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Comprehensive genetic analysis reveals the genetic structure and diversity of *Calanthe hoshii* (Orchidaceae), an endemic species of the Ogasawara Islands: Implications for appropriate conservation of a critically endangered species

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Abstract

The Ogasawara Islands, isolated from the continent throughout their geological history, harbor abundant endemic species. However, 32% of the native plant species are on the Japanese Red List, signaling their endangered status. Among these endangered species, *Calanthe hoshii* stands out as highly susceptible to extinction. Two wild and 89 ex situ individuals were alive in 2020, but the last known wild individual died in October 2021, indicating this species may have become extinct in the wild. Despite the conservation efforts under the Act for the Conservation of Endangered Species of Wild Fauna and Flora, the lack of essential information regarding genetic diversity and population structure among the remaining individuals poses a significant obstacle to developing effective conservation measures. In this research, we conducted a genetic analysis of the wild and ex situ individuals, which revealed notably reduced genetic diversity between individuals, with individual heterozygosity levels (1.0×10^{-5}) measuring one-ninth of those observed within a closely related species *Calanthe triplicata* (1.5×10^{-4}). Comprehensive genetic analysis revealed that *C. hoshii* consisted of three genetic clusters of different sizes: cluster 1 comprised 95% of the total population, while clusters 2 and 3, with few individuals, were only found in the ex situ populations at Koishikawa Botanical Garden. Since extant *C. hoshii* maintains a remarkably low genetic diversity at both individual and population levels, it is necessary to consider future management strategies, such as artificial breeding among different clades identified in this study, to safeguard the stability and resilience of this species.

KEYWORDS

ex situ conservation, genetic conservation, genetic diversity, Ogasawara Islands, RAD-seq

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1 | INTRODUCTION

The Ogasawara Islands are oceanic islands located in the northwestern Pacific Ocean, approximately 1000 km south of the Japanese archipelago (Figure 1). Of the 441 native taxa of vascular plants, 161 (37%) are considered endemic (Ministry of the Environment, Japan, 2010). However, the habitats of these native plants face increasing threats due to human impacts, including changes in the environments caused by historical settlements, java cedar (*Bischofia javanica*) plantations (Abe et al., 2020), a decline in pollinators damaged by green anoles (*Anolis carolinensis*), and the impacts of tourism (Kachi, 2010). Consequently, out of the 441 native taxa, 144 are currently classified as endangered, necessitating conservation efforts from individual species to entire ecosystems (Ministry of the Environment, Japan, 2010).

Calanthe hoshii (Orchidaceae) is a perennial herb species endemic to Hahajima Island, one of the Ogasawara Islands, Japan. It was discovered in the late 1960s and described as a new species in 1983 (Kobayashi, 1983). Even at its initial discovery, there were six wild individuals at two sites: four on Mt. Chibusa and two at Sekimon (Toyoda, 2014). They continued to decline due to various factors, including illegal excavation, feeding damage inflicted by giant African land snails (*Lissachatina fulica*), habitat loss resulting from the spread of alien trees such as java cedar, and natural disasters (Hirai, 2013). In 2004, *C. hoshii* was designated as a "national endangered species of wild fauna and flora" under the Act for the Conservation of Endangered Species of Wild Fauna and Flora. Concurrently, it was also included as one of the target species among 16 vascular plant species for the protection and reproduction programs of nationally rare species of wild fauna and flora (Ministry of Environment, Japan, & Ministry of Agriculture, Forestry and Fisheries, Japan, 2004). However, of the two wild individuals known in 2020, one died in that year (sample ID: YI-0293), and the other died in October 2021 (sample ID: YI-0292) (Ministry of Environment, unpublished). The wild population of this species may have become extinct. Koishikawa Botanical Garden (Graduate School of Science, University of Tokyo, Japan) is leading ex situ conservation efforts of this species by vegetative propagation by division. Other botanical gardens, including the Botanic Gardens of Toyama, Jindai Botanical Garden, and Tsukuba Botanical Garden, are also engaged in ex situ conservation initiatives. These programs aim to reintroduce ex situ propagated plants back to the three planting sites on Hahajima Island, further bolstering the conservation of this species (Ministry of Environment, Japan, 2015).

In comparison with the closely related species *Calanthe triplicata*, *C. hoshii* exhibits inherent vulnerability, characterized by an increased accumulation of detrimental mutations within its genome and lower adaptability to environmental changes (Hamabata et al., 2019). The vulnerability of this species was demonstrated in its populations in the wild; between 2008 and 2010, flowering and fruiting were observed in the planted population of Mt. Funaki, resulting in nearly 100 natural seedlings, all of which ended up dead within 5 years. In 2015, an attempt was made to grow seedlings at Koishikawa Botanical Garden and Tsukuba Botanical Garden. Out of the 10 seedlings (sample ID: YI-1534-1, YI-1534-2), two survived, and one eventually flowered in 2021, suggesting that this species can grow up to maturity under artificial management. However, there is a growing concern that the suitable wild habitat, temperature below 30°C and humidity of more than 80% (Yukawa, personal communication), may no longer exist.

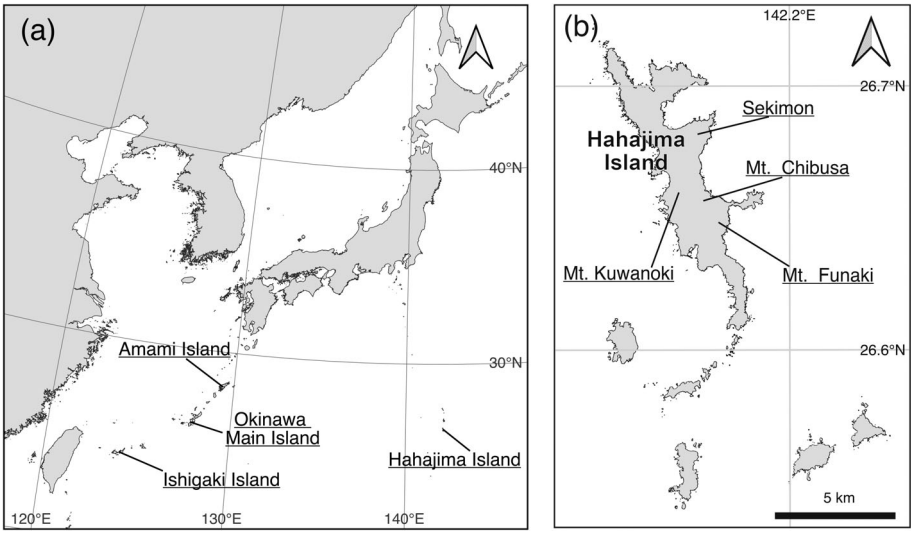


FIGURE 1 Map showing the collection sites of *Calanthe hoshii* and *Calanthe triplicata*, including Nansei Islands (Amami Island, Okinawa Main Island, Ishigaki Island) and Ogasawara Islands (a), and Ogasawara Islands, including Hahajima Island (b). [Correction added on 12 August 2024, after first online publication: Figure 1 in this version has been replaced.]

TABLE 1 Collection sites and number of *Calanthe hoshii* and *Calanthe triplicata*.

Species	Collection site	Status	Number
Calanthe hoshii	Mt. Chibusa (MC), Hahajima Island, Ogasawara Islands	Wild	2
	Mt. Kuwanoki (MK), Hahajima Island, Ogasawara Islands	Reintroduced	12
	Mt. Funaki (MF), Hahajima Island, Ogasawara Islands	Reintroduced	43
	Confidential site (CH), Hahajima Island, Ogasawara Islands	Reintroduced	3
	Tsukuba Botanical Garden (TB), National Museum of Nature and Science, Tokyo	Ex situ	2
	Koishikawa Botanical Garden (KB), Graduate School of Science, the University of Tokyo	Ex situ	19
Calanthe triplicata	Shino River, Amami Island	Wild	20
	Amurogama, Amami Island	Wild	1
	Mt. Omoto, Ishigaki Island	Wild	2
	Mt. Sankaku, Okinawa Main Island	Wild	1

At Koishikawa Botanical Garden, efforts have been ongoing since 1985 to collect and propagate plant individuals from the wild. The propagation comprised a maximum of seven individuals: four collected from Mt. Chibusa, two from Sekimon by Koishikawa Botanical Garden, and one from Mt. Funaki donated by a local resident. The one from Mt. Funaki is probably not described in the paper by Kobayashi (1983). However, considering the initial situation of propagating without identifying individuals in the 1990s, the actual number utilized for propagation may be fewer than seven. Despite the pressing need for conservation measures for this species, the lack of information concerning genetic diversity and the genetic differences between individuals impedes the development of appropriate and effective conservation strategies (Nakahama et al., 2023). In this study, we conducted a genetic analysis involving 81 individuals of *C. hoshii*, encompassing both wild and ex situ conservation populations, to (1) measure the genetic diversity of *C. hoshii* compared with its closely related species *C. triplicata*, (2) elucidate the phylogenetic structure of individuals from wild and ex situ conservation populations of *C. hoshii*, and (3) provide critical information for constructing a sustainable conservation strategy for *C. hoshii*.

2 | MATERIALS AND METHODS

2.1 | Plant materials and sampling strategy

We collected a total of 81 leaf samples of *C. hoshii* from 2014 to 2021, which comprised two wild individuals from Mt. Chibusa, 58 from wild planted areas (12 from Mt. Kuwanoki, 43 from Mt. Funaki, and three from an undisclosed location), 19 individuals cultivated at Koishikawa Botanical Garden, and two individuals grown at Tsukuba Botanical Garden. The two wild individuals from Mt. Chibusa (sample ID: YI-0292, YI-0293) were collected in 2014 before they died in 2020 and 2021. For *C. triplicata*, we collected leaves from 21 individuals on Amami Island, two on Ishigaki Island, and one on Okinawa Main Island (Tables 1 and S1).

2.2 | DNA extraction, restriction site-associated DNA sequencing (RAD-seq), and multiplexed inter-simple sequence repeat (ISSR) genotyping by sequencing (MIG-seq)

Genomic DNA was extracted from silica-dried leaves using the modified cetyltrimethylammonium bromide (CTAB) method (Porebski et al., 1997). After extraction, the concentration of extracted DNA was quantified using the Qubit dsDNA BR Assay kit (Invitrogen, USA). We used the protocol outlined by Sakaguchi et al. (2015) for double-digest RAD-seq, which itself is a modification of the original double-digest RAD-seq proposed by Peterson et al. (2012). In brief, EcoRI and BglII were used for restriction digestion during library preparation. After PCR amplification of DNA, the sequencing libraries were purified. The libraries were sequenced using 150-bp paired-end reads in a single lane of an Illumina HiSeq2000 (Illumina, San Diego, CA, USA). Subsequently, the MIG-seq was employed to supplement the sequence data for several samples that lacked sufficient information from the RAD-seq. While MIG-seq generates a smaller quantity of data, it represents a cost-effective approach to enhance the dataset. MIG-seq libraries were

constructed following the protocol of Suyama et al. (2022). This protocol mainly includes four steps. In the first step, we amplified ISSR from genomic DNA by multiplexed polymerase chain reaction (PCR) with MIG-set primer set-1 (Suyama & Matsuki, 2015). Polymerase chain reaction was performed in a MiniAmp Plus Thermal Cycler (Thermo Fisher Scientific, Applied Biosystem, CA, USA), following the condition described in the original protocol. The 1st PCR step was performed to amplify ISSR regions from genomic DNA. In the second step, each first PCR product was purified/equalized using AMPure XP Beads (Beckman Coulter Inc, USA) following the protocol of Hosomichi et al. (2014). In the third step, indexes and Illumina sequencing regions were added to the purified first PCR product, and performed the second PCR as described in the original protocol of Hosomichi et al. (2014). In the fourth step, the second PCR product from each sample was mixed in equal amounts, and size was selected using AMPure XP beads for the final size of >350 bp. Using an Illumina MiSeq and a MiSeq Reagent kit v3 (150 cycles) (Illumina Inc, USA), 80-bp paired-end sequencing was performed. The first 17 bases (simple sequence repeat [SSR] and anchor regions) in both reads were skipped using the "DarkCycle" option. Sequencing data obtained in this study have been submitted to the DDBJ Sequence Archive (DRA) under accession numbers PSJDB17223 and PSJDB17870. [Correction added on 12 August 2024, after first online publication: The last sentence in section 2.2 has been added.]

2.3 | De novo mapping and variant calling

Firstly, regarding RAD-seq data, three samples (YI-1484, YI-1504, and YI-0115-11) were excluded due to their small file size, each less than 1/100 of the largest file size. The quality filtering and adapter trimming of the raw sequence data were carried out using trimmomatic-0.39 (Bolger et al., 2014); the parameters for RAD-seq data were as follows: "TruSeq3-PE-2.fa:2:30:10 AVGQUAL:30 MINLEN:150 CROP:150 SLIDINGWINDOW:4:20." The parameters for MIG-seq data were as follows: "TruSeq3-PE-2.fa:2:30:10 AVGQUAL:20 SLIDINGWINDOW:4:15 MINLEN:80." Three datasets were generated for the following analysis: dataset (A) including *C. hoshii* and *C. triplicata* using RAD-seq data; dataset (B) including only *C. hoshii* using RAD-seq data; and dataset (C) including only *C. hoshii* using MIG-seq data. The variants were detected using Stacks 2.62 (Catchen et al., 2013). The parameters for Stacks were set as follows: "denovo_map.pl: –M 2-m 3–n 1, populations: –write-single-snp –p 1 –R 0.5 –max-obs-het 0.5 –min-maf 0.05" for dataset (A) and (B), and "denovo_map.pl: –M 3 –m 8 –n 5, populations: –write-single-snp –p 1 –R 0.5 –max-obs-het 0.5 –min-maf 0.05" for dataset (C). The variants were filtered, and the genetic distance was calculated using Tasssel5 (Bradbury et al., 2007). For dataset (A), 18 individuals, comprising less than 20% of sites, were filtered out, including one of the last individuals, YI-0293. For dataset (B), 21 individuals, comprising less than 40% of sites, were filtered out, including one of the last wild individuals, YI-0293. For dataset (C), one individual, comprising less than 25% of sites, was filtered out. After filtration, these data files were used for subsequent analysis, and the individual observed heterozygosity was generated in dataset (A) using Stacks.

3 | PHYLOGENETIC ANALYSIS

To determine the optimal model for phylogenetic tree construction, we employed modeltest-ng version 0.2.0 (Darriba et al., 2020), and GTR + G4 and GTR + I + G4 were selected for datasets (A) and (B), respectively. Phylogenetic trees were generated using RAxML-NG v. 1.1.0 (Kozlov et al., 2019), with 500 bootstrap replicates using the maximum likelihood methodology.

3.1 | Population structure analysis

To identify the population structure of *C. hoshii*, subsequent analysis was conducted using datasets (B) and (C). Population structure was performed using Structure version 2.3.4 (Pritchard et al., 2000). The software was run at *K* values of 1–7, allowing 10 runs per *K* value to estimate the optimal number of groups (*K*). The parameters were set for the burn-in periods as 50 000 and at 100 000 Markov Chain Monte Carlo (MCMC). Delta *K*, an ad-hoc statistic, was used to estimate the optimal value of *K* (Evanno et al., 2005) using Structure Harvester (Earl & vonHoldt, 2012).

4 | RESULTS

4.1 | Dataset characteristics

A total of 676 706 170 reads with 102 182 631 670 bases were obtained from RAD-seq data, and 18 429 998 reads with 1 474 399 840 bases were obtained from MIG-seq data. After variant calling and filtration, we obtained 5851 single-nucleotide polymorphisms (SNPs) from 72 individuals of *C. hoshii* and 15 *C. triplicata* for dataset (A), 4470 SNPs from 58 individuals of *C. hoshii* for dataset (B), and 129 SNPs from 80 individuals of *C. hoshii* for dataset (C).

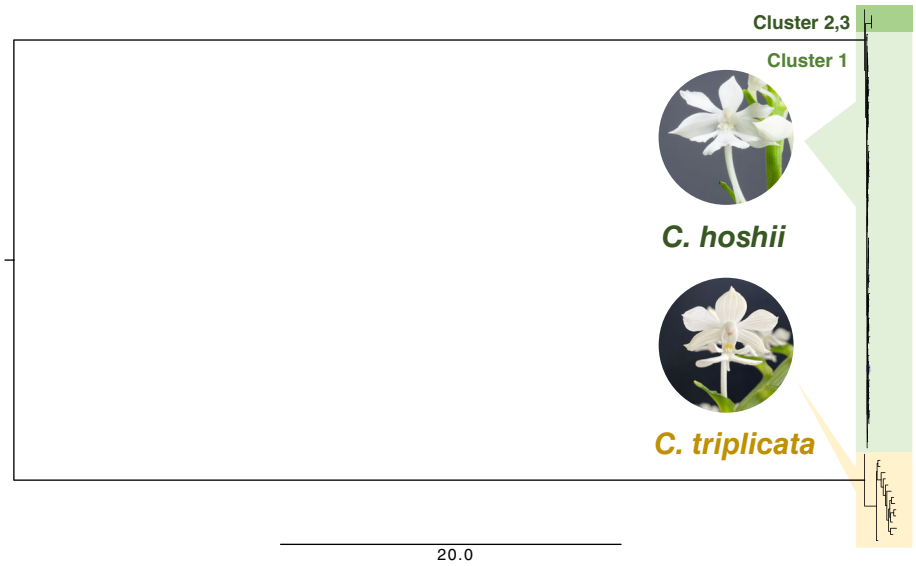


FIGURE 2 Phylogenetic tree of *Calanthe hoshii* and *Calanthe triplicata* based on 5851 single-nucleotide polymorphisms (SNPs) inferred from RAxML-ng using model GTR + G4, visualized by FigTree version 1.4.4. Clusters 2 and 3, indicated by dark green area, correspond to those shown in Figures 4 and 5.

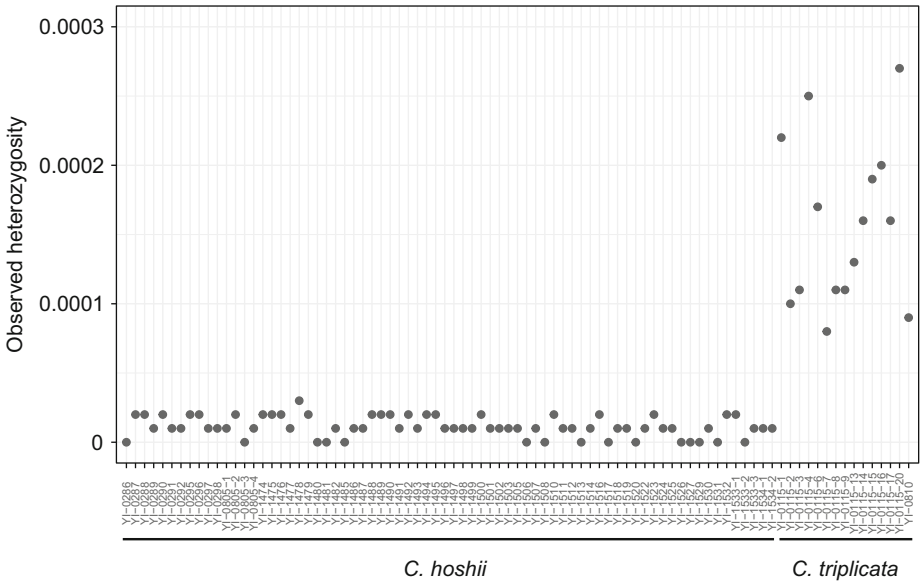


FIGURE 3 The observed heterozygosity of *Calanthe hoshii* and *Calanthe triplicata* calculated by Stacks version 2.62 using 5851 single-nucleotide polymorphisms (SNPs).

4.2 | Phylogeny and genetic diversity of *C. hoshii* and *C. triplicata*

Calanthe hoshii was clearly differentiated from *C. triplicata*, and samples of *C. hoshii* exhibited shorter branch lengths than those of *C. triplicata* (Figures 2 and S1). The average genetic distances between intraspecies samples of *C. hoshii* and *C. triplicata* were 0.067 and 0.145, respectively, while the interspecies average distance between *C. hoshii* and *C. triplicata* was 0.783.

The observed heterozygosity of each individual, calculated by dividing the heterozygous base sites with all detected sites, is 0–3.0 $\times$ 10^{-5} (average 1.0 $\times$ 10^{-5}) for *C. hoshii*, and 8.0 $\times$ 10^{-5}–2.7 $\times$ 10^{-4} (average 1.5 $\times$ 10^{-4}) for *C. triplicata* (Figure 3). This indicates *C. hoshii* presents significantly lower genetic diversity at the individual level compared with *C. triplicata* (Wilcoxson rank sum test with continuity correction, $p = 2.6 \times 10^{-10}$).

4.3 | Phylogenetic relationship and population structure among *C. hoshii* samples

The intraspecies phylogenetic tree of *C. hoshii* shows sharp distinctions between the three clusters with high bootstrap values (Figure 4).

According to the delta *K* value, the optimal number of ancestries (*K*) was estimated as two (Figure S2). Cluster 1 comprised most of the extant individuals. However, four samples were assigned to different clusters. Three of

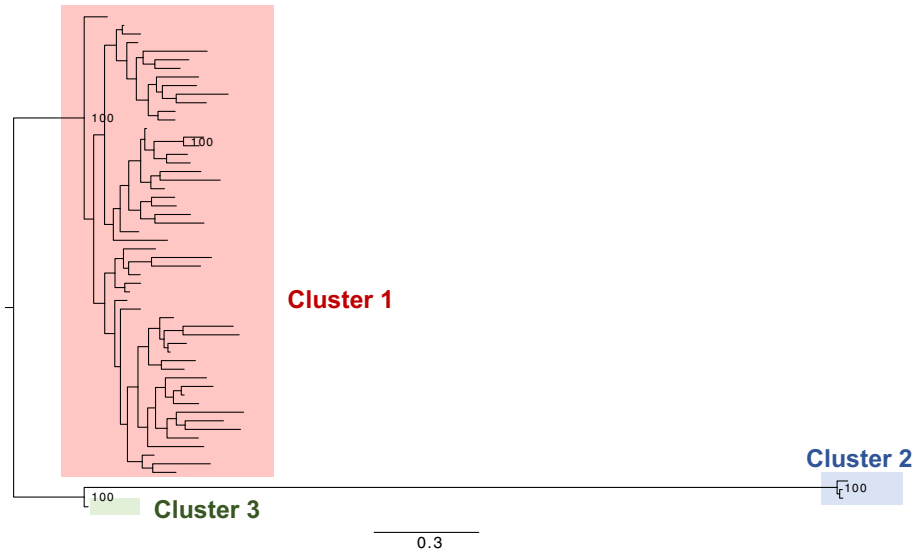


FIGURE 4 Phylogenetic tree of *Calanthe hoshii* based on 4470 single-nucleotide polymorphisms (SNPs) inferred from RAxML-ng using model GTR + I + G4 with bootstraps 500, visualized by FigTree version 1.4.4. Bootstraps greater than 90 are shown in the figures.

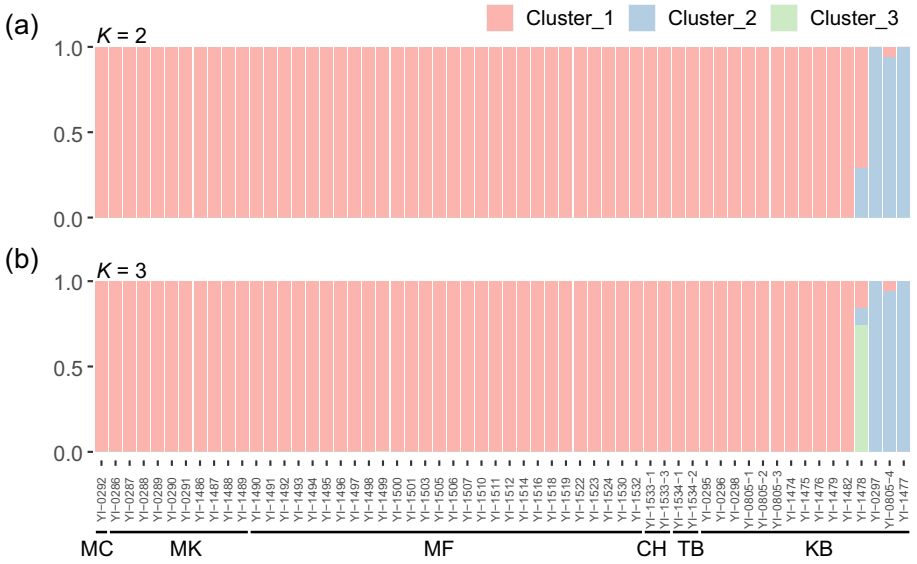


FIGURE 5 Bayesian clustering results by STRUCTURE analysis of *Calanthe hoshii*. The bar plot shows 58 individuals at (a) $K = 2$ and (b) $K = 3$. Abbreviations at the bottom indicate the collection sites: MK, Mt. Kuwanoki; MC, Mt. Chibusa; MT, Mt. Funaki; CH, the Confidential site in Hahajima Island; TB, Tsukuba Botanical Garden; KB, Koishikawa Botanical Garden.

these samples were assigned to cluster 2, and sample YI-1478 occupied a notable position between clusters 1 and 2 in the phylogenetic tree (Figure S3), forming a distinct cluster (Figure 5). Therefore, the following text describes the argument with $K = 3$: With $K = 3$, 54 of the 58 individuals were assigned to cluster 1, with three individuals to cluster 2 and one to cluster 3 (Figure 5). MIG-seq analysis showed that all the samples that were excluded through the filtering of RAD-seq analysis belonged to cluster 1 (Figure S4). As a result of RAD-seq and MIG-seq analyses, all individuals growing on Hahajima Island belonged to cluster 1, whereas those cultivated in Koishikawa Botanical Garden included individuals from clusters 1, 2, and 3.

The net nucleotide distance, which measures the difference in allele frequencies between clusters, revealed values of 0.2518 between clusters 1 and 2, 0.1135 between clusters 1 and 3, and 0.2403 between clusters 2 and 3 based on RAD-seq data. These values correspond to the results of the phylogenetic tree analysis (Figure 4). The FST values were 0.3623, 0.6728, and 0.8472 for clusters 1, 2, and 3, respectively, indicating greater divergence of clusters 2 and 3 from their common ancestor.

5 | DISCUSSION

5.1 | Phylogeny and genetic diversity of *C. hoshii* and *C. triplicata*

Phylogenetic analysis has shown that *C. hoshii* is clearly differentiated from *C. triplicata* (Figure 2) and exhibits reduced genetic diversity of *C. hoshii* at the individual and population levels compared with *C. triplicata*. The

issue of low genetic diversity within individuals was inferred by a previous study (Hamabata et al., 2019), while our study diverges from this by providing a comprehensive examination that elucidates the prevalence of low genetic diversity at the population level and low genetic variation among individuals across nearly all extant *C. hoshii* (Figures 2 and 3). The genetic diversity observed in both wild individuals and natural seedlings from the reintroduction site (YI-0298, YI-1534-1, and YI-1534-2) was found to be comparable to that of other individuals, indicating a potential progression of inbreeding within the species over time, thereby sustaining consistently low genetic diversity (Figure 3). *Calanthe hoshii* had a small population size even at the time of its discovery in the late 1960s, and its prolonged existence as a small population on the oceanic island has likely led to reduced genetic diversity. Other endemic species in the Ogasawara Islands, such as *Crepidiastrum grandicollum*, *Crepidiastrum linguaefolium*, *Crepidiastrum ameristophyllum* (Ito & Ono, 1990), *Pittosporum parvifolium*, *Pittosporum chichijimense*, *Pittosporum beecheyi* (Ito et al., 1997), eastern buzzard (*Buteo japonicus toyoshimai*) (Nagai et al., 2019), *Distylium lepidotum* (Yagi et al., 2019), and *Rhododendron boninense* (Isagi et al., 2020), have low genetic diversity with small population sizes.

The sample set of *C. hoshii* analyzed in this study includes clones which may have led to a reduction in the genetic diversity of *C. hoshii* due to filtering for high heterozygosity and minor alleles. Since there is no record of which individual of *C. hoshii* is the clone, the optimal value for the parameters is unknown. However, while this analysis does not fully address the issue of clones, our findings align with those of Hamabata et al. (2019), who analyzed the individual-level genetic diversity of *C. hoshii* and *C. triplicata* using RNA-seq data. Their study, conducted with a different method from ours, also reported significantly lower genetic diversity in *C. hoshii* compared with *C. triplicata*. Thus, it appears that the genetic diversity of *C. hoshii* is inherently lower than that of *C. triplicata*.

5.2 | Population structure and phylogenetic relationship among *C. hoshii* samples

The structure analysis indicated that *C. hoshii* consists of two or three clusters. When applying K to three, 54 of the 58 individuals subjected to analysis were assigned to cluster 1, while clusters 2 and 3 consisted of only three individuals and one individual, respectively (Figures 4 and 5). It is plausive that individuals in cluster 3 originated from the crossing between those in clusters 1 and 2, based on the results of its phylogenetic position (Figure 4) and $K = 2$ (Figure 5). However, we consider that a definitive argument cannot be made based on the results of the present analysis. Furthermore, the phylogenetic tree (Figure 2), which includes 19 *C. hoshii* individuals filtered out from the intraspecific phylogeny (Figure 4) and the structure analysis (Figure 5), shows that all 19 individuals fall within cluster 1, suggesting that only a few individuals belong to clusters 2 and 3. Additionally, the MIG-seq result indicates that all the samples that were excluded during the filtering of RAD-seq analysis belong to cluster 1, as shown in the MIG-seq analysis (Figure S4). While most *C. hoshii* plants for ex situ conservation were collected during the 1980s and lack precise information regarding their original growing locations, wild individuals from Mt. Chibusa and those historically collected from the same location are grouped within cluster 1 (Table S1, Figures 5 and S4). Furthermore, two out of three individuals assigned to cluster 2 originated from Mt. Funaki, while the remaining individual originated from Mt. Chibusa (Table S1, Figures 5 and S4). Unfortunately, information regarding cluster 3 is unavailable (Table S1). Considering these findings, we hypothesize the existence of two clusters at Mt. Chibusa. However, the differentiation patterns on the island remain unclear due to the lack of precise habitat information.

5.3 | Conclusions and further conservation directions

Considering the reduced genetic diversity within *C. hoshii*, as revealed in this analysis, inbreeding depression may pose a significant risk in the future, along with generational changes (Wright et al., 2008). The observation that seedlings produced in reintroduction areas on Hahajima Island do not thrive in the wild environment is consistent with the fact that *C. hoshii* is considered inherently vulnerable, attributed to the accumulation of detrimental mutations within its genome (Hamabata et al., 2019). This vulnerability could also be accelerated by inbreeding depression. Additionally, it is important to note that this species is susceptible to hot and dry weather. Recent climate change, such as rising temperature and aridity, has become more intense on Hahajima Island (Matsuyama, 2023). Another possible cause of the decline in the reintroduced populations of *C. hoshii* is infection by viruses caused by the *Calanthe* mild mosaic potyvirus (CalMMV) (Gara et al., 1998) and Cymbidium Mosaic Virus (CyMV) (Ogasawara Islands World Natural Heritage Regional Scientific Committee, 2012) during ex situ propagation (Ministry of Environment, unpublished

observation). High susceptibility to virus infection may result from reduced genetic diversity (Zhu et al., 2000). Since arbitrary crosses may potentially diminish the genetic diversity of *C. hoshii*, it is imperative to implement seed-based propagation measures that aim to increase genetic diversity through controlled crossbreeding between different clusters to counter the impact of these viruses. This study revealed the phylogenetic relationships among extant individuals of *C. hoshii* and found that they belong to three genetic clusters. Of the three clusters found in *C. hoshii*, cluster 1 represented 95% of the total number of individuals, and only a small number of individuals in clusters 2 and 3 were cultivated in Koishikawa Botanical Garden, indicating clusters 2 and 3 should be propagated and cultivated in other botanical gardens to reduce the risk of genetic loss and ensure the continued variability of ex situ populations. For the appropriate conservation of this species, artificial breeding must be conducted using the information on genetic clusters revealed in this study.

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CONFLICT OF INTEREST STATEMENT

We declare that there is no conflict of interest.

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SUPPORTING INFORMATION

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