# Ecotype variation in the endemic tree *Callicarpa subpubescens* on small oceanic islands: genetic, phenotypic, and environmental insights

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**Abstract**

*Callicarpa subpubescens*, endemic to the Ogasawara Islands, is suggested to have multiple ecotypes in the Hahajima Islands, specifically in the central part of the Ogasawara Islands. In this study, associations between genetic groups and spatial distribution, habitat, leaf morphology, size structure, and flowering time of each genetic group were investigated on Hahajima and the satellite Imoutojima Islands. Genetic groups were identified using EST-SSR markers, revealing four ecotypes named based on morphological features: Dwarf (D), Glabrescent (G), Tall (T), and Middle (M), with M being a result of the hybridization of G and T. Ecotype D, adapted to dry environments, is characterized by small tree size, dense thick leaves with abundant hairs, and is distributed in dry scrub. Ecotype G, adapted to understory of mesic forests, lacks leaf hairs. Ecotype T, adapted to the canopy of mesic forests, has hairy leaves and is tall in tree height. Ecotype M, adapted to the canopy of mesic scrub or edges of mesic forests, has hairy leaves but with a shorter tree height than ecotype T. Flowering peaks differed among all ecotype pairs except G and M, but the flowering times more or less overlapped among all ecotypes, suggesting that pre-mating isolation among ecotypes is not perfect. Post-mating isolation is considered absent, as there were no differences in the results, germination, and survival rates of one-year seedlings among inter- and intra-ecotype crossings. The existence of such ecotypes provides valuable insights into the ongoing speciation processes

adapting to the oceanic island environments.

**Keywords**

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

## 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 (Gillespie et 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; Hoballah et al. 2007), morphology (Yang et al. 2007), odor (Okamoto et al. 2015), and/or flowering phenology (Martin et al. 2007) etc. In contrast, the latter prevents 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.

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), although the majority of outcomes resulting from hybridization are maladaptive (Seehausen et al. 2008, Todesco et al. 2016). 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 improve 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

94 Pacific Ocean off the coast of Japan, approximately 1,000 km south of
95 Tokyo (Fig. S1). This island chain comprises four distinct groups: the
96 Mukojima, Chichijima, and Hahajima Islands (collectively called the Bonin
97 Islands) and the Volcano Islands. Their total land area is small (~80 $km^2$),
98 but their endemic species rates are as high as 40% for vascular flora (Ono et
99 al. 1986) and more than 90% for land snails (Tomiyama and Kurozumi
100 1991). The elevation of the Hahajima Islands is the highest among the
101 Bonin Islands, and their topography is also more varied relative to the
102 others. Furthermore, cloud cover and fog frequently occur at high
103 elevations, which allow the area to develop endemic mesic scrub that reach
104 1–2 m in height (Shimizu 1992).
105 The genus *Callicarpa* (Lamiaceae) in the Ogasawara Islands
106 includes three recognized endemic species, *Callicarpa parvifolia*, *C. glabra*,
107 and *C. subpubescens*, which are considered to represent an adaptive
108 radiation (Ono 1991). All three are dioecious, despite most *Callicarpa*
109 species being hermaphroditic (Kawakubo 1990). *Callicarpa parvifolia* and
110 *C. glabra* are distributed only in the Chichijima Islands, while *C.*
111 *subpubescens* is distributed widely across the Ogasawara Islands, including
112 on the Volcano Islands. Pollen dispersal occurs via insects, such as endemic
113 small bees, introduced honey bees (Abe 2006), and endemic *Xylocopa* bees
114 (Setsuko S. personal observation). The sizes of fruits and seeds are
115 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. Furthermore, a common garden of *C. subpubescens* on Hahajima Island showed multiple phenotypic groups with diverse flowering phenology and leaf morphology (Ogasawara Environmental Planning Laboratory 2023). This variation was consistent with observations from greenhouse cultivation of cutting-propagated seedlings (Setsuko S. personal observation), indicating a genetic basis for these phenotypic differences rather than plasticity. The observed phenotypic variations in *C. subpubescens* on Hahajima Island are indicative of genetically distinct groups, possibly representing distinct ecotypes ongoing speciation. In this context, an ecotype is defined as a population within a single species with differing phenotypes, locally adapting to specific habitats, and the observed phenotype differences being genetically

138 determined (Turesson 1922a, b).

139 In this study, we conducted a detailed genetic analysis of *C.*
140 *subpubescens* throughout the Hahajima and satellite Imoutojima Islands,
141 which is located approximately 5.6 km south-southeast of the southern tip of
142 Hahajima Island (Fig. S1). To do so, we analyzed samples from populations
143 and isolated trees using 14 expressed sequence tag (EST)–based SSR
144 markers. We aimed to answer the following questions: 1) Is there any
145 correspondence between genetic groups and their spatial distribution,
146 habitat, leaf morphology, and size structure within each genetic group? If so,
147 2) Are there pre- and post-mating reproductive isolation mechanisms
148 between these groups? 3) How are each of these groups adapting to the local
149 environment?

150

151 **Materials and Methods**

152 **Sample collection**

153 We comprehensively sampled leaves of 602 and 114 trees from the
154 Hahajima and Imoutojima Islands (hereafter collectively referred to as the
155 Hahajima Islands), respectively (Fig. 1). In low-density areas, we sampled
156 as many trees as possible, while in high-density areas, we selectively
157 sampled several representative trees exhibiting typical morphologies. Trees
158 included nine populations taken from the Hahajima Islands that were
159 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.

**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 S1, which were 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. 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 Oosterhout et 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 S1, were used. This selection was made because the sampled trees, especially isolated ones, were difficult to assign to specific populations (Fig. 1). Additionally, admixed populations were excluded because outliers from the source population may be obscured by each other when using admixed populations (Yelmen et al. 2021).

**Genetic analysis**

We used the Bayesian clustering program STRUCTURE version 2.3.4 (Falush et al. 2007; Pritchard et al. 2000) to identify genetic groups of all sampled 716 *C. subpubescens* trees in the Hahajima Islands, then checked whether these genetic groups corresponded to phenotypic groups. 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 20 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 $\Delta K$ values (Evanno

et al. 2005). Next, we checked for the existence of minor clusters using the online version of CLUMPAK (Kopelman et al. 2015). Trees assigned at $Q \geq 0.9$ to each cluster were then considered to be core trees, and trees with $Q < 0.9$ were considered to be intermediate trees (Kato et al. 2014; Li et al. 2021). We also conducted a principal coordinate analysis (PCoA) using all trees to investigate the relationships between genetic groups. PCoA was conducted using GenAlEx version 6.501 (Peakall and Smouse 2012).

**Characteristics of genetic groups**

To investigate the habitat of each genetic group, we extracted the forest type of trees that were categorized as core trees from a vegetation map (Fig. 1) sourced from the Biodiversity Center of Japan (1999-), and obtained elevation and slope data for all core 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 *C. subpubescens* core tree, 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 *C. subpubescens* core tree and at a nearby open site: rPPFD = (PPFD above the canopy / PPFD at open site) × 100.

To characterize the leaf morphology of each genetic group, we sampled two to five intact leaves from a total of 28 core trees (i.e., seven trees per group). 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

248 principal component analysis (PCA) to test the morphological aggregation
249 of leaves of each genetic group.
250 To characterize size distribution of trees of each genetic group, we
251 measured the maximum stem length, the maximum diameter at breast height
252 (DBH), and counted the number of stems per tree. Our samples included a
253 total of 81 trees (i.e., 13–24 trees per group) that were categorized as core
254 trees.
255
256 **Pre- and post-mating reproductive barriers**
257 To determine whether pre-mating isolation exists among genetic groups, the
258 flowering phenology of 57 trees (i.e., 9–18 trees per group) that were
259 categorized as core trees were investigated. The number of flowering cymes
260 of each tree was counted once a month for eight months (i.e., May 2014 to
261 January 2015).
262 To determine whether post-mating isolation exists among genetic
263 groups, we conducted artificial inter-crossings via pollination between
264 different genetic groups, and intra-crossing via pollination within the same
265 genetic groups. These experiments used plants derived from cutting-
266 propagated seedlings raised in a greenhouse. For inter-cross pollination, a
267 total 16 cymes from five maternal plants of group D were crossed with two
268 paternal plants of group G (G × D) (Table S2, see Table 1 for group names).
269 For intra-cross pollination, a total of 18 cymes from five maternal plants of

group D were crossed with eight paternal plants of group D (D × D). 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 (Table S2). Their germination was monitored for six months. 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 S2). 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 groups. In addition to seedlings derived from artificial crossings (i.e., G × D and D × D), natural pollinated seeds of group G were sown and grown under the same conditions. Note that these comparisons were specifically limited to the offspring of artificial crosses (G × D and D × D) and seeds of group G. Subsequently, an EST-SSR analysis was conducted on seedlings of group G using the same STRUCTURE method as applied to the 716 trees in the Hahajima Islands, and only seedlings assigned with a $Q \geq 0.9$ to group G were included in 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 ($\theta$% vol.) was calculated using the following equation: $\theta = -27.8V^5 + 30.3V^4 - 0.7V^3 - 9.0V^2 + 3.8V$. 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 S1). 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 S1). 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.

### Genetic groups of *Callicarpa subpubescens* in the Hahajima Islands

The STRUCTURE analysis showed that $\Delta K$ was highest at $K = 4$, with second highest peak at $K = 7$ (Fig. 2a). The log-likelihoods converged and reached a plateau around $K = 7$ (Fig. 2b). As explained in the results below, four phenotypic groups of *C. subpubescens* were recognized in the Hahajima Islands. These groups were named "G: Glabrescent," "T: Tall," "D: Dwarf," and "M: Middle" based on their phenotypic characteristics (Table 1). $K = 4$ corresponded to the phenotypic groups as shown below (Fig. 2c). $K = 7$ differentiated between four subtypes of group G, which were found in the northern, central, and southern parts of Hahajima Island as well as in Imoutojima Island, and this differentiation was likely caused by isolation by distance. Thus, we determined that $K = 4$ could represent the phenotypic groups of *C. subpubescens* in the Hahajima Islands, and hereafter, the genetic groups are also referred to as G, T, D, and M. When $K = 4$, the neighbor-joining tree revealed relatively small $F_{ST}$ divergence between clusters G and M, which were 0.08 and 0.18, respectively. In contrast, clusters T and D exhibited larger $F_{ST}$ values of 0.39 and 0.27, respectively (Fig. 2d). When $K = 7$, the neighbor-joining tree showed a clustering pattern where the four clusters representing group G in blue, red, pink, and light blue were closely grouped. The $F_{ST}$ values for the four G clusters ranged from 0.08 to 0.21, with the pink cluster representing the group G on Imoutojima Island showing the highest value. The $F_{ST}$ values for the other clusters were similar to those observed at $K = 4$.

In a PCoA analysis using all trees, two axes (1 and 2) that explained 20.1% of the variation and generally separated the four core trees, although intermediate trees were scattered throughout the entire plot (Fig. 3a). In axes 1 and 3, explaining 17.3% of the variation, an overlap was observed between core trees of G and M, while core trees of T showed an overlap with core trees of D (Fig. 3b). In a STRUCTURE analysis at $K = 3$, we observed a genetic cluster that corresponded to core trees of M at $K = 4$ that showed an admixed pattern of clusters G and T, and most trees had higher $Q$ values of cluster T than cluster G (Fig. 2c).

**Spatial distribution, habitat, and morphological traits of genetic groups**

Core trees of D had the narrowest distribution range and was allopatrically distributed with other core trees (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 S2). No overstory trees existed in 11% of the locations of core trees of D, while 82% had alien *Leucaena leucocephala* trees in the overstory; regardless, the mean rPPFD was as high as 88% (Table 1). On the other hand, core trees of 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 core trees of G inhabited mesic forests that had a mean height of 7.8 m. All core trees of G examined were present in the understory of mesic forests, with a mean rPPFD of 22%.

Next, core trees of T and M were both distributed on the ridges of the central mountain, but core trees of T (el. 302.2 ± 58.3 m) were distributed at a lower elevation than those of M (el. 394.0 ± 24.3 m). 88% of core trees of T inhabited mesic forests with a mean height of 7.9 m, while those of M were more concentrated in higher elevation areas and inhabited both mesic scrub (31%) and mesic forests (69%) with a mean forest height 3.9 m. Core trees of 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 core trees of M 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 core trees of T and 86% for those of M.

Next, PCA was conducted using eleven leaf morphological traits of core trees for each genetic group. 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. 4). The distribution of each plot was not clearly separated, but different genetic groups did not overlap with each other. Core trees of M were located between core trees of G and T. The leaf morphology of core trees of G was characterized by large leaf area, few hairs on either side of the leaf, and few leaf serrations (Fig. S3). The leaf morphology of core trees of D featured small, rounded, and thick leaves with many hairs. Core trees of T were

characterized by short petioles and many hairs. Finally, core trees of M had moderate-sized leaves and a moderate number of hairs compared to the other groups.

The maximum stem length of core trees significantly differed among all genetic group pairs except between groups G and M ($p < 0.05$, Fig. 5a). In addition, DBH of core trees was significantly different among groups except between groups G and D, and G and M ($p < 0.05$, Fig. 5b). The largest maximum stem length and DBH value were found in group T (mean: 7.1 m and 9.8 cm, respectively), followed by 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 group D (mean: 7.7) than other groups (mean: 1.1–1.5; $p < 0.05$, Fig. 5c).

**Pre- and post-mating reproductive isolation among groups**

The main flowering times of core trees of G and M were almost the same from June to July and showed the same peaks in July. The flowering time of core trees of T was from July to December with a peak in October (Fig. 6). The flowering time of core trees of D was long, lasting from July to January (except in September, probably due to the above-average temperatures from late August to early September), with two peaks in August and November. This period overlapped with most trees of the other groups. Flowering

patterns were similar to those reported by Sugai et al. (2019), except that core trees of D showed a higher number of flowering trees in July and August in our study. Although we found differences in peak flowering among all group pairs except groups G and M, the flowering times more or less overlapped among all groups.

We found no significant difference in fruit set rates between inter-cross G × D and intra-cross D × D ($p = 0.63$, Fig. S4a). 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. S4b), and that the seedling mortality rates were not significantly different between inter- and intra-cross seedlings ($p = 0.68$, Fig. S4c). We note that the genetic distance between groups G and D is rather large (Fig. 2d). Volumetric soil water contents when seedlings began to wilt were found to be significantly lower in intra-cross D × D seedlings than in core seedlings of G, while inter-cross G × D seedlings showed intermediate values that did not significantly differ from core seedlings of G and intra-cross D × D seedlings (Fig. S7d).

## Discussion

### Classifications and properties of ecotypes of *Callicarpa subpubescens*

There was a clear correspondence between genetic groups and phenotypic

groups (Table 1). Similar relationships are observed in both common garden and greenhouse, confirming the presence of four ecotypes of *C. subpubescens*, namely Glabrescent (G), Tall (T), Dwarf (D), and Middle (M), in the Hahajima Islands. Ecotype D is distributed in dry scrub on steep cliffs. Currently, many trees are found beneath the canopy of the alien tree species *L. leucocephala*, but they would constitute the canopy layer in the dry scrub without the alien trees. Trees are small with an average height of 1.5 m. It is characterized small, thick leaves with abundant hairs, and it flowers over an extended period from summer to winter. Ecotype G is widely distributed across the entire island, in the understory of mesic forests. The tree is moderate in size with an average height of 3 m. It is characterized by very few leaf hairs, and its flowering peaks during the summer. Ecotype T is distributed on the central ridges of the island and constitutes the canopy of mesic forests. The tree size is large with an average height of 7 m. It is characterized by abundant leaf hairs, and it has flowering peaks during the autumn. Ecotype M is distributed along the central ridges of the island, like ecotype T, but occupies higher elevations than ecotype T. It constitutes the canopy of mesic scrub or is found at the edge of mesic forests. The tree size is moderate with an average height of 3.5 m. Leaf hair density is also moderate, and it has flowering peaks during the summer.

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 trees collected in this study likely allowed us to clearly identify ecotype M as a separate genetic cluster. The results of STRUCTURE and PCoA analyses suggest that ecotype M may have resulted from an admixture between ecotypes G and T (Figs. 2, 3). Alternatively, there is the possibility that ecotype M might be ancestral, with ecotypes T and G differentiate from ecotype M due to founder effects (Lawson et al., 2018). However, given that the genetic diversity of ecotypes T and G is rather higher than that of ecotype M (Sugai et al., 2019), founder effects seem unlikely. Furthermore, an admixture analysis and neighbor-net tree 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. 2023). According to RAD-Seq analysis, it has been revealed that ecotype G in the Hahajima Islands originated from *C. subpubescens* in the Chichijima Islands, located 45 km to the north (Setsuko et al. 2023). Therefore, it is presumed that hybridization occurred through secondary contact between the originally distributed ecotype T and the later-colonizing ecotype G in the Hahajima Islands. A PCA analysis of leaf morphology revealed that ecotype M showed a distribution intermediate between ecotypes G and T (Fig. 4).

Moreover, the maximum stem length and DBH of ecotype M was also intermediate between ecotypes G and T (Fig. 5).

**Pre- and post-mating isolation among ecotypes**

The main flowering times between ecotypes G and M were in summer and mostly overlapped, while those between ecotypes G and T, as well as M and T, generally did not overlap, except for some trees (Fig. 6). The genetic distances between G and M are small, and between G and T, and between M and T are greater (Fig. 2d, Sugai et al. 2019), suggesting that the magnitude of flowering time differences between ecotypes contributes to the genetic distances between them, as has been shown by other studies (Gustafsson and Lönn 2003, Stanton and Shore 1997). However, despite the generally different flowering times between ecotypes T and G, there is some overlap in certain trees. Considering the occurrence of ecotype M, which is a hybrid origin between the summer-flowering ecotype G and the autumn-flowering ecotype T, it can be inferred that pre-mating isolation is incomplete even among ecotypes with different flowering times.

On the other hand, the flowering time of ecotype D was long, ranging from summer to winter and generally overlaps with the flowering times of other ecotypes. However, in the STRUCTURE analysis, individuals of ecotype D showed little admixture with other ecotypes (Fig. 2c), and the genetic distance between ecotype D and other ecotypes was great (Fig. 2d,

Sugai et al. 2019). This is likely because ecotype D is narrowly distributed on dry scrub on steep cliffs, and is allopatric. Pollen dispersal is usually limited by distance (Adams 1992), and as result, genetic distances are expected to be greater between ecotype D and other ecotypes. In summary, there is almost no pre-mating isolation between ecotypes G and M, incomplete pre-mating isolation based on flowering times between ecotypes T and G, as well as T and M, and incomplete pre-mating isolation based on geographic distance between D and the other ecotypes.

Post-mating isolation could only be measured between ecotypes G and D. However, as no differences were observed in fruit set, germination, and seedling mortality rates between inter-ecotype and intra-ecotype crossings, it is presumed that post-mating isolation is at least not present until the seedling stage. Nevertheless, there is also the possibility that individuals resulting from inter-ecotype crosses may be infertile, so further verification is necessary. Additionally, the presence or absence of post-mating isolation between other ecotype pairs should also be investigated.

**Adaptation of ecotypes to local environment**

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 core seedling of G (Fig. S4d). The maximum stem length and DBH of

512 ecotype D were low (Fig. 5) and it had smaller, thicker leaves that contained
513 many hairs (Fig. S3). Previous studies have identified a negative correlation
514 between drought tolerance and tree height (McGregor et al. 2021), and
515 small, thick, trichome-rich leaves are also known to be an adaptation to dry
516 areas (Ilyas et al, 2021; Tsujii et al. 2016). Taken together, these facts
517 suggest that ecotype D is adapted to lower soil moisture and this may be
518 why it inhabits soil types that the other ecotypes do not. In the Bonin
519 Islands, several endemic tree species are known to exhibit different genetic
520 groups within a single species that are distributed in mesic forest and dry
521 scrub (Sugai et al. 2022; Tsuneki et al. 2014). This suggests that genetic
522 differentiation by soil moisture conditions is probably a common pattern of
523 differentiation in the Bonin Islands.
524 Ecotype G, which is distributed throughout the understory of the
525 mesic forests, has almost no leaf hairs, whereas ecotypes T and M, which
526 grow in bright areas and constitute the forest canopy, have hairs on their
527 leaves. Ecotype D, that also have hairs on their leaves, would have
528 originally constituted the canopy of dry scrub, although at present the upper
529 layer is covered by alien trees. Leaf hairs are known to reduce
530 photoinhibition caused by strong sunlight (Ripley et al. 1999). Growing
531 ecotype G in a sunny location causes leaf burn and atrophy, while no such
532 phenomenon occurs in the other three hairy ecotypes (Setsuko S. personal
533 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-

556 tolerant than ecotype T due to its parentage from ecotype G, which is
557 distributed in the mesic forest understory. If this is the case, ecotype M may
558 be an example of adaptive introgression (Suarez-Gonzalez et al. 2018). On
559 the other hand, ecotype M inhabited mesic scrub and mesic forests with low
560 forest height, and did not inhabit mesic forests with high forest height,
561 where ecotype T inhabited. The reason why ecotype M cannot survive in
562 those areas may be because ecotype M, which has ecotype G as a parent,
563 cannot grow as tall as ecotype T. To test these possibilities, future studies are
564 needed. Specifically, common garden experiments are needed to determine
565 whether differences in shade tolerance and growth rates are between
566 ecotypes T and M, as well as which genes ecotype M has acquired from its
567 parental ecotypes.

568 In summary, ecotype D is adapted to dry environments, while
569 ecotype T is adapted to the canopy of mesic forests, characterized by humid
570 environments. This suggests that adaptive radiation has led to the ecological
571 divergence of *C. subpubescens* in the Hahajima Islands. Additionally,
572 ecotype G, originating from the Chichijima Islands, is adapted to the
573 understory of mesic forests, a humid and dim environment where other
574 ecotypes are not present. The ecotype M, derived from the hybrid between
575 ecotypes G and T, formed a hybrid zone in the mesic scrub. The presence of
576 four ecotypes within a single tree species on small islands of approximately
577 20 $km^2$ in size may be attributed not only to adaptive radiation, but also to

hybridization facilitated by immigration of allopatrically differentiate ecotypes from adjacent islands and subsequent secondary contact among ecotypes.

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**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 supporting information.

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781 **Figure and table legends**

782 Fig. 1 Spatial distribution of core and intermediate trees (left) and a
783 topographic and a vegetation map of Hahajima and Imoutojima Islands
784 (right). The G, T, D and M are referring to the ecotypes indicated in Table 1.
785 Letters indicated by arrows on the left map are the population names and
786 locations reported in Sugai et al. (2019). The three-digit numbers on the
787 right map indicate elevations.

788

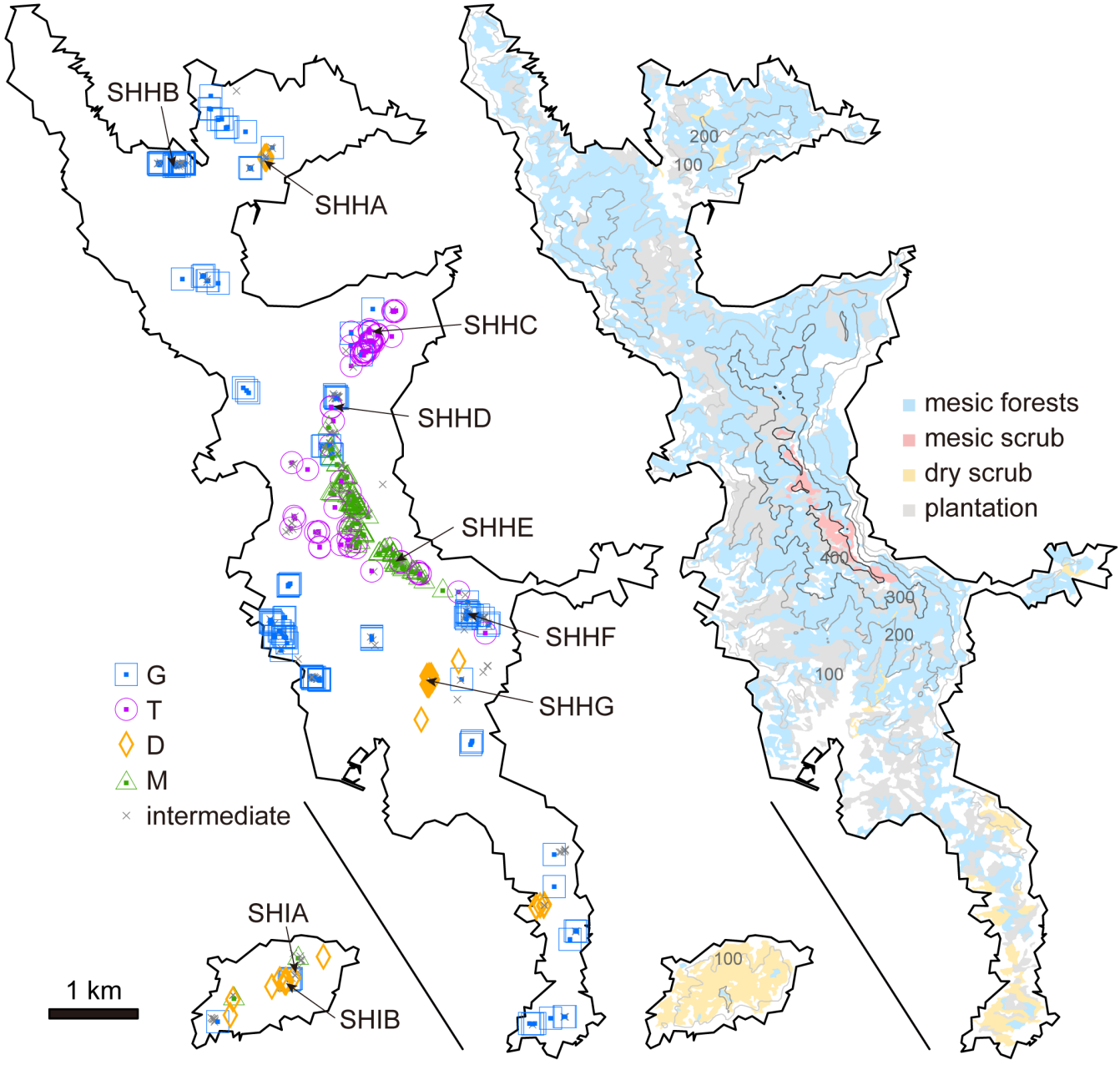
789 Fig. 2 Results of STRUCTURE analysis. (a) $\Delta K$, representing the number of
790 clusters ranging 2–9, (b) changes in log likelihood ranging 1-10, (c) bar
791 plots showing clustering of all trees from $K = 2$ to 7, and (d) neighbor-
792 joining trees show relationships among each cluster at $K = 4$ and 7. Vertical
793 columns of the bar plots represent individuals and bar height are
794 proportional to the posterior mean of the estimated admixture proportion,
795 and individuals are aligned roughly from north to south by population. The
796 solid gray lines below the bar plot X-axis indicate populations used in Sugai
797 et al. (2019). The gray dashed line refers to trees on satellite Imoutojima
798 Island; all other trees are on Hahajima Island. Letters in the circles of
799 neighbor-joining trees (G, T, D, and M) refer to the ecotypes as indicated in
800 Table 1, the colors of the circles correspond to the colors of the bar plot, and
801 numbers beside the circles indicate the $F_{ST}$ values of each cluster.

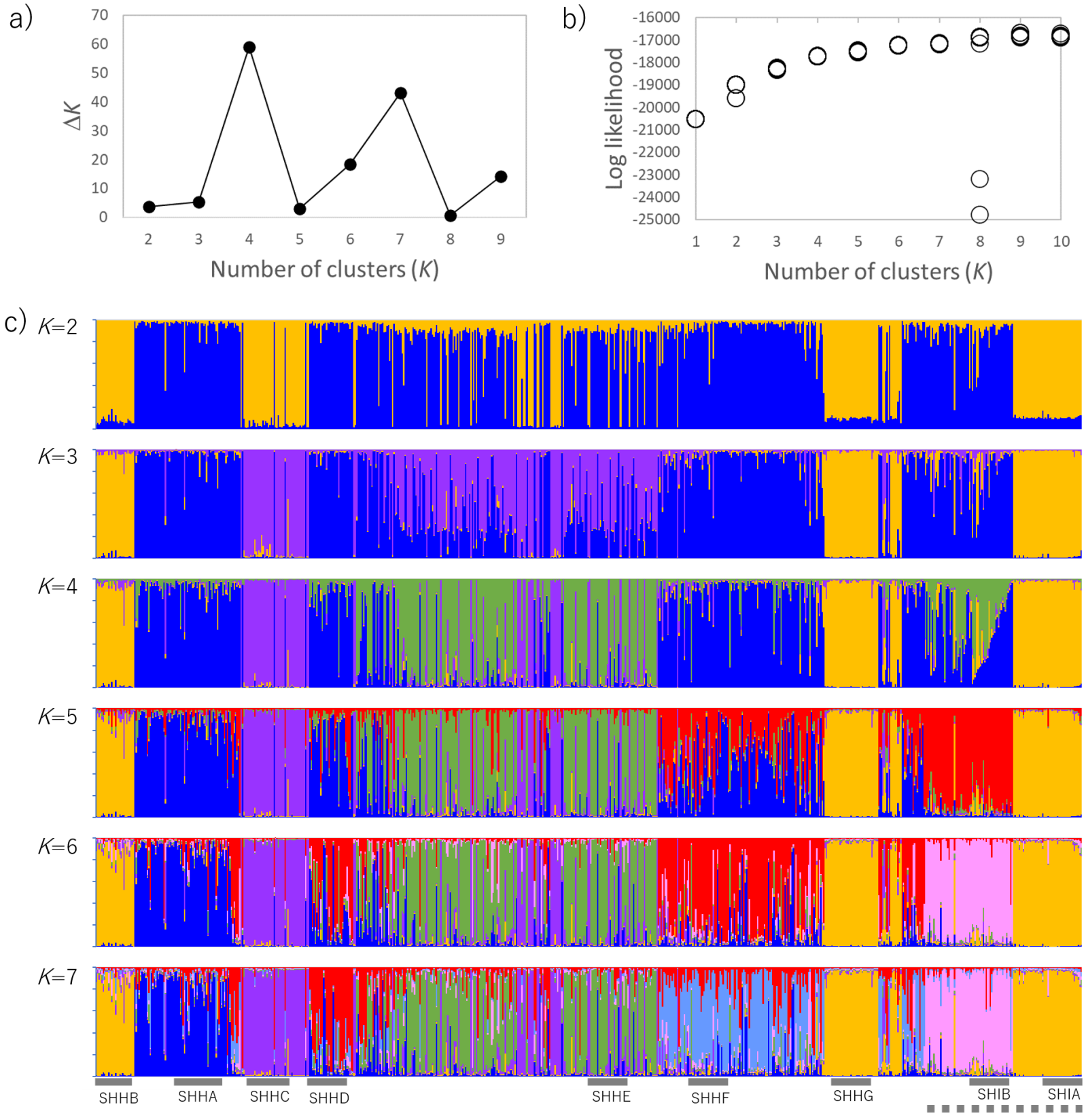
802

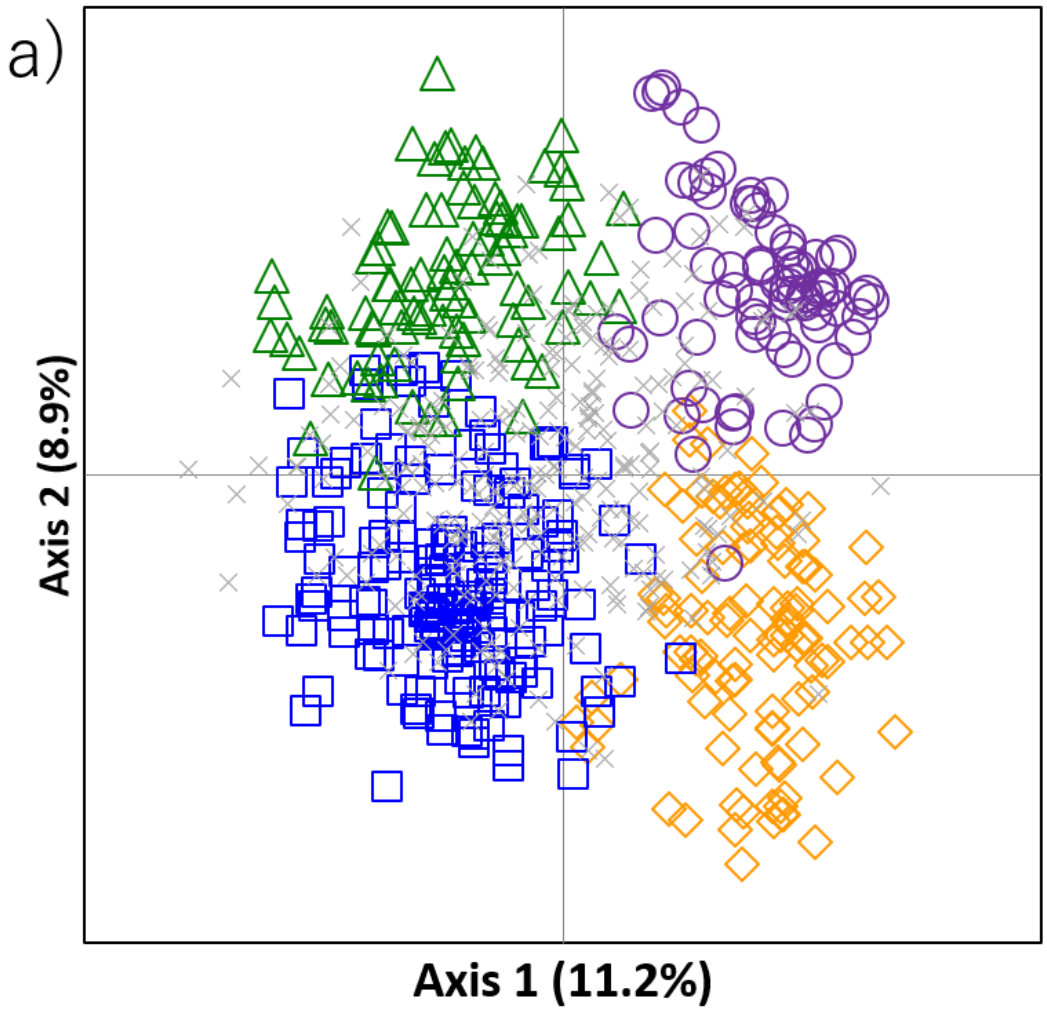
803 Fig. 3 Principal coordinate analysis (PCoA) scatter plots of all trees for the
804 1st and 2nd principal coordinate axes (a) and the 1st and 3rd principal
805 coordinate axes (b). Core trees (trees assigned at $Q \geq 0.9$ in STRUCTURE
806 analysis) for ecotype G, T, D, and M, as shown in Table 1, are represented as
807 blue squares, purple circles, yellow diamonds, and green triangles,
808 respectively, with the intermediate trees (trees assigned at $Q < 0.9$ in
809 STRUCTURE analysis) among ecotypes indicated by gray x signs.
810
811 Fig. 4 Distributions of the first and second principal components (i.e., PC1
812 and PC2) for eleven leaf morphological traits. Hair_U; leaf hair density on
813 the upper surface, Hair_L; leaf hair density on the lower surface, Serration;
814 number of serrations, LA; leaf area, LMA; leaf mass per area. G, T, D, and
815 M refer to the ecotypes as indicated in Table 1.
816
817 Fig. 5 Size distribution of core trees of each ecotype. Different letters
818 indicate significant differences among ecotypes ($p < 0.05$, pairwise t-test
819 with Bonferroni correction). G, T, D, and M refer to the ecotypes as
820 indicated in Table 1.
821
822 Fig. 6 Temporal changes in the number of flowering cymes of each core tree
823 (a), daily precipitation, and the daily maximum temperature, along with the
824 maximum temperatures over the past 10 years from June 2014 to January

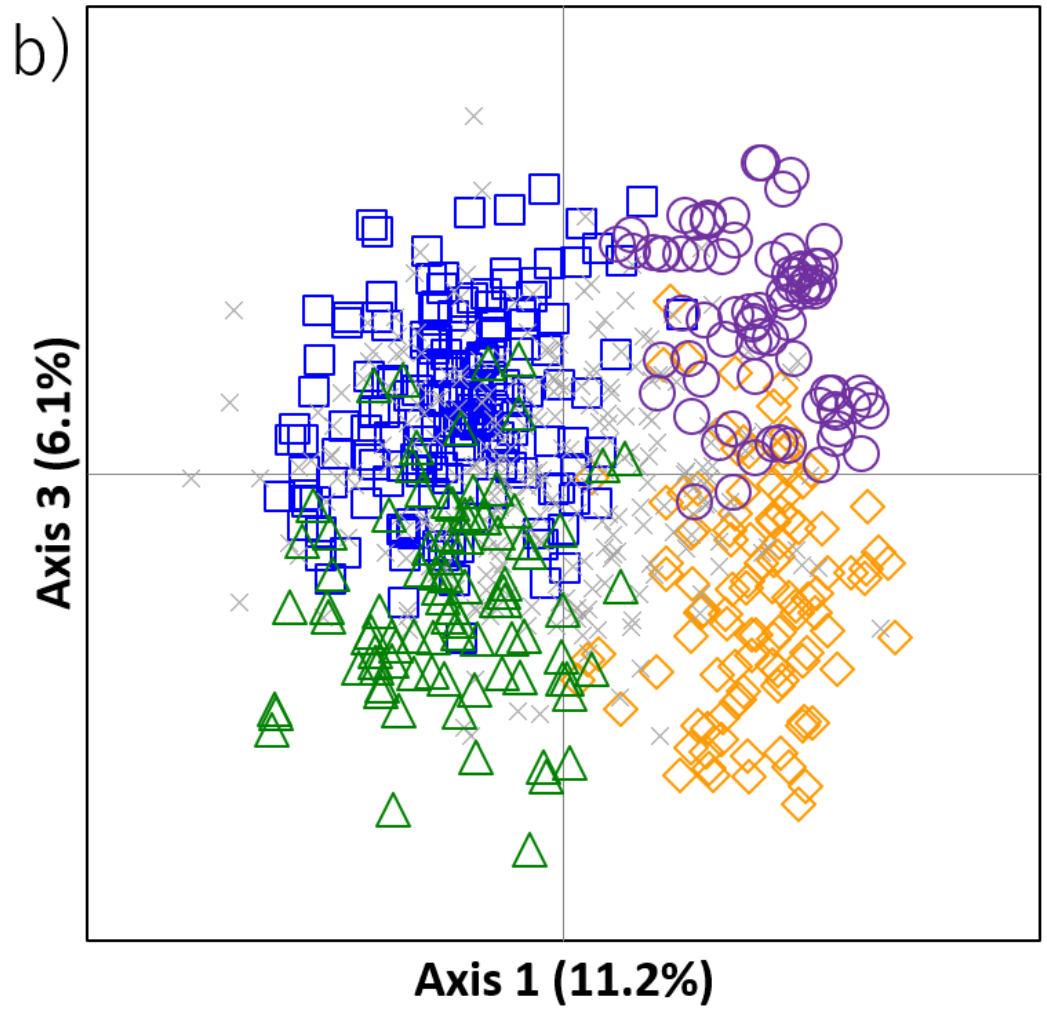
2015 on Hahajima Island (Japan Meteorological Agency 2024) (b). (a) Each horizontal line indicates an individual, and darker colors indicate a greater number of flowering cymes. G, T, D, and M refer to the ecotypes as indicated in Table 1.

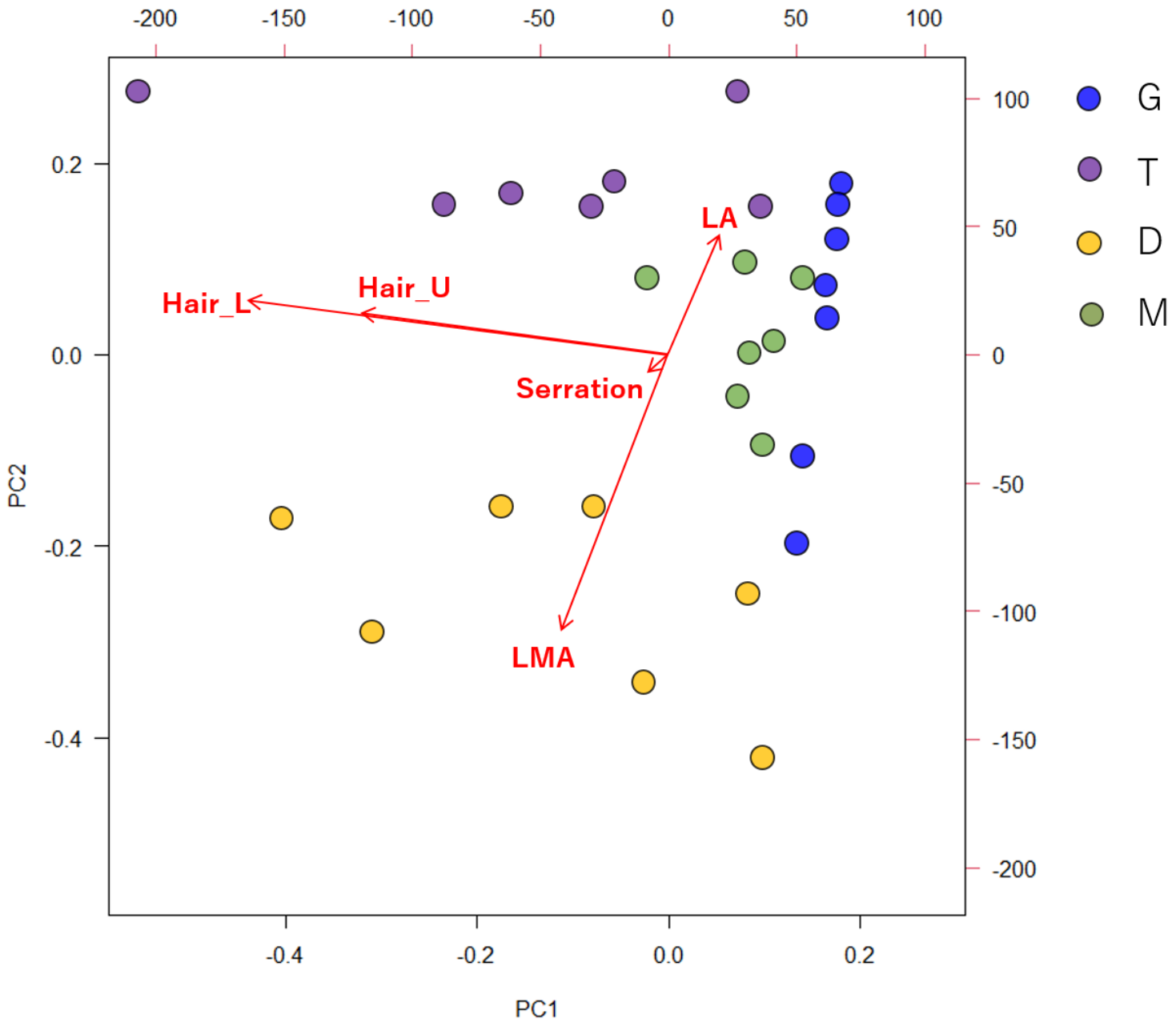
Table 1 Name, ID, and characteristics of each ecotype

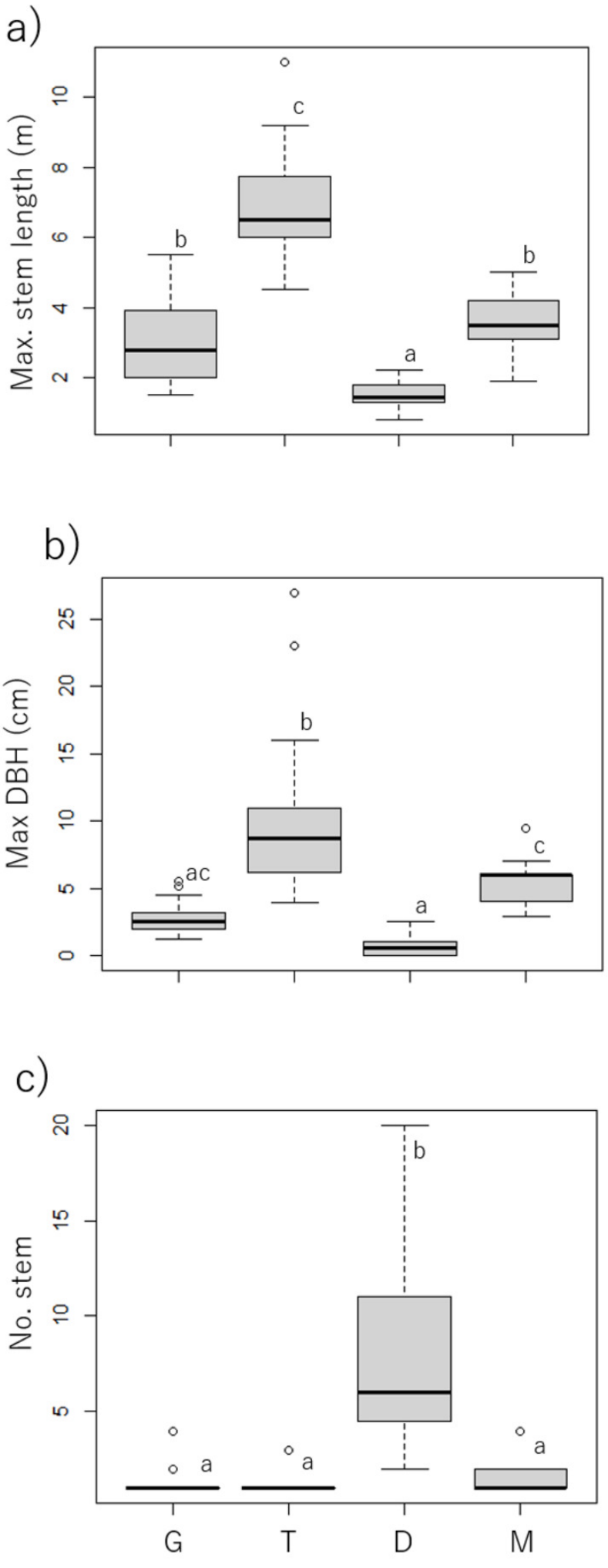

SHHB
SHHA
SHHC
SHHD
SHHE
SHHF
SHHG
SHIA
SHIB
G
T
D
M
intermediate
1 km
mesic forests
mesic scrub
dry scrub
plantation
200
100
400
300
200
100
100


a)
ΔK
Number of clusters (K)
b)
Log likelihood
Number of clusters (K)
c)
K=2
K=3
K=4
K=5
K=6
K=7
SHHB
SHHA
SHHC
SHHD
SHHE
SHHF
SHHG
SHIB
SHIA
d)
K=4
T 0.392
0.181
M
0.273
D
G
0.079
K=7
T 0.412
M 0.188
G 0.208
0.278
D
0.093
G
G 0.101
G
0.082


a)
Axis 2 (8.9%)
Axis 1 (11.2%)


b)
Axis 3 (6.1%)
Axis 1 (11.2%)


-200
-150
-100
-50
0
50
100
0.2
0.0
-0.2
-0.4
PC2
PC1
-0.4
-0.2
0.0
0.2
100
50
0
-50
-100
-150
-200
LA
Hair_L
Hair_U
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)
No. stem
5
10
15
20
a
a
b
a
G
T
D
M

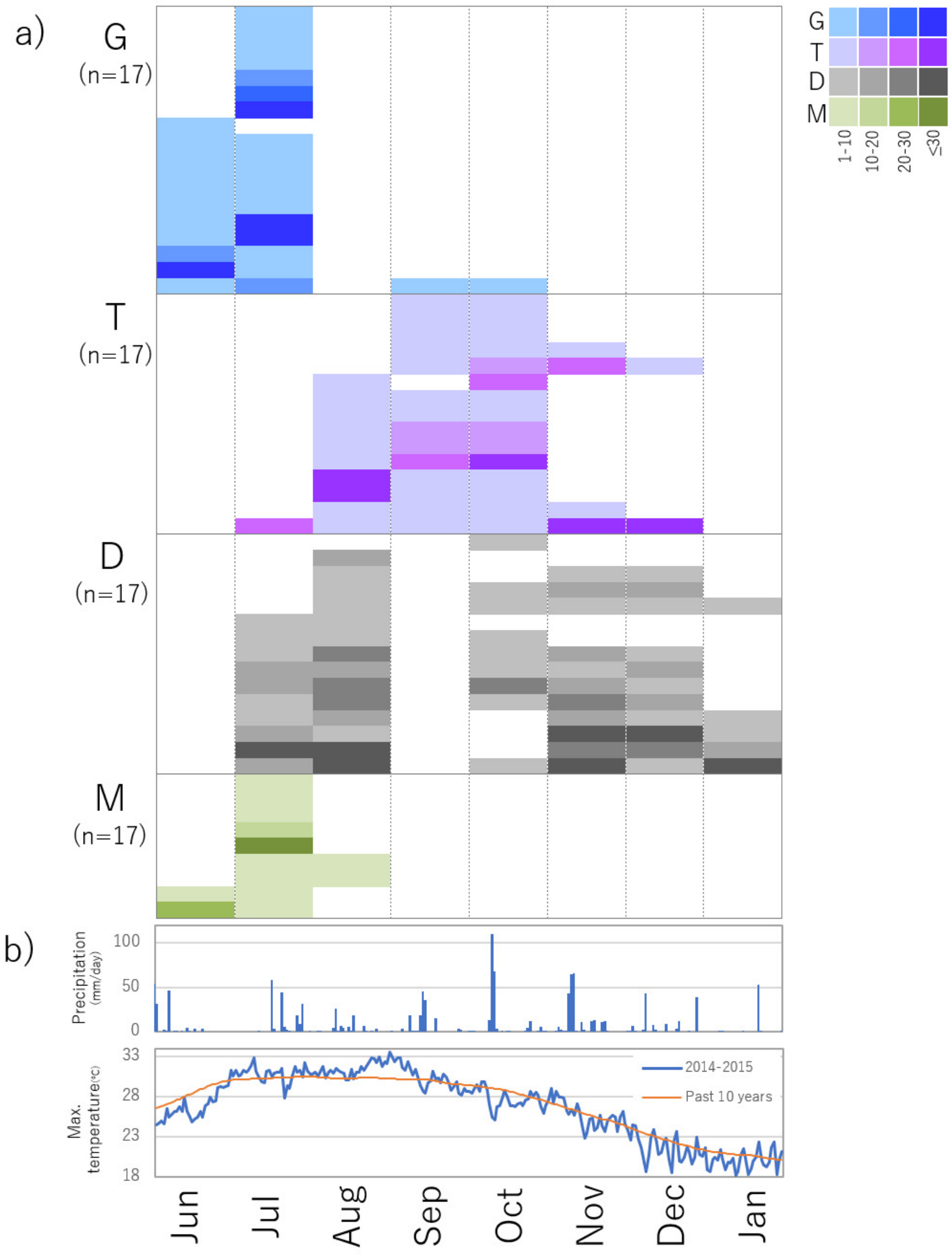

a)
G
(n=17)
T
(n=17)
D
(n=17)
M
(n=17)
G
T
D
M
1-10
10-20
20-30
≤30
b)
Precipitation (mm/day)
100
50
0
Max. temperature(℃)
33
28
23
18
2014-2015
Past 10 years
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan

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

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