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Range-wide sampling reveals cryptic lineages but largely conserved mycorrhizal associations in the Japanese fairy lantern *Thismia kobensis*

Kenji Suetsugu^{1,2}  | Kazuma Takizawa¹ | Kazuki Inui¹ | Akihiro Nishimura¹ | Shun K. Hirota^{3,4}

¹Department of Biology, Graduate School of Science, Kobe University, Kobe, Hyogo, Japan

²Institute for Advanced Research, Kobe University, Kobe, Hyogo, Japan

³Botanical Gardens, Osaka Metropolitan University, Osaka, Japan

⁴Graduate School of Symbiotic Systems Science and Technology, Fukushima University, Fukushima, Fukushima, Japan

Correspondence

Kenji Suetsugu, Department of Biology, Graduate School of Science, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe, Hyogo 657-8501, Japan.

Email: kenji.suetsugu@gmail.com

Societal Impact Statement

The jewel-like flowers of *Thismia* are as rare as they are beautiful, often recorded from only a single site per species. Access to 15 populations of *T. kobensis* has enabled an uncommon, range-wide assessment of morphology, genetics, and fungal partners. Our analyses showed that *T. kobensis* comprises three cryptic lineages that rarely exchange genes, despite their nearly identical flowers. Across most of its range, two lineages consistently associate with the same fungal lineage, whereas a small northern lineage depends on different fungi. These findings highlight the need to conserve each lineage separately, with special attention to the vulnerable northern group.

Summary

- *Thismia* (Thismiaceae) includes some of the most elusive angiosperms, with many restricted to their type localities. Limited dispersal may foster cryptic diversity within isolated populations, complicating species delimitation. Although many *Thismia* species display high mycorrhizal specificity, whether such specificity is consistent within species remains unclear due to the paucity of comparative population-level studies. *Thismia kobensis*, with 15 extant populations, offers a valuable model for investigating population structure and mycorrhizal associations.
- We examined inner floral morphology and genome-wide SNPs to evaluate morphological and genetic differentiation across all known *T. kobensis* populations. Population genetic analyses, including Mantel tests, were conducted to identify factors that shape genetic structure, and fungal barcoding was used to assess associations between plant genotypes and fungal partners.
- MIG-seq data identified three genetic groups with minimal gene flow, despite the largely uniform floral morphology. One group was widespread, whereas the other two were confined to the northern Kinki region. Mantel tests detected no significant isolation-by-distance patterns, suggesting restricted dispersal even across short distances. Genetic structure showed limited correspondence with

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mycorrhizal associations: VTX00113 predominated overall, while the rarest group primarily associated with VTX00106 and VTX00295.

- These findings suggest that *T. kobensis* may comprise multiple cryptic species or distinct evolutionary lineages. The presence of VTX00113 in two genetic groups indicates a conserved fungal association, whereas the distinct partners in the rarest group may reflect genetic divergence mediated by host shifts. Additional data on fungal communities in surrounding soils and autotrophic plants are needed to clarify partner selectivity among groups.

KEYWORDS

arbuscular mycorrhizal fungi, biogeography, genetic structure, Glomeraceae, mycoheterotrophy, mycorrhizal specificity, specialization

1 | INTRODUCTION

Thismia is a genus of mycoheterotrophic monocots often called fairy lanterns due to their vivid flowers, which typically emerge just above the soil surface yet remain hidden within the leaf litter (Dančák et al., 2020; Roberts et al., 2003). These plants are notable for their rarity and highly restricted distributions (Thorogood & Mat Yunoh, 2021). To date, more than 100 species have been described, with at least 55 known only from their type localities and 38 recorded solely from the first collection (Dančák et al., 2020; POWO, 2025).

However, recent studies suggest that *Thismia* species may be more widespread than previously assumed. For example, *T. brunneomitra*, initially believed to occur at a single site, has since been documented across a 300-km range in Brunei, Sarawak, and Sabah, Malaysia (Dančák et al., 2020). Similarly, *T. neptunis*, once considered extinct and endemic to its type locality, was rediscovered at six sites in Sarawak, Malaysia (Dančák et al., 2020; Sochor et al., 2018). Genetic analyses reveal pronounced intraspecific differentiation among geographically distant populations, occasionally approaching interspecific levels despite morphological resemblance (Dančák et al., 2020). Given the high incidence of cryptic diversity, limited seed dispersal via rain splash, and persistent taxonomic uncertainty (Coelho et al., 2021; Dančák et al., 2020), molecular approaches are indispensable for clarifying evolutionary and ecological dynamics in this genus.

Fairy lanterns rely entirely on arbuscular mycorrhizal (AM) fungi for carbon and other nutrients. Many *Thismia* species exhibit high mycorrhizal specificity, typically associating with a single or a few AM fungal lineages (Gomes et al., 2017; Guo et al., 2019; Merckx et al., 2017; Suetsugu & Okada, 2025a). However, as most species are known from a single locality, it remains unclear whether such narrow associations are maintained across populations of a single species. Studies confined to limited sites may overlook geographic variation in fungal partners, especially as restricted gene flow in isolated mycoheterotrophic populations may foster divergence in fungal preferences (Taylor et al., 2004; Waterman & Bidartondo, 2008). For instance, populations of the partially mycoheterotrophic orchid *Epipactis helleborine* from dune and forest habitats exhibited higher seedling

establishment in their native environments, possibly reflecting distinct mycorrhizal preferences (Jacquemyn et al., 2018).

Such local adaptation may promote not only ecotype formation but also speciation. Disruptive selection on fungal partner use, where plant performance depends on the associated fungal lineage, may drive specialization and reinforce reproductive isolation (Jacquemyn et al., 2024; Suetsugu et al., 2023). The radiation of *Afrothismia* appears to be linked to specialization on distinct Glomeraceae lineages, with subsequent host tracking that generates phylogenetic congruence between plant and fungus (Merckx & Bidartondo, 2008). Similarly, Suetsugu et al. (2022) found that genetically similar *Oxygyne* individuals tend to associate with more closely related fungal communities. Several studies further suggest that mycorrhizal associations can aid species delimitation in closely related partially or fully mycoheterotrophic plants with high fungal specificity (Freudenstein & Barrett, 2014; Suetsugu et al., 2023; Suetsugu & Okada, 2025b). These findings support the idea that mycorrhizal associations are shaped by evolutionary history and may contribute to speciation (Jacquemyn et al., 2024).

While genus- or species-level analyses have revealed broad coevolutionary patterns, intraspecific studies are essential to identify finer-scale patterns such as geographic mosaics of coevolution (Thompson, 2005). Although geographic variation in fungal associations is well-documented in orchidaceous and ericaceous mycoheterotrophic plants (Barrett et al., 2010, 2022; Dowie et al., 2016; Taylor et al., 2004), it has rarely been examined in AM mycoheterotrophs, particularly in *Thismiaceae*, where most species are extremely rare (Merckx et al., 2017).

In this context, *T. kobensis*, with 15 extant populations, provides an excellent model for examining the relationship between genetic structure and mycorrhizal interactions within a single *Thismia* species. It is most closely related to the Taiwanese *T. huangii* (Suetsugu et al., 2023). Both belong to *Thismia* sect. *Labiothismia*, which comprises 14 species, including *T. abei*, *T. taiwanensis*, and *T. americana* (Nuraliev & Sennikov, 2024; Suetsugu et al., 2023). Within *Thismia* sect. *Labiothismia*, fungal partners have been identified in *T. huangii* and *T. abei*, which predominantly associate with VTX00113 and

VTX00106/VTX00295, respectively (Merckx et al., 2017; Suetsugu & Okada, 2025a). Given that four of the five closely related Oceanian *Thismia* species primarily associate with VTX00345 (Merckx et al., 2017), it is intriguing to examine whether fungal strain sharing also occurs within *Thismia* sect. *Labiothismia*. This can be tested by determining whether *T. kobensis* associates with VTX00113, VTX00106, or VTX00295.

Originally described from a single locality and once considered extinct (Suetsugu et al., 2018), *T. kobensis* is now documented at 15 populations across ~250 km (Inui et al., 2025; Okada et al., 2024; Suetsugu et al., 2023). Unlike most *Thismia* species, which are typically confined to undisturbed primary forests (Thorogood & Mat Yunoh, 2021), *T. kobensis* also occurs in moderately disturbed sites, including *Cryptomeria* and *Chamaecyparis* plantations and secondary forests. This ecological breadth may reflect relatively low specificity, as the ability to associate with diverse fungi may facilitate local partner use and alleviate habitat constraints (Ogura-Tsujita & Yukawa, 2008). However, high specificity does not necessarily limit distribution if preferred fungal partners are widespread (Ogura-Tsujita & Yukawa, 2008). It is therefore important to test whether *T. kobensis* associates with a broader set of fungal lineages than *T. huangii* or *T. abei*, which have narrower ranges and fewer populations. Moreover, given the frequent cryptic diversity in *Thismia* (Dančák et al., 2020), the wide distribution of *T. kobensis* may represent a complex of cryptic taxa with distinct fungal associations. If so, this critically endangered species would be further subdivided, reducing effective population sizes and heightening conservation needs. Integrated analyses of genetic structure and fungal partnerships are therefore essential.

By analyzing inner floral characters, MIG-seq-based genome-wide SNPs, and molecular identification of mycorrhizal fungi from

individuals across all known populations, this study examines the relationship between population differentiation and mycorrhizal associations in *T. kobensis*. Specifically, we address the following questions: (i) To what extent does *T. kobensis* exhibit population differentiation, and is this primarily explained by isolation by distance? (ii) Does *T. kobensis* display high specificity toward its mycorrhizal partners, and is this consistent across genetically divergent groups? (iii) How do the mycorrhizal communities of *T. kobensis* compare with those of its close relatives *T. abei* and *T. huangii*?

2 | MATERIALS AND METHODS

2.1 | Study species, sites, and sampling scheme

Thismia kobensis is a semisubterranean species with creeping vermiform roots. Its erect stem, about 1 cm tall, bears around six translucent white, scale-like leaves, including three involucre bracts that are narrowly triangular to ovate and up to 5 mm long (Suetsugu et al., 2023). The largest leaves, or involucre bracts, are positioned just below the solitary pale orange flowers. The perianth tube is hexagonal-prismatic at the apex and urceolate at the base, measuring 8.0–9.0 mm in length (Figure 1). The roots possess a two-layered epidermis: The outer exoepidermis is largely devoid of fungal hyphae, while the inner endoepidermis contains thick, figure-eight-shaped hyphae that remain mostly undigested (Okada et al., 2024). In contrast, the inner cortex harbors highly degraded fungal coils, suggesting active carbon transfer to the plant (Feller et al., 2022). Although *T. kobensis* and *T. huangii* share floral traits such as a dark orange annulus, truncate connectives with hairs, and the absence of

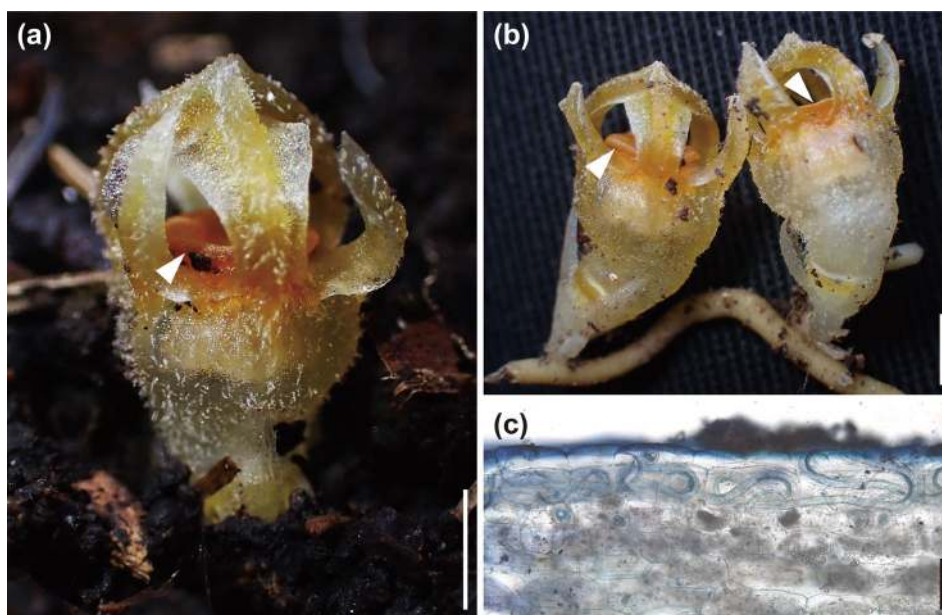


FIGURE 1 *Thismia kobensis* and its mycorrhizal interactions. (a) Flowering plant. (b) Habit showing creeping, vermiform roots. (c) Longitudinal section of the root showing both intact and degenerated fungal coils within cortical cells. Arrows indicate short, laterally expanded annuli. Scale bars: 3 mm (a–b) and 100 μm (c). Photographs by Kazuma Takizawa (a), Kenji Suetsugu (b), and Hidehito Okada (c).

nectaries, *T. kobensis* differs in having a short, laterally expanded annulus ($\sim 1.3 \times 0.4$ mm vs. 0.4×0.7 mm, narrow and erect) and numerous short hairs per stigma lobe (vs. a single long hair in *T. huangii*; Suetsugu et al., 2023).

From May 2021 to May 2024, samples were obtained from 51 individuals representing all 15 known extant populations of *T. kobensis*, encompassing a range of vegetation types (Table S1). Populations were defined as sites separated by at least 2 km. Despite recent discoveries of new populations (Inui et al., 2025; Okada et al., 2024; Suetsugu et al., 2023), *T. kobensis* remains extremely rare, with fewer than 10 flowering individuals in many populations, and is listed as Critically Endangered in the Japanese Red List (Ministry of the Environment, Government of Japan, 2025). To minimize disturbance, only small root fragments (~ 1 cm) were collected from each individual for molecular analyses. In addition, one whole plant per population was dissected to examine inner floral structures, as species-level identification in *Thismia* requires these characters (Dančák et al., 2020; Sochor & Hroneš, 2018). Morphological observations and measurements, particularly of diagnostic traits distinguishing *T. kobensis* from *T. huangii*, were performed using a stereomicroscope (M165C; Leica Microsystems, Wetzlar, Germany). Voucher specimens were deposited in the herbarium of the National Museum of Nature and Science (TNS), Japan.

2.2 | Phylogenomic analysis

Genetic profiles among *T. kobensis* individuals were assessed using MIG-seq, a microsatellite-associated DNA sequencing method analogous to RAD-seq (Suyama & Matsuki, 2015). Genomic DNA was extracted from surface-sterilized root fragments, following the CTAB method (Doyle & Doyle, 1990). MIG-seq libraries were prepared from 46 individuals representing 15 populations, following Suyama et al. (2022). Sequencing was conducted on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) using a MiSeq Reagent Kit v3 (150 cycles). Sequence data have been deposited in the DDBJ Sequence Read Archive (accession number PRJDB37516).

After primer removal and quality filtering, 9,232,030 reads ($200,696 \pm 4,671$ per sample) were retained from 10,051,628 raw reads ($218,514 \pm 5,040$ per sample). SNP discovery was carried out with Stacks v2.60 (Rochette et al., 2019) using parameters: minimum depth for stack creation (m) = 3, maximum distance between stacks (M) = 2, and allowed mismatches between loci (n) = 2. SNP sites with high heterozygosity ($H_o \geq 0.6$) and those with fewer than three minor alleles were removed. SNPs in linkage disequilibrium were filtered using the --indep-pairwise command in PLINK 1.90 (Chang et al., 2015), with parameters set to 50, 10, and 0.1.

Genetic differentiation among populations was evaluated using Neighbor-Net and STRUCTURE analyses. In Stacks, SNPs with a minimum proportion threshold ($R = 0.5$) yielded 399 SNPs from 46 samples. A Neighbor-Net network based on uncorrected p distances was generated in SplitsTree4 v4.14 (Huson & Bryant, 2006), excluding ambiguous sites. Population structure was analyzed in STRUCTURE

v2.3.4 (Pritchard et al., 2000), with 30 independent runs for $K = 1-10$, each with a 100,000-step burn-in followed by 100,000 MCMC iterations. The optimal K was determined using the Delta K method (Evanno et al., 2005) in Structure Harvester (Earl & von Holdt, 2012) and visualized in CLUMPAK (Kopelman et al., 2015).

Based on these analyses, all *T. kobensis* specimens, except one unassigned individual, were classified into three genetic groups (Groups 1–3; see below). To further evaluate genetic differentiation, population genetic parameters, including observed heterozygosity (H_o), expected heterozygosity (H_e), mean inbreeding coefficient (F_{is}), nucleotide diversity (π), and number of private alleles, were calculated for each population or group using Stacks.

Pairwise F_{ST} values were calculated based on Weir and Cockerham (1984) using the R package hierfstat (Goudet, 2005), with 95% confidence intervals from 1000 bootstrap replicates. Populations with only one sample were excluded from F_{ST} calculations. To test for correlations between genetic distance (linearized F_{ST} [$F_{ST}/(1 - F_{ST})$]) and geographic distance, Mantel tests were conducted using the R package vegan (Oksanen et al., 2025). Analyses were run for all combinations, excluding analyses of Group 3 (two closely located populations) alone: (1) all individuals, (2) Group 1, (3) Group 2, (4) Groups 1 and 2, (5) Groups 2 and 3, and (6) Groups 1–3 combined.

Finally, gene flow was estimated using the online tool divMigrate (Sundqvist et al., 2016), based on the effective number of migrants (N_m ; Alcalá et al., 2014), which quantifies the relative magnitude of bidirectional gene flow between population pairs using genetic differentiation and a hypothetical migrant pool. Populations with only one sample were excluded from the divMigrate analysis.

2.3 | Molecular analysis of mycorrhizal fungi

A fungal metabarcoding library was generated from 49 samples of *T. kobensis* spanning 15 populations. DNA was extracted with the CTAB method. Based on morphological evidence of Glomeromycotina-type AM associations (Okada et al., 2024), PCR amplification was carried out with the Glomeromycotina-compatible AMV4.5NF/AMDGR primer set (Sato et al., 2005), with 3–6-mer Ns fused to the primers. Amplifications were performed with Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, United States) following Suetsugu et al. (2025).

Amplicons were pooled and purified with the AMPure XP Kit (Beckman Coulter, CA, United States), and DNA fragments around 500 bp were selected using E-Gel SizeSelect (Thermo Fisher Scientific, Waltham, MA, United States). Sequencing was performed on an Illumina MiSeq platform using a MiSeq Reagent Micro Kit v2 (300 cycles, Illumina, United States) with an 8-pM loading concentration and a 10% PhiX spike-in. Sequencing data have been deposited in the NCBI Sequence Read Archive (Accession Number PRJNA1257301).

Bioinformatic analyses were performed using Claident v0.9.2024.06.1 (Tanabe & Toju, 2013), following Suetsugu et al.

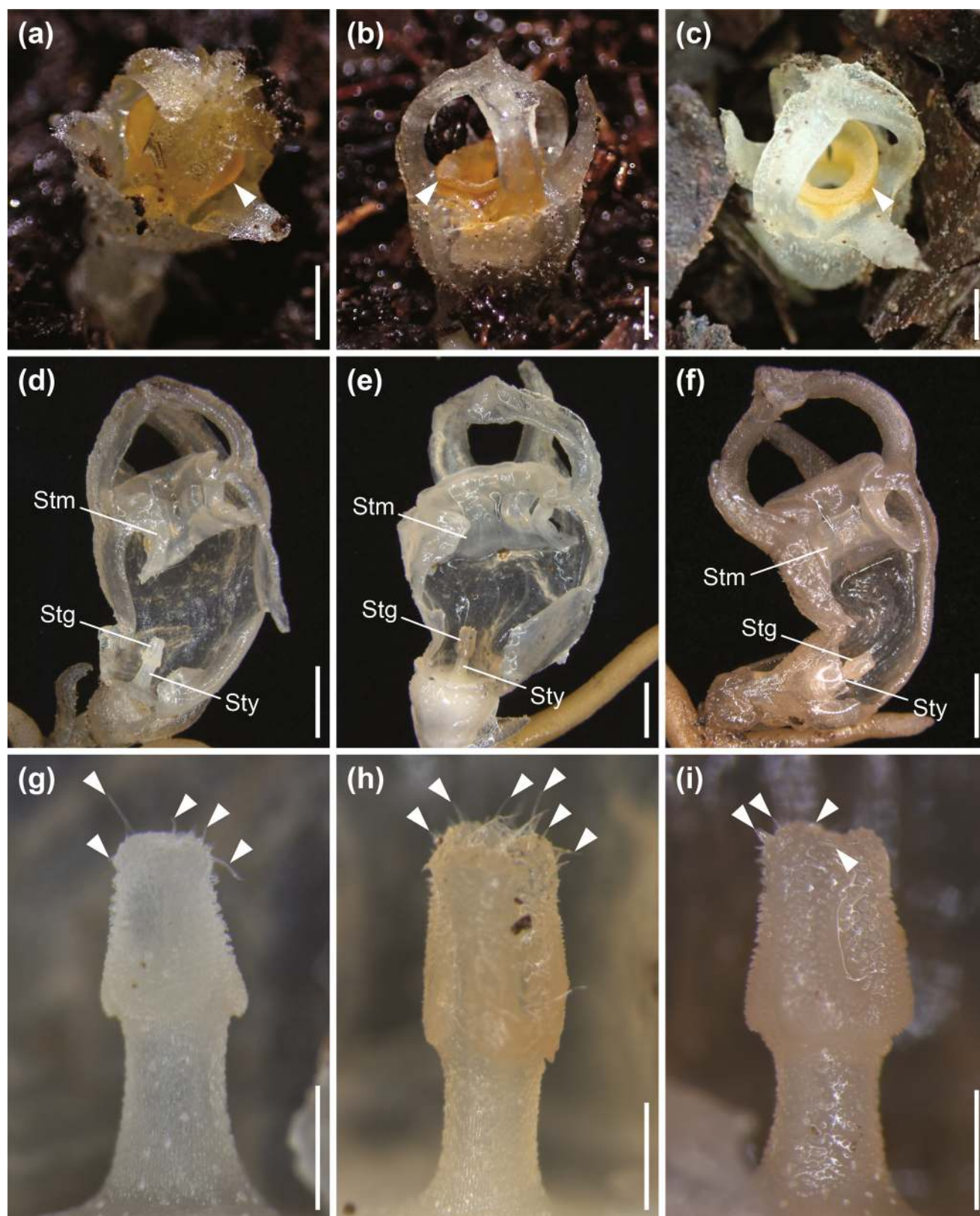


FIGURE 2 Representative samples of *Thismia kobensis* from distinct genetic groups (Group 1: a, d, g; Group 2: b, e, h; Group 3: c, f, i). (a, d, g) *T. kobensis* from Nose-cho, Toyono-gun, Osaka Prefecture (Kazuma Takizawa and Kazuki Inui T35). (b, e, h) *T. kobensis* from Tamba-shi, Hyogo Prefecture (Kazuma Takizawa and Kazuki Inui T37). (c, f, i) *T. kobensis* from Tambasasayama-shi, Hyogo Prefecture (Kazuki Inui and Masaki Saito T34). (a–c) Flowering plants. Arrows indicate short, laterally expanded annuli. (d–f) Longitudinal sections of perianth tubes showing stamens (Stm), styles (Sty), and stigmas (Stg). (g–i) Styles and stigmas. Arrows indicate numerous short hairs on each stigma lobe. Scale bars: 2 mm (a–f) and 500 μ m (g–i). Photographs by Kazuma Takizawa (a–b, d–i) and Masaki Saito (c).

(2025). Briefly, high-quality reads were clustered into operational taxonomic units (OTUs) at a 97% similarity threshold. Taxonomic assignments were performed using Claident, and only OTUs assigned to the subphylum Glomeromycotina were retained. Rare VTXs, defined as those with one read per PCR amplicon or ≤ 50 reads across all data matrices, were excluded. To standardize sequencing depth, abundance-based rarefaction was applied, normalizing all samples to 3,567 reads. OTUs were assigned to VTXs in the MaarJAM database (Öpik et al., 2010) with thresholds of $\geq 90\%$ identity, e -value $\leq 1e-50$, and $\geq 80\%$ query coverage (Wall et al., 2020). The resulting VTX-level community matrix was used for downstream analyses.

β -diversity of mycorrhizal communities in *T. kobensis* and *T. abei* (based on previously published mycorrhizal data) was evaluated using the Bray–Curtis dissimilarity index in the vegan package and weighted UniFrac distance matrices (WUDMs) in the GUniFrac package (Chen & Chen, 2021). Data for *T. abei* were retrieved from the NCBI SRA (Accession No. PRJNA1213262). *T. huangii* could not be included because only Sanger data are available (Merckx et al., 2017). Because WUDMs incorporate phylogenetic relationships among VTXs, these were inferred from VTX type sequences in the MaarJAM database (Öpik et al., 2010). Sequences were aligned using MAFFT v7.475 (Katoh & Standley, 2013), and a maximum likelihood (ML) tree was reconstructed in IQ-TREE v2.2.2 (Nguyen et al., 2015) under the HKY + F + I model (selected via BIC), with 1000 ultrafast bootstrap replicates (Hoang et al., 2018).

Hierarchical clustering based on these indices was performed using average linkage with hclust (the stats package in R) to evaluate the effect of plant identity on AM fungal structure. PERMANOVA was used to test for differences in community composition among groups (*T. kobensis* Groups 1–3 and *T. abei*) with adonis2 in vegan (10,000 permutations). Pairwise PERMANOVAs with FDR-adjusted p values were calculated using the pairwiseAdonis package (Martinez Arbizu, 2020).

3 | RESULTS

3.1 | Morphological and molecular differentiation

Morphological analysis confirmed that all specimens shared diagnostic traits of *T. kobensis*, including a short, laterally expanded annulus (more than 1 mm wide and less than 0.5 mm tall) and numerous short hairs on each stigma lobe (Suetsugu et al., 2023). Accordingly, all individuals unambiguously belonged to *T. kobensis* when compared with described species. Although floral tube hair density and flower color varied within and among populations, no consistent trends were observed across the three genetically divergent groups (see below). Nevertheless, individuals in Group 2 often exhibited a more cuboid or cylindrical stigma shape, in contrast to the pyramidal to truncated-pyramidal form in Groups 1 and 3 (Figure 2), suggesting that the possibility of an undescribed taxon cannot be entirely ruled out.

Neighbor-Net analysis identified three genetic groups (Groups 1–3) within *T. kobensis*, with one specimen (T20–1) from

Tambasayama (TBS-B, Hyogo Prefecture) positioned between Groups 2 and 3 (Figure 3a). STRUCTURE analysis ($K = 3$, based on the largest ΔK value) also revealed three clusters, largely corresponding to those defined by the Neighbor-Net analysis (Figure 3b). Nonetheless, STRUCTURE analysis indicated that specimen T20–1 contained both the rarest cluster (corresponding to Group 3) and the most prevalent cluster (primarily associated with Group 1), making its assignment uncertain. Another specimen (T10–1) from Kita-ku (KIT-B, Hyogo Prefecture), assigned to Group 1 in Neighbor-Net, also harbored both the dominant cluster and the rarest cluster, but given its Neighbor-Net placement and a minor Group 3 component, it was retained in Group 1. Notably, most specimens, including those from the easternmost site (Mori population, Shizuoka Prefecture), belonged to a single group (Group 1), whereas the other two groups were confined to the northern Kinki region (Hyogo and Kyoto Prefectures) (Figure 3b).

Nucleotide diversity (π) and expected heterozygosity (H_E) were highest in the wide-ranging Group 1 ($\pi = 0.127$, $H_E = 0.124$), followed by Group 2 ($\pi = 0.113$, $H_E = 0.100$) and Group 3 ($\pi = 0.062$, $H_E = 0.046$) (Table 1). Within individual populations, F_{IS} values were generally near zero, consistent with random mating. In contrast, Group 1 as a whole had a much higher F_{IS} (0.340), likely reflecting the Wahlund effect, as genetically distinct populations were analyzed together.

Genetic differentiation among the three groups was substantial; F_{ST} estimates ranged from 0.703 to 0.880 between Groups 1 and 2, 0.670 to 0.856 between Groups 1 and 3, and 0.826 to 0.869 between Groups 2 and 3. Although the 95% confidence intervals overlapped, the minimum between-group F_{ST} was higher than the maximum within-group F_{ST} . Likewise, divMigrate analyses indicated very limited gene flow (Table S2), consistent with over 100 private alleles in both Groups 1 and 2 (Table 1). High F_{ST} values were also found among populations within the same group, suggesting restricted gene flow even at that scale (Tables S3 and S4). Consequently, Mantel tests showed no significant correlation between genetic and geographic distances across all datasets (Table S5).

3.2 | Patterns of mycorrhizal associations

Fungal community analysis revealed that *T. kobensis* roots were mainly colonized by *Rhizophagus* VTX00113 (160,891 reads; 92.05% of total reads; Figure S1). Nearly all Group 1 individuals primarily harbored VTX00113, with 37 of 38 individuals showing over 95% of reads assigned to this variant. This strong association (131,976 reads; 97.37% of Group 1 total) across populations spanning ~ 250 km highlights its highly specific symbiosis. All Group 2 individuals ($n = 7$) were also dominated by VTX00113 (24,961 reads; 99.97%). One individual with uncertain group assignment based on MIG-seq was also associated with VTX00113 (3567 reads; 100.00%).

In contrast, all Group 3 individuals ($n = 3$) were dominated by two *Rhizophagus* variants: VTX00295 (7702 reads; 71.97%) and VTX00106 (2604 reads; 24.33%), with only a minor fraction of

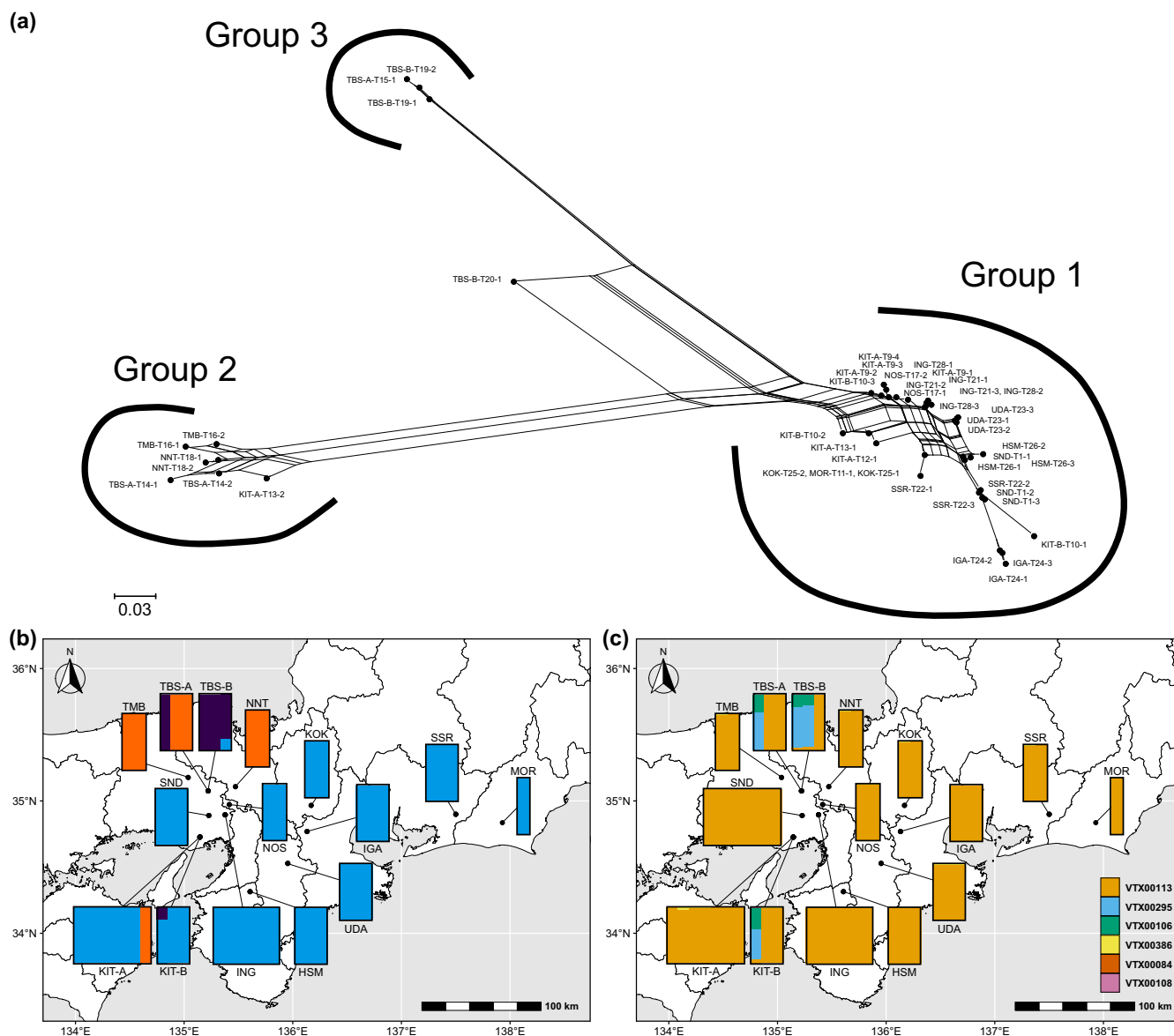


FIGURE 3 Genetic profiles and mycorrhizal associations in *Thismia kobensis*. (a) Neighbor-Net network of *T. kobensis* individuals based on uncorrected p distances. Branch lengths represent the average number of substitutions per site. (b) Map showing sampling sites and sample sizes, with plant genetic structure inferred in STRUCTURE v2.3.4. (c) Map showing sampling sites and sample sizes, with mycorrhizal associations identified through metabarcoding. Each point in (b) and (c) represents a sampling locality. The width of each chart reflects the sample size per population, and the color of each chart indicates genetic clusters in (b) and associated fungal lineages in (c). Sample ID details are provided in Table S1.

VTX00113 (387 reads; 3.62%) (Figure 3c). These individuals were collected from secondary broadleaf forests dominated by *Eurya japonica* and *Camellia japonica*, whereas those associated with VTX00113 mainly occurred in *Cryptomeria japonica* plantations (Table S1). One Group 1 specimen was mainly associated with VTX00295 (1915 reads; 53.69%) and VTX00106 (1394 reads; 39.08%), rather than VTX00113 (249 reads; 6.98%) (Figure 3c).

Hierarchical clustering based on Bray-Curtis dissimilarity and weighted UniFrac distances showed that most *T. kobensis* individuals associated with VTX00113 formed a highly uniform cluster, with some samples nearly identical across sites. In contrast, all Group

3 individuals, together with one Group 1 individual dominated by VTX00295 and VTX00106, clustered with *T. abei* (Figure 4). Searches of VTX00295, VTX00106, and VTX00113 in the MaarjAM database returned 233, 9, and 1150 records, respectively, spanning diverse ecosystems (Table S6).

PERMANOVA revealed no significant differences in mycorrhizal composition between Groups 1 and 2 or between Group 3 and *T. abei* ($p = 1$). In contrast, significant differences were found between *T. abei* and Group 1, *T. abei* and Group 2, and between Groups 1 and 3 ($p < 0.01$ for all). A marginal difference was also detected between Groups 2 and 3 ($p = 0.05$; Table S7).

TABLE 1 Population statistics of *Thismia kobensis* based on MIG-seq data.

Groups/populations	H_D	H_E	F_{IS}	π (calculated from variant positions)	Number of private alleles	Number of samples
Group 1	0.048	0.124	0.340	0.127	178	35
SND	0.057	0.045	0.012	0.065	1	3
KIT-A	0.048	0.068	0.073	0.078	3	6
KIT-B	0.046	0.074	0.089	0.098	1	3
MOR	0.039	0.019	0.000	0.039	0	1
NOS	0.041	0.034	0.015	0.051	2	2
ING	0.044	0.042	0.010	0.049	6	6
SSR	0.057	0.039	-0.011	0.050	1	3
UDA	0.046	0.036	0.008	0.051	2	3
IGA	0.058	0.040	-0.009	0.052	31	3
KOK	0.048	0.031	0.000	0.048	3	2
HSM	0.066	0.040	-0.015	0.057	7	3
Group 2	0.069	0.100	0.103	0.113	107	7
KIT-A	0.033	0.017	0.000	0.033	0	1
TBS-A	0.074	0.044	-0.006	0.070	3	2
TMB	0.063	0.037	-0.011	0.055	3	2
NNT	0.082	0.049	-0.005	0.078	2	2
Group 3	0.072	0.046	-0.014	0.062	12	3
TBS-A	0.070	0.035	0.000	0.070	0	1
TBS-B	0.067	0.039	-0.009	0.061	6	2

4 | DISCUSSION

Community profiling using metabarcoding revealed that most *T. kobensis* individuals consistently associated with a narrow lineage of AM fungi, specifically VTX00113 in the MaarjAM database. This stable association, observed across genetically divergent and geographically distant populations, indicates strong fungal specialization on this mycobiont.

Large-scale studies combining population genetics and fungal preferences in mycoheterotrophic plants remain scarce but provide valuable insights into both conservation genetics and symbiotic specificity. For example, *Epipogium aphyllum*, pollinated by bumblebees and dispersing dust-like seeds by wind, shows high genetic diversity with little geographic structure, likely because gene flow counteracts genetic drift in small, isolated populations (Minasiewicz et al., 2022). Its fungal partners (*Inocybe* spp., occasionally *Hebeloma*) likewise show no spatial structuring, reflecting effective seed dispersal and the wide distribution of their ectomycorrhizal hosts. These results suggest that some mycoheterotrophic plants can maintain stable fungal associations across broad regions, at least when both seed dispersal capacity and fungal host availability are high.

In contrast, *T. kobensis* was expected to show pronounced genetic structure and potentially geographically variable fungal associations, given its limited pollen and seed dispersal via fungal gnats and rain splash, respectively (Coelho et al., 2021; Guo et al., 2019; Suetsugu & Sueyoshi, 2021). Yet most individuals, including those from the easternmost site (Mori population), belonged to a single genetic cluster (Group 1), whereas individuals from northern Kinki formed distinct groups. The two smaller clusters (Groups 2 and 3) were restricted to range margins,

where reduced gene flow may foster mutation accumulation (Yang et al., 2016). Although filtering by minor allele frequency may have lowered detection of private alleles in small groups, Group 2 still harbored a comparable number of private alleles to that of Group 1. These genetic differences may also reflect local adaptation, such as cold tolerance (Zhen & Ungerer, 2008), as *T. kobensis* is the northernmost Asian member of the predominantly tropical genus *Thismia* (Suetsugu et al., 2023).

Although all individuals are classified as *T. kobensis* under current taxonomy, their divergence suggests that this taxon may comprise multiple species under the biological species concept, which defines species as reproductively isolated populations (Mayr, 1992). High F_{ST} values and low migration rates estimated by divMigrate indicate three distinct evolutionary lineages. Mantel tests detected no significant correlation between genetic and geographic distances, even within individual groups. Given some differentiation among populations within the same group, the absence of isolation by distance likely reflects highly localized gene flow that restricts connectivity even among nearby sites. While we do not propose formal taxonomic changes here due to limited sampling of Groups 2 and 3, individuals in Group 2 consistently had a more cuboid or cylindrical stigma than those in Groups 1 and 3. These subtle differences may represent diagnostic traits requiring formal description, a possibility that merits further investigation.

Despite clear genetic divergence, most individuals (especially in Groups 1 and 2) consistently associated with VTX00113, showing that mycoheterotrophic plants may exhibit little geographic variation in fungal partners even under restricted dispersal. In contrast, individuals from the rarest group (Group 3) primarily associated with VTX00295 and VTX00106. Because Group 3 is known from only two

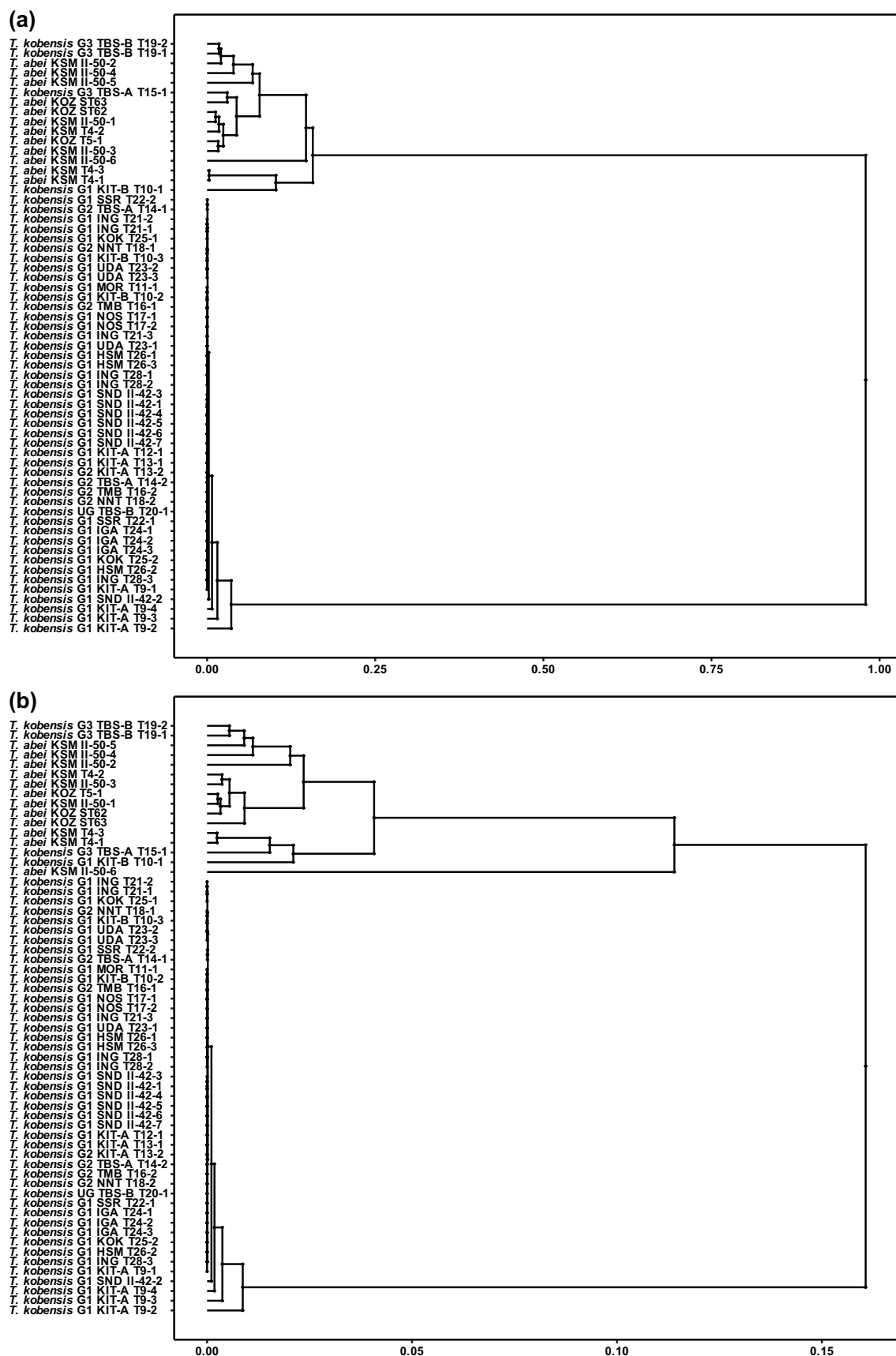


FIGURE 4 Hierarchical clustering of mycorrhizal communities in *Thissmia kobensis* and its close relative *Thissmia abei*, based on the Bray-Curtis dissimilarity index (a) and weighted UniFrac distance matrix (b). G1–G3 correspond to genetic groups identified by MIG-seq. UG indicates samples for which MIG-seq did not resolve genetic groups. Details corresponding to each sample ID are provided in Table S1.

small populations, its mycorrhizal associations remain tentative. One explanation is that it represents an ecotype or cryptic species with distinct host preferences (Jacquemyn et al., 2024; Thompson, 2005). Alternatively, the pattern may reflect environmental filtering by local fungal communities, as Group 3 occurred in evergreen broadleaf forests, whereas *T. kobensis* elsewhere was mainly found in *Cryptomeria japonica* plantations. These mechanisms are not mutually exclusive and may jointly reinforce the distinctness of Group 3.

Given that one Group 1 individual was associated with VTX00295 and VTX00106, whereas all Group 2 individuals, despite similar genetic divergence, were exclusively linked to VTX00113, we infer that fungal availability may influence associations more strongly than host genetics. Nonetheless, distinct mycorrhizal preferences and environmental filtering may act together. Further studies of mycorrhizal communities in additional individuals, combined with surveys of surrounding autotrophic plants and soils, will help clarify the relative roles of environment and host genetics.

Intriguingly, VTX00113, the dominant partner of *T. kobensis*, is also the main associate of its closest relative, *T. huangii* from Taiwan (Merckx et al., 2017), suggesting that their ancestor likely shared this AM fungus and that speciation did not disrupt fungal partnerships. Similarly, four of five closely related *Thismia* species from Australia and New Zealand associate with comparable *Rhizophagus* assemblages (Merckx et al., 2017). Phylogenetic conservatism in mycorrhizal interactions is also evident in *Oxygyne* (Thismiaceae), *Afrothismia* (Afrothismiaceae), *Voyria* (Gentianaceae), and *Epirixanthes* (Polygalaceae) (Bidartondo et al., 2002; Mennes et al., 2015; Merckx & Bidartondo, 2008; Suetsugu et al., 2022). The association with VTX00295 and VTX00106 in some *T. kobensis* individuals may likewise reflect phylogenetic signals, as *T. abei*, the outgroup to *T. kobensis* and *T. huangii*, primarily associates with these fungi but sometimes with VTX00113 (Suetsugu & Okada, 2025a). Notably, VTX00106 and VTX00295 are also the main associates of *Relictithismia kimotsukiensis*, the sole species of the monotypic genus *Relictithismia*. Broader surveys of mycorrhizal associations within section *Labiolithismia*, coupled with ancestral state reconstruction, are therefore needed.

Species with highly specialized ecological interactions are often considered vulnerable to disturbance (Kiers et al., 2010). However, our findings show that *T. kobensis*, despite its high mycorrhizal specificity, can persist in disturbed habitats, including secondary forests and plantations. This adaptability may be enabled by the wide distribution and low habitat specificity of its dominant partner. BLAST searches showed that VTX00113 occurs across temperate and tropical habitats worldwide, including anthropogenic sites such as plantations, lawns, golf courses, road verges, and even polluted areas (Öpik et al., 2010). It has also been reported from moderately disturbed temperate forests in Japan, including secondary forests and conifer plantations (Miyake et al., 2020). However, this VTX is not dominant in *Chamaecyparis* plantations and secondary forests where *T. kobensis* often occurs. In a broad survey of these habitats, VTX00113 appeared in only 5 of 39 plots and ranked 18th in sequence abundance (Miyake et al., 2020). The consistent association with this fungal lineage across *T. kobensis* populations strongly suggests specialization, which may partly limit the ecological resilience of the species.

In summary, this study demonstrates strong genetic divergence within *T. kobensis*, which may justify recognition as multiple species under the biological species concept or, at minimum, as distinct evolutionary units (Groups 1–3). Groups 2 and 3, located at the northern range edge, contain few individuals and populations, highlighting the need for targeted conservation. Most individuals, even from genetically divergent populations, consistently associated with VTX00113, indicating a stable mycorrhizal partnership across genetic and geographic variation. Although the rarest group was mainly linked to VTX00295 and VTX00106, it remains uncertain whether this reflects local fungal availability or partner selection. Further studies, including fungal community surveys in soils and autotrophic plants, as well as artificial crosses among groups and in situ seed baiting, are required to clarify whether mycorrhizal associations contribute to the emergence of distinct evolutionary lineages.

AUTHOR CONTRIBUTIONS

K.S. designed the study and drafted the initial manuscript. K.I., K.T., and K.S. collected materials. A.N., K.S., and S.K.H. performed the laboratory work. K.T., S.K.H., and K.S. analyzed the data. All authors revised and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

MIG-seq data and fungal community data have been deposited in the Sequence Read Archive under Accession Numbers PRJDB37516 and PRJNA1257301, respectively. Additional supporting information is available in the supporting information.

ORCID

Kenji Suetsugu  <https://orcid.org/0000-0002-7943-4164>

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SUPPORTING INFORMATION

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