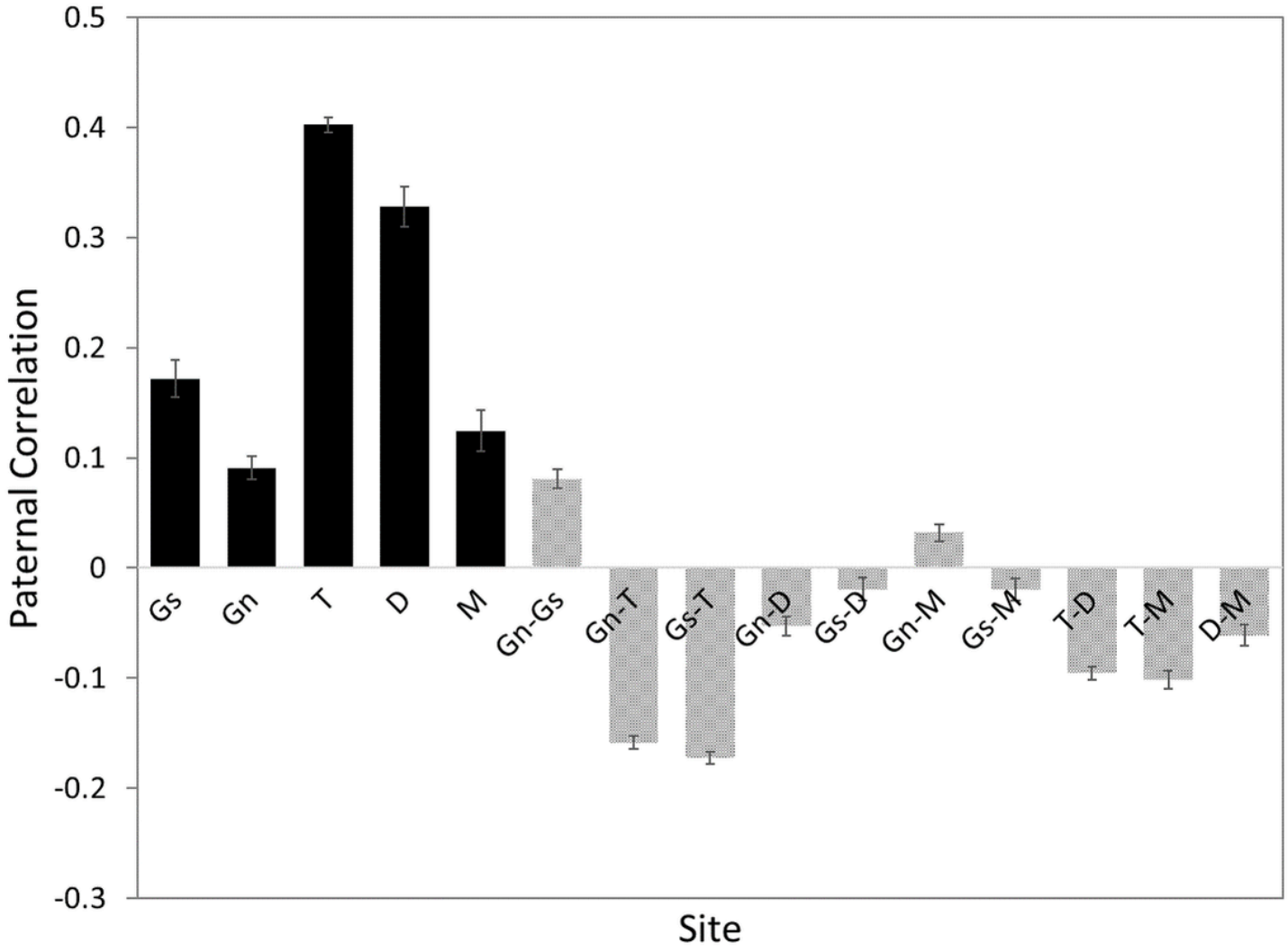

Figure 6

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

Supplementary Files

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- SupplementaryTablesXXXFigsHDY23A0142.pdf