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Reticulate evolution, introgression, and recent diversification in *Epimedium* sect. *Macroceras*

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ABSTRACT

Hybridization can hinder or promote diversification, and growing genomic evidence suggests that it can facilitate adaptation and speciation. Despite recent progress, however, the quantitative contribution and temporal scope of hybridization to diversification remain poorly understood. The genus *Epimedium* is a recently diverged lineage, and sect. *Macroceras* largely consists of endemic species in Japan that are distributed across diverse environments, including limestone, serpentine, coastal habitats, heavy-snow regions, and regions with mild winters. Although natural hybridization and hybrid species have been reported in this section, molecular evidence demonstrating the contribution of hybridization to lineage diversification is limited. We reconstructed phylogenetic relationships using genome-wide single-nucleotide polymorphism (SNP) data from *Epimedium* sect. *Macroceras* and tested for genomic signatures consistent with hybridization. Phylogenetic analyses suggest that *E. koreanum* from Korea is sister to Japanese *Epimedium* lineages, consistent with an initial colonization of Japan from the Korean Peninsula. The analyses also revealed complex relationships among Japanese species and frequent signals of historical interspecific introgression. Our results are consistent with a history of recent diversification in sect. *Macroceras* accompanied by introgressive hybridization, which may have contributed to diversification across heterogeneous environments in Japan. This study provides the first genome-wide insights into the evolutionary history of *Epimedium* sect. *Macroceras* and reveals complex reticulate relationships among the lineages.

1. Introduction

Hybridization is a "creative evolutionary force" that shapes plant diversity (Soltis and Soltis, 2009). While polyploidization resulting from hybridization is a major driver of speciation, homoploid hybridization also has diverse consequences for species diversification (Schley et al., 2022), ranging from evolutionary novelty (Rieseberg et al., 2007) to lineage extinction due to homogenization (Seehausen et al., 2008; Todesco et al., 2016). Recent genomic studies have increasingly revealed reticulate phylogenies among taxa with the same ploidy, underscoring the role of hybridization in plant diversification (Goulet et al., 2017; Stubbs et al., 2023; Wang et al., 2022).

Interspecific hybridization is more likely at range boundaries, where related taxa come into contact. In such cases, species boundaries between related species are often porous or semipermeable, allowing introgression of foreign alleles (Harrison and Larson, 2014). While hybrids tend to exhibit lower mean fitness (Barton and Hewitt, 1989, 1985), adaptive introgression can occur when introgressed alleles are favored by selection in admixed populations, with accumulating empirical support in plants (Mimura et al., 2024; Rendón-Anaya et al., 2021; Suarez-Gonzalez et al., 2018; Whitney et al., 2010). Such processes may facilitate shifts in ecological differentiation and persistence under changing environmental conditions.

Hybridization can also lead to homoploid hybrid speciation, which is the origin of a new species via hybridization without genome duplication (Abbott et al., 2013; Mallet, 2007; Rieseberg, 1997; Soltis and

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Soltis, 2009). Given that reproductive isolation in homoploid hybrids must arise without the instant ploidy barrier typical of allopolyploidy, homoploid hybrid speciation has often been viewed as relatively rare and contingent on a narrow set of ecological and genomic conditions (Blanckaert and Bank, 2018; Buerkle et al., 2000; Schumer et al., 2014). Nonetheless, accumulating syntheses and case studies indicate that hybrid lineages can attain ecological or reproductive distinctiveness (Lexer and Widmer, 2008; Rieseberg et al., 2003; Yakimowski and Rieseberg, 2014). Theoretical and empirical studies suggest that hybridization can facilitate adaptive radiation by generating novel trait combinations and expanding ecological opportunities (Kagawa and Seehausen, 2020; Seehausen, 2004). However, how often hybridization contributes to diversification in homoploid lineages and which ecological and genomic factors govern this process remain poorly understood (Peñalba et al., 2024).

Epimedium Tourn. ex L. is a relatively young genus that has undergone recent radiation, with an estimated divergence time of approximately 2.11 Mya (Guo et al., 2022; Zhang et al., 2023). It includes about 62 species and is the largest herbaceous genus in Berberidaceae (Stearn, 2002; Zhang et al., 2022). This genus is a taxonomically challenging group because of its high morphological inter- and intraspecific variation (Xu et al., 2019; Zhang et al., 2022). Because *Epimedium* sect. *Macroceras* exhibits self-incompatibility and weak intrinsic reproductive isolation (Suzuki, 1983), these taxonomic difficulties may reflect processes such as shared ancestral polymorphisms resulting from recent diversification and/or hybridization.

Epimedium sect. *Diphyllon*, mainly distributed in China, is the largest section in the genus (about 51 species in central-southeastern China) and has been suggested to have undergone recent evolutionary radiation, with divergence estimated at approximately 1.14 Mya (Guo et al., 2022; Zhang et al., 2022). *Epimedium* sect. *Macroceras* is another group (seven species) distributed primarily in Japan and the neighboring regions of East Asia. The separation of this section from the rest of the genus is thought to have occurred later than that of sect. *Diphyllon* (Zhang et al., 2007). Despite its recent diversification, sect. *Macroceras* occurs across a variety of environments in Japan, including limestone, serpentine, and coastal slopes. Some species of sect. *Macroceras* are thought to be of hybrid origin based on morphological studies. For example, *E.* × *setosum* Koidz. (or *E. setosum*), occurring mainly on limestone soils in western Honshu, has been proposed to represent a hybrid species based on morphological polymorphism and intermediate traits between *E. diphyllum* Lodd. ex Graham and *E. sempervirens* Nakai ex F.Maek (Horie et al., 2012; Kurosaki, 1981; Maekawa, 1955). Another example is *E. trifoliolatobinatum* (Koidz.) Koidz. (orth. corr. of *E. trifoliatobinatum* under ICN Art. 60), which has been suggested as a hybrid-origin species between *E. diphyllum* and *E. grandiflorum* C.Morren (Maekawa, 1955; Suzuki, 1982, 1978). The subspecies of *E. trifoliolatobinatum* (subsp. *maritimum*) is distributed exclusively on coastal slopes with occasional seawater splashes (Suzuki, 1982), whereas *E. trifoliolatobinatum* var. *trifoliolatobinatum* is often found in serpentine soils (Suzuki, 1990). These ambiguous morphological variations may reflect relatively frequent hybridization and recent diversification within this taxonomic group. However, molecular evidence for hybridization or introgression in sect. *Macroceras* remains limited, and phylogenetic relationships within the section remain poorly resolved.

In this study, we aimed to clarify patterns of lineage divergence and evaluate genomic signatures consistent with introgression in *Epimedium* sect. *Macroceras* in Japan. Specifically, we aimed to (1) infer genome-wide phylogenetic relationships among and within currently recognized species and reconstruct genomic lineage relationships; (2) estimate divergence times among major lineages; (3) examine how phylogenetic patterns correspond to morphological differentiation and habitat variation; and (4) test for genomic signatures consistent with introgression among genetically inferred lineages while confirming the homoploid status of these taxa. To address these objectives, we used genome-wide single nucleotide polymorphisms (SNPs) to evaluate whether the evolutionary history of sect. *Macroceras* is consistent with gene flow, recognizing that recent divergence can blur the distinction between incomplete lineage sorting and introgression. Given the taxonomic complexity of *Epimedium* and shallow genomic divergence, we distinguished between traditionally recognized taxonomic species used for phylogenetic and population structure analyses and operational genomic lineage groups used in downstream analyses.

2. Material and methods

2.1. Plant materials

We collected samples from 54 localities in Japan and two localities in Korea, representing ten taxa from six species of sect. *Macroceras*, including subspecies and varieties (Table S1, Fig. 1). Six of the seven species of sect. *Macroceras* are distributed in Honshu, Kyushu, Shikoku Islands of Japan, and four of them are endemic to Japan. Most Japanese *Epimedium* (sub)species are distributed in western Japan. The taxonomy of *Epimedium* is complex. For example, *E. grandiflorum* has three varieties with distinct distributions: var. *thunbergianum*, mainly in eastern Japan, var. *grandiflorum* in western Japan (western Honshu and Shikoku) and var. *higoense* in Kyushu, Japan. *Epimedium sempervirens* mainly occurs along the Japan/East Sea. Although white-flowered plants are sometimes treated as var. *rugosum*, we treated all as *E. sempervirens*. *Epimedium diphyllum* subsp. *diphyllum* is distributed in Shikoku and Kyushu, and *E. diphyllum* subsp. *kitamuranum* is found in eastern Shikoku, where some populations are morphologically polymorphic. *Epimedium trifoliolatobinatum* var. *trifoliolatobinatum* is found almost exclusively in serpentine soils in western Japan, and its subspecies, *E. trifoliolatobinatum* subsp. *maritimum*, is found in limited locations along the coasts of western Shikoku and eastern Kyushu. *Epimedium* × *setosum* is also distributed in a relatively limited area in western Japan. *Epimedium koreanum*, with cream-yellow petals, is mainly distributed in eastern and northern mountainous sites in Japan. Two allopatric populations of *E. koreanum* from the Korean Peninsula were also included in this study. In addition, we included *E. sagittatum* (Siebold and Zucc.) Maxim. as an outgroup species in sect. *Diphyllon*. The remaining species of sect. *Macroceras*, *E. macrosepalum* Stearn, is distributed in eastern Russia (Zhang et al. 2022) and could not be included in this study. Species identification was based on the traditionally recognized morphological characteristics and geographic distributions. Leaflet and flower morphologies were observed at the collection sites or in the greenhouse from fully mature and expanded shoots and flowers. Leaflet types were categorized as described by Horie et al. (2012). Habitats were categorized as limestone soil, serpentine soil, coastal areas, or other habitats based on field observations at each sampling site and geological information obtained from Geological Map Navi (the Geological Survey of Japan, AIST). The mean annual temperature (MAT) of the localities was estimated using ClimateAP (Wang et al., 2017).

2.2. Draft sequences of Epimedium

The draft genome of *E. diphyllum* was sequenced using a short-read library with an Illumina NovaSeq 6000 (Illumina Inc.) and HiFi sequencing with Sequel IIe (PacBio, Inc.). All filtered reads were used to estimate the genome size (3.82 Gb) using Genomescope v2.0 (Vurture et al., 2017) and genome consensus assembly using hifiasm v0.19.8 (Cheng et al., 2021). We employed the consensus assembly method, which resulted in better results (longer N50) than the phased assembly method. The final draft genome sequence contained a total of 3,908,879,247 bp with 780 contigs and an N50 of 8.8 Mbp. The coverage of core genes was 94.6% (single-copy BUSCOs: 87.4%; duplicated BUSCOs: 7.2%), based on BUSCO v5.7.1 (Simão et al., 2015). The draft genome was annotated using GINGER (Taniguchi et al., 2023). We used RNA-seq-based (mapped and *de novo*), homology-based, and ab initio-based methods with three RNA-seq datasets from flower, leaf, and

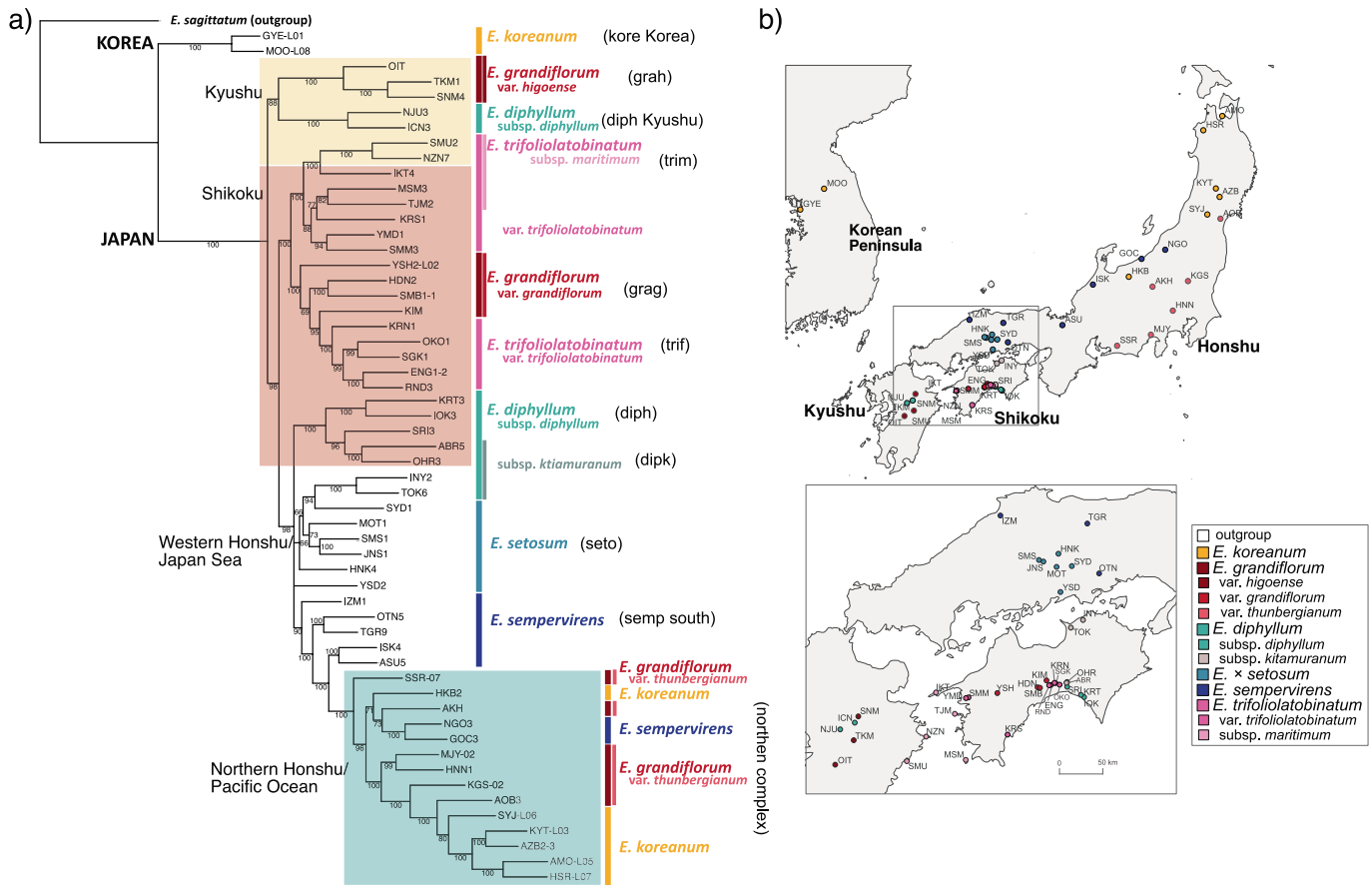

Fig. 1. Phylogenetic tree and sampling locations of genus *Epimedium* sect. *Macroceras*. (a) 50% majority-rule maximum-likelihood tree inferred using IQ-TREE2 under MFP + ASC (ModelFinder with ascertainment-bias correction). Node labels indicate UFBoot support (%) based on 1,000 replicates. The lineage groups used in subsequent analyses are indicated in parentheses. (b) Sampling localities for all specimens except *E. sagittatum*. Map labels show population names only, whereas species identity is indicated by the color scheme used in panel (a).

root samples of *E. diphyllum* collected in this study. This resulted in 33,206 genes, an average of 4.59 exons per gene, and an average CDS length of 1,127.4 bp, with a BUSCOs completeness of 94.5%.

2.3. SNP detection using GRAS-Di

SNP genotype data were obtained using Genotyping by Random Amplicon Sequencing-Direct (GRAS-Di) technology (Toyota Motor Corporation, Japan). GRAS-Di was used to obtain genome-wide SNP data without requiring prior genomic resources (Enoki and Takeuchi, 2018). This PCR-based reduced-representation approach uses random primers to amplify numerous loci across the genome, enabling the recovery of thousands of SNPs. Genome-wide SNP datasets generated using this approach have been widely used to resolve shallow phylogenetic relationships and population genetic structure in non-model organisms (Hosoya et al., 2019; Ikeda et al., 2020). Plant DNA was extracted from fresh leaves for SNP detection using DNeasy Plant Kit (QIAGEN, Germany). Paired-end GRAS-Di libraries were sequenced on an Illumina NovaSeq platform (2 × 150 bp; Illumina Inc.). Sequencing data were quality-checked and filtered for adapters and low-quality bases using Trimmomatic ver. 0.39 (Bolger et al., 2014) with the following parameters: ILLUMINACLIP:2:30:10, LEADING:20, TRAILING:20, SLIDINGWINDOW:4:15, and MINLEN:35. The filtered reads were mapped to the draft genome sequences of *E. diphyllum* using BWA (Li and Durbin, 2009) with default settings. We then stacked the mapped reads from all individuals and extracted SNPs using the Stacks 2.41 pipeline (Catchen et al., 2013; Rochette et al., 2019), with a maximum allowed difference of M = 2 and a maximum of two mismatched sites when merging the putative loci of each sample (n = 2). We then filtered the SNPs using the 'populations' module in Stacks v2.41. Genotypes with a read depth < 10 were treated as missing. We retained loci present in at least 80% of the samples, with a minimum allele count of 3 and a maximum observed heterozygosity of 0.60. To ensure that only biallelic SNPs were retained, we further filtered the dataset using bcftools v1.21 (Danecek et al., 2021), resulting in 23,169 SNPs. The final filtered SNP array was used for subsequent analysis.

For analyses requiring approximately independent markers, we generated LD-pruned SNP datasets using PLINK v1.9 (Chang et al., 2015) by removing SNPs in high linkage disequilibrium ($r^2 > 0.2$) with a sliding window of 50 SNPs, advanced by 20 SNPs. Because SNP density can be uneven in reduced representation data, LD pruning was performed using a variant count window. Two LD-pruned datasets were generated for different analyses. First, a dataset including the outgroup (*E. sagittatum*), which belongs to a different section, was used for species tree inference with SVDQuartets and divergence time estimation using SNAPPER. This dataset contained 10,576 SNPs. Second, for genetic structure and introgression analyses, the outgroup was excluded prior to LD pruning using the same parameters, resulting in 10,627 SNPs. This dataset was used for SplitsTree4, PCA/ADMIXTURE, *f*-branch statistics and TreeMix analyses. LD pruning was performed separately for each dataset to ensure that marker independence was evaluated within the set of taxa used in each analysis.

2.4. Phylogenetic inference and genetic structure

Maximum likelihood phylogenetic trees were constructed using IQ-

TREE2 v2.4.0 (Minh et al., 2020) with *E. sagittatum* (sect. *Diphyllon*) used as the outgroup. All concatenated SNP data were used to infer the phylogeny. The best-fit substitution model was selected using ModelFinder implemented in IQ-TREE2 with Ascertainment Bias Correction (MFP + ASC). Branch support values for the nodes were calculated using 1,000 ultrafast bootstrap replicates.

Principal component analysis (PCA) was performed using the R package SNPRelate based on the LD-pruned SNP dataset after removing the outgroup species to visualize the major axes of genetic variation among individuals. Individuals were plotted along the first two principal components (PC1 and PC2). Population structure was further inferred using ADMIXTURE v1.3.1 (Alexander et al., 2009) with the same dataset. We tested K values from 1 to 10 with 10 independent runs for each K, using different random seeds. Cross-validation error was estimated using 10-fold cross-validation. The optimal K was determined based on the lowest cross-validation error across replicate runs.

2.5. Definition of genomic lineages for analysis

Maximum-likelihood phylogenetic reconstruction revealed several instances of non-monophyly within traditionally recognized species (Fig. 1). Because downstream analyses, such as lineage tree inference and gene flow tests, depend on the definition of analysis units, we avoided enforcing taxonomic species monophyly. Instead, we defined operational genomic lineages primarily based on the major phylogenetic structure recovered in the ML tree and concordant clustering patterns, without requiring strict reciprocal monophyly. Taxa from northern Japan lacking clear genomic differentiation were treated as a single operational lineage ("northern complex"), including *E. grandiflorum* var. *thunbergianum* (grat), northern *E. sempervirens* (semp), and Japanese *E. koreanum* (kore). Southern *E. sempervirens* was treated separately (semp south) because it is paraphyletic and recovered as sister to the northern complex. In contrast, *E. grandiflorum* var. *higoense* (grah) and var. *grandiflorum* (grag) were treated as separate lineages because *E. grandiflorum* was polyphyletic. The remaining taxonomic units were *E. diphyllum* subsp. *diphyllum* (diph), subsp. *diphyllum* from Kyushu (diph Kyushu) and subsp. *kitamuranum* (dipk), *E.* × *setosum* (seto), *E. trifoliolatobinatum* var. *trifoliolatobinatum* (trif) and subsp. *maritimum* (trim), and *E. koreanum* from Korea (kore Korea). The final operational genomic lineages used were kore (Korea), diph, diph (Kyushu), dipk, seto, semp (south), trif, trim, grah, grag, and northern complex. These units were used for SVDQuartets with SNAPPER time-calibration, *f*-branch statistics, TreeMix, and BioGeoBEARS analyses. This operational definition was used solely for genomic analyses and does not represent a formal taxonomic revision.

2.6. Lineage tree with divergent-time estimation

We inferred the lineage tree from the pruned SNP dataset using SVDQuartets implemented in PAUP* v4.0a (Chifman and Kubatko, 2014) under the multispecies coalescent model. We used the lineage groups defined above, rather than enforcing strict species monophyly. All quartets were evaluated using the dataset including the outgroup species (*E. sagittatum*), and node support was assessed using 200 nonparametric bootstrap replicates. In addition, an unrooted phylogenetic network was estimated using the Neighbor-Net method implemented in SplitsTree4 (Huson and Bryant, 2006), based on uncorrected P distances calculated from the SNP dataset, including ambiguous state codes (i.e., heterozygous sites).

Divergence times were estimated under the multispecies coalescent using SNAPPER implemented in BEAST v2.7.7 (Bouckaert et al., 2019), which directly models SNP data. The lineage tree topology inferred from SVDQuartets was fixed during the analysis, and a Yule speciation prior was used. Because SNAPPER is computationally intensive, we used a reduced dataset of 886 high-quality biallelic and unlinked SNPs with minimal missing data. Time calibration was implemented using a secondary calibration on the stem node of the focal lineage with a lognormal prior (median = 2.11 Ma, 95% interval = 1.88–2.35 Ma), corresponding to a previously reported divergence estimate (Guo et al., 2022). Two independent MCMC runs were conducted with different random seeds, each run for 500,000 MCMC steps, with parameters and trees sampled every 1,000 steps. Convergence and mixing were assessed using Tracer v1.7.2 (Rambaut et al., 2018). After removing the first 20% of the samples as burn-in, the two runs were combined. All parameters showed effective sample sizes (ESS) greater than 300 in each run and > 600 in the combined dataset. A maximum clade credibility (MCC) tree with mean node heights was generated using TreeAnnotator, implemented in BEAST v2.7.7.

2.7. Introgression across species

To elucidate the evolutionary relationships and introgression within the phylogenetic tree, we first inferred a maximum likelihood tree using TreeMix v1.13 (Pickrell and Pritchard, 2012) based on the pruned SNP dataset. Because the sect. *Diphyllon* species (*E. sagittatum*) showed a high proportion of missing SNP genotypes in the GRAS-Di dataset, it was excluded from this analysis. Instead, *E. koreanum* from the Korean Peninsula, which represents the lineage sister to all Japanese *Epimedium* species in the phylogenetic tree, was used as the outgroup. Previously defined lineage groups (Section 2.5) were used for this analysis. We first ran ten bootstrap replicates for each model, with the number of migration edges (m) ranging from 1 to 15. The optimal number of migration edges was estimated using the Evanno method in the R package OptM (Fitak, 2021). TreeMix analysis was performed using 500 bootstrap replicates with an optimum number of migration edges. Bootstrapped lineage group trees were generated by resampling the pruned SNPs, with each replicate re-optimized under the maximum likelihood framework implemented in TreeMix. Allele frequencies for each lineage group were calculated from available genotypes, and missing data were ignored when estimating allele counts. A consensus tree of the bootstrap replicates was constructed using CONSENSE implemented in PHYLIP v3.697 (Felsenstein, 2014). We used BITE v1.2.0008 (Milanesi et al., 2017) to visualize migration edges inferred by TreeMix and to plot the residual covariance matrix.

We also calculated f_b statistics based on Patterson's *D* statistics (Patterson et al., 2012) using Dsuite v0.5 (Malinsky et al., 2021) to test for deviations from a strictly bifurcating species tree that may be consistent with introgression among lineages while accounting for incomplete lineage sorting (ILS). *D* statistics were calculated using the ABBA–BABA framework for sets of three populations or taxa and one outgroup (i.e., ((P1,P2),P3),outgroup) (Durand et al., 2011). We used the same pruned SNP data and outgroup (*E. koreanum* from Korea) as in the TreeMix analysis. Individuals were grouped according to the operational genomic lineages defined in Section 2.5, and allele frequencies were calculated from available genotypes while ignoring missing data. All possible trios of lineage groups were tested based on the lineage tree inferred using SVDQuartets.

2.8. Ancestral range estimation

Ancestral ranges were estimated using BioGeoBEARS implemented in R (Matzke, 2014), based on the lineage groups defined above. Lineage distributions were coded across seven geographic regions (A–G). Each taxon was assigned to a single area based on its current distribution. Although extant taxa occupy single areas, widespread ancestors are plausible; we set the maximum range size to three (max_range_size = 3) as a conservative constraint and verified that results were qualitatively robust to the model with a larger range size (max_range_size = 7). We fitted the DEC, DIVALIKE, and BAYAREALIKE models, as well as their "+J" models that allow founder-event speciation. Model fit was compared using AICc weights. Ancestral range estimates were conducted using the time-calibrated lineage tree.

2.9. Estimation of genome size and polyploidy

To test for homoploidy, the genome sizes of the 10 accessions collected in Japan were compared using flow cytometry (Quantum P Flow Cytometer, Quantum Analysis). Young and fresh leaves were cut and stained with standard DAPI (4′,6-diamidino-2-phenylindole) using Quantum Stain NA UV2 (Quantum Analysis). All measurements were processed with the internal standard of diploid *Calanthe discolor* LindI. (6.36 Gb/1C) at CytoTechs Inc., Japan. In addition, to assess ploidy across all individuals using sequencing data, allele depth ratios were calculated for heterozygous biallelic SNPs from the GRAS-Di dataset. SNPs were filtered by read depth (DP $\geq$ 12) and genotype quality (GQ $\geq$ 20). Allele frequency (AF) and minor allele fraction (MAF) distributions were examined for each individual. The absence of distinct peaks at 0.25 or 0.75 was interpreted as consistent with diploidy.

3. Results

3.1. Phylogenetic structure of sect. Macroceras

Genome-wide SNP data provided a well-resolved overview of relationships within *Epimedium* sect. *Macroceras*. The mean genotype missing rate across individuals in sect. *Macroceras* was 0.104, whereas the outgroup species *E. sagittatum* showed a much higher missing genotype rate (0.783; Table S2). Phylogenetic analyses consistently revealed a clear geographic structure among the Japanese lineages (Fig. 1). *Epimedium koreanum* from the Korean Peninsula was recovered as the lineage sister to all Japanese lineages, whereas Japanese individuals identified as *E. koreanum* clustered with other Japanese species.

Among the Japanese taxa, three major clades were identified. One was recovered as sister to the remaining Japanese species and comprised only taxa distributed in Kyushu, including *E. grandiflorum* var. *higoense* and *E. diphyllum* subsp. *diphyllum* (Fig. 1). The remaining two clades were the western and eastern-northern clusters. The western clade included *E. trifoliolatobinatum* and *E. grandiflorum* var. *grandiflorum*, which are mostly distributed on Shikoku Island. The eastern-northern clade consisted of *E. diphyllum*, *E.* × *setosum*, *E. sempervirens*, *E. koreanum,* and *E. grandiflorum* var. *thunbergianum*. Some morphologically identified species formed polytomy in the phylogenetic tree. *Epimedium diphyllum* subsp. *diphyllum* formed a polytomy (Kyushu and Shikoku lineages). *Epimedium grandiflorum* formed a polytomy among its three varieties, which broadly corresponded with their geographic distributions. In particular, *E. grandiflorum* var. *thunbergianum*, Japanese populations of *E. koreanum*, and northern individuals of *E. sempervirens* formed a clade (hereafter referred to as the "northern complex"; see Methods 2.5).

Phylogenetic network analysis, excluding the outgroup, further revealed reticulate relationships among species of sect. *Macroceras* (Fig. 2a). The overall structure was broadly consistent with the ML tree, although the network suggested a closer relationship between Korean *E. koreanum* and the northern complex than that indicated by the ML phylogeny. Species identified based on morphology largely clustered together, although some taxa appeared polyphyletic, and individuals tended to cluster according to their geographic proximity. Principal component analysis (PCA) also revealed a pattern that was broadly consistent with the ML phylogeny and network (Fig. 2b). In contrast, ADMIXTURE analysis detected little structure, with K = 1 showing the lowest cross-validation error, although genetic structure was observed when K $\geq$ 2 (Fig. S1).

3.2. Morphological and environmental variation

The morphologies of the leaflet and floral spur varied among the species distributed in western Japan (Fig. 3). Floral spurs were present in most species and lineages, including *E. koreanum* from the Korean Peninsula, whereas spur loss was observed in a subset of the Japanese western lineages. Species occurring in eastern and northern Japan, including the northern complex lineages, share the same leaflet architecture, with typically biternate compound leaves. This leaflet architecture is also observed in *E. grandiflorum* var. *higoense* and var. *grandiflorum* inwestern Japan, including Kyushu, and in Korean *E. koreanum*. Mapping the mean annual temperature of the sampling locations onto the phylogeny revealed that Japanese *Epimedium* species likely originated in colder environments and subsequently expanded into warmer habitats. Some of these lineages occur in distinct habitats, including serpentine soils and coastal areas.

3.3. Lineage tree inference

The species tree (i.e., lineage tree) inferred using SVDQuartets for the 11 genomic lineage groups and an outgroup (Fig. 4) was partially discordant with the phylogenetic tree inferred using the ML method. In the lineage tree, the northern complex was recovered as sister to the

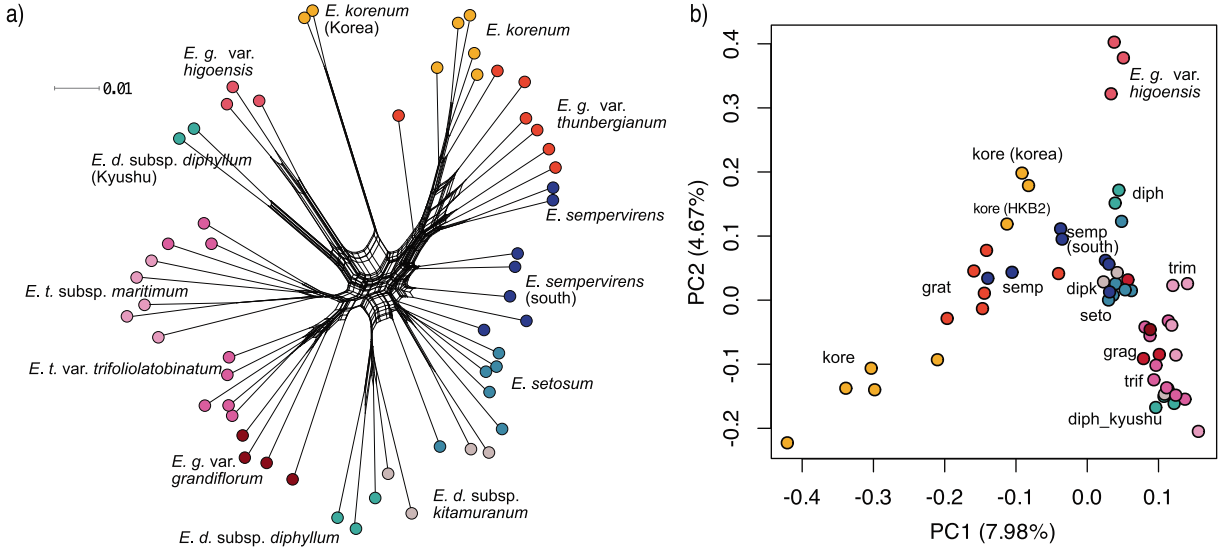

Fig. 2. Genetic structure of sect. *Macroceras* based on genome-wide SNPs. (a) Unrooted phylogenetic network inferred using the Neighbor-Net method implemented in SplitsTree4. (b) Principal component analysis (PCA) based on genome-wide SNPs. Colored circles represent species or subspecies (see Fig. 1a).

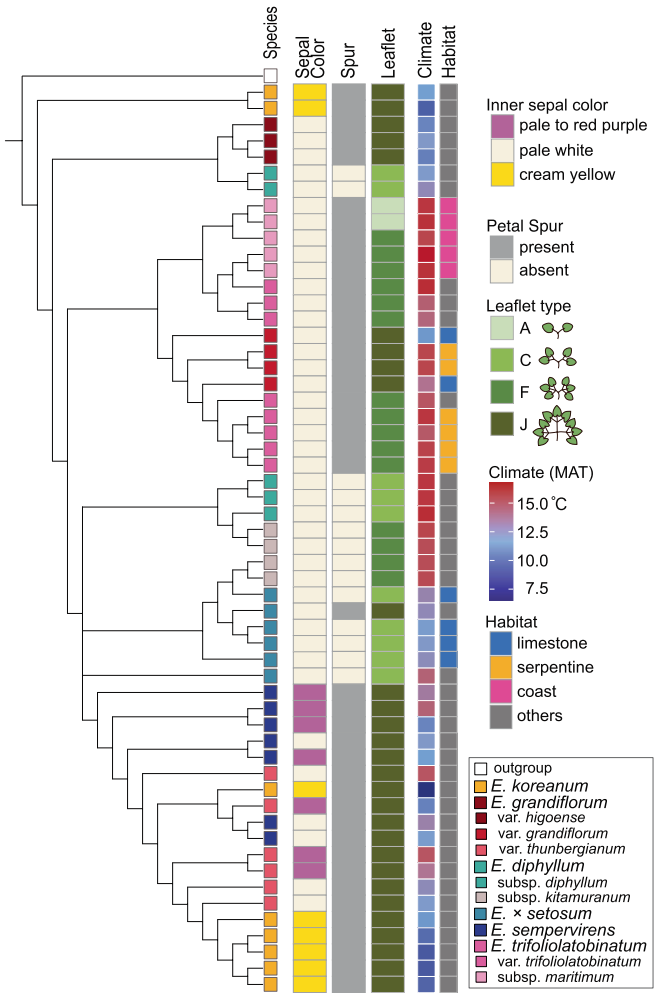

Fig. 3. Phylogenetic distribution of morphological traits and habitat characteristics. Morphological data were obtained from field observations and greenhouse surveys. Leaflet types follow Horie et al. (2012). Climate variables were extracted from ClimateAP (Wang et al., 2017). Habitat categories include soil type (limestone or serpentine), coastal habitats, and other habitat types.

remaining Japanese *Epimedium*. This pattern is consistent with the network analysis, in which *E. koreanum* from Korea and individuals belonging to the northern complex showed a close relationship. The overall topology was otherwise similar to the ML phylogenetic tree, except for southern *E. sempervirens*. In the lineage tree, southern *E. sempervirens* appeared to be more closely related to *E. diphyllum* and *E.* × *setosum* than to the northern complex, although this clade received a relatively low level of support. Divergence-time estimation using SNAPPER indicated that the split between the Korean and Japanese lineages of sect. *Macroceras* occurred at approximately 1.31 Mya, and diversification of Japanese *Epimedium* began around 0.95 Mya (Fig. 4).

Among the candidate models tested in BioGeoBEARS, the DIVALIKE + J model was selected as the best-fitting model based on the AICc using the SVDQuartets lineage tree (Table S3). Increasing the maximum range size to seven did not affect the results (data not shown). The ancestral range reconstruction did not clearly resolve whether the most recent common ancestor of the Japanese clade was located in northern Japan or Kyushu Island (Fig. S2). At the root of the Japanese lineage, multiple areas, including the Korean Peninsula, northeastern Japan, and Kyushu, showed comparable probabilities, indicating uncertainty in the early biogeographic history of Japanese lineages. However, BioGeoBEARS reconstructs ancestral ranges based on a bifurcating phylogeny. Subsequent analyses revealed evidence of introgression among lineages, suggesting that the evolutionary history of sect. *Macroceras* cannot be fully explained by a simple bifurcating tree.

3.4. Introgression and hybridization among lineages

The optimum number of migration edges was estimated to be four, and historical introgression events were detected using TreeMix (Fig. 5a, Fig. S3). The model with four migration edges inferred introgressions: (1) between Korean *E. koreanum* and the northern complex, (2) between Korean *E. koreanum* and an ancestral node of *E. sempervirens* and the northern complex, (3) between an ancestral branch of the northern complex and *E. grandiflorum* var. *grandiflorum*, and (4) between an ancestral branch of *E. diphyllum* and an ancestral branch of *E. trifoliolatobinatum* var. *trifoliolatobinatum* and *E. grandiflorum* var. *grandiflorum*. The TreeMix species tree based on 500 bootstrap replicates was generally concordant with the ML phylogenetic tree estimated above. Residuals from the TreeMix analysis suggested additional potential introgression, for example, between *E. trifoliolatobinatum* subsp. *maritimum* and *E. diphyllum* in Kyushu (Fig. S4).

We also found significant introgression among Japanese *Epimedium* species based on *D* statistics (*D* values ranging from 0.002 to 0.247 and

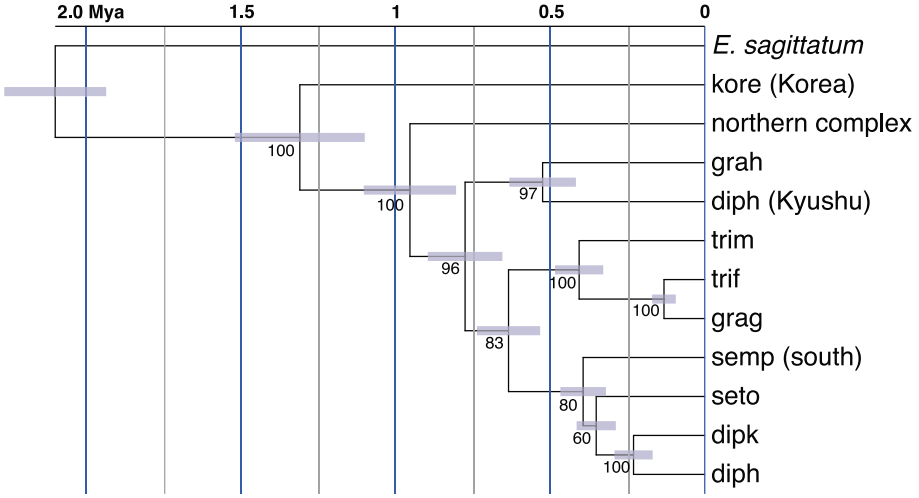

Fig. 4. Time-calibrated lineage tree of sect. *Macroceras*. The topology was fixed to the lineage tree inferred using SVDQuartets, and divergence times were estimated using SNAPPER. Node bars indicate 95% highest posterior density (HPD) intervals for node ages, and numbers at nodes indicate quartet support (%) from the SVDQuartets analysis. The abbreviations for the lineage groups are shown in Fig. 1a.

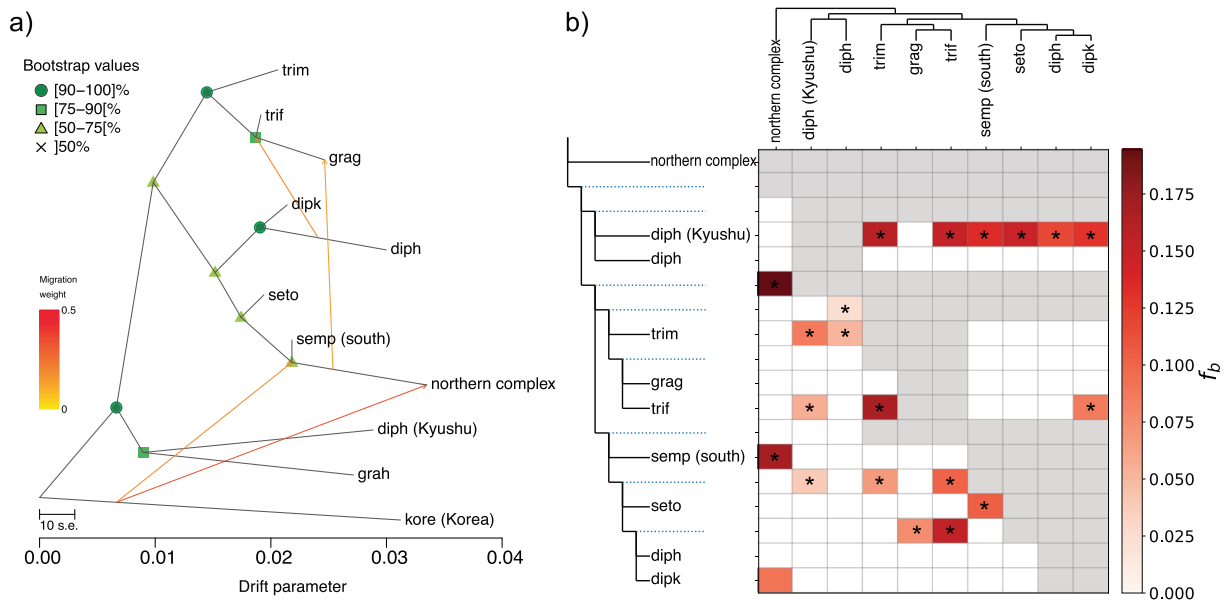

Fig. 5. Migration edges (TreeMix) and *f*-branch statistics (Dsuite) from the pruned SNPs. (a) Estimated migration edges (m = 4) inferred by TreeMix on the consensus tree. The consensus tree was constructed from 500 bootstrap replicates using CONSENSE (PHYLIP v3.697). The results for alternative numbers of migration edges are shown in Fig. S4. (b) *f*-branch (f_b) heat map from Dsuite based on the lineage tree inferred using SVDQuartets. Cells represent excess allele sharing between lineage branches. Colors indicate f_b values; nearly all colored cells have $|Z| \geq 3$ (marked with '*'). The abbreviations for lineage groups follow Fig. 1a.

Z-scores ranging from 0.06 to 9.00). The *f*-branch statistic (f_b) was used to quantify signals of gene flow associated with specific branches of a phylogenetic tree, summarizing multiple *D*- and f_4-ratio tests. While the major gene flow signals inferred by the *f*-branch statistic were broadly concordant with the TreeMix results, the *f*-branch analysis suggested more widespread gene flow among species (Fig. 5b). For instance, *E. diphyllum* in Kyushu showed introgression with most of the western lineages.

3.5. Genome size and polyploidy

A draft genome assembly of *Epimedium* estimated a genome size of approximately 3.82 Gb. Based on flow cytometry, the genome sizes were broadly similar across the ten Japanese *Epimedium* taxa (Table S4). Interspecific differences in genome size reached approximately 9%, with *E. trifoliolatobinatum* subsp. *maritimum* having the largest genome size. Minor allele frequency (MAF) distributions were examined to assess potential polyploidy. Across all *Macroceras* individuals in this study, the allele frequency distribution showed a single dominant peak at approximately 0.5 (Fig. S5).

4. Discussion

4.1. Reticulate phylogeny with introgression of Epimedium sect. Macroceras in Japan

We present, to our knowledge, the first genome-wide SNP survey of relationships within sect. *Macroceras*, a group of herbaceous species widely distributed across the Japanese archipelago. Although the ML tree and phylogenetic network largely recovered clusters corresponding to morphologically identified taxa, some species were polyphyletic and showed evidence of reticulation. This pattern indicates partial incongruence between morphology and phylogeny. The phylogenetic relationships among Japanese *Epimedium* species were strongly structured geographically, with individuals from nearby regions tending to cluster together regardless of their morphological differences. Rapid diversification, followed by secondary contact and hybridization, may have contributed to the reticulate evolutionary patterns and morphological polymorphism in sect. *Macroceras*, contributing to the long-recognized taxonomic complexity of this genus (Wang et al., 2017; Zhang et al., 2022). Such reticulate patterns are common in recently diverged lineages owing to processes including hybridization, incomplete lineage sorting, and population demography (Naciri and Linder, 2015).

Our divergence time estimates suggest that the split between the Korean and Japanese *Epimedium* lineages occurred approximately 1.31 Mya, whereas diversification within Japan began approximately 0.95 Mya. These estimates broadly coincide with the Mid-Pleistocene Transition. The divergence time estimates should be interpreted with caution because our calibration did not rely on fossil constraints, and the geographic origin of the Japanese lineages remains uncertain. Therefore, the estimated dates should be regarded as approximate time frames rather than precise estimates of divergence. Nevertheless, our estimates broadly overlap with those reported in previous studies: diversification within sect. *Diphyllon* was estimated to have begun around 1.14 Mya (Guo et al., 2022). This temporal overlap suggests that diversification in *Epimedium* may have occurred around the Mid-Pleistocene Transition, when glacial cycles became longer and more severe (Chalk et al., 2017). Climatic oscillations during this period likely promoted repeated range shifts, secondary contact, and hybridization among populations. Although fossil evidence does not indicate increased speciation rates during the Quaternary (Willis and Niklas, 2004), molecular evidence suggests that climatic fluctuations during this period facilitated diversification in many plant lineages, particularly in mountainous regions and in herbaceous species (Kadereit and Abbott, 2021). Repeated glacial–interglacial cycles may therefore have contributed to the geographic structure and reticulate evolutionary history observed in Japanese *Epimedium*.

The geographic origin of Japanese sect. *Macroceras* within the archipelago remains uncertain. The SVDQuartets lineage tree recovered the northern complex as sister to the remaining Japanese lineages, whereas the ML tree placed the Kyushu lineage as sister to the remaining Japanese clade. Such incongruence among phylogenetic analyses may reflect incomplete lineage sorting or historical introgression, both expected in recently diverged lineages, but may also be influenced by uncertainties related to lineage delimitation and sampling design. Nevertheless, the Kyushu lineage was consistently recovered in a position close to the root of the Japanese clades across the ML tree, the TreeMix consensus tree, and the phylogenetic network, suggesting that

western Japan may have contributed to the early diversification of sect. *Macroceras*.

This close relationship between the Korean and Kyushu lineages is consistent with a western entry into Japan via the Korean Peninsula, providing a possible explanation for the observed phylogenetic patterns. Under this scenario, an ancestral lineage from the Korean Peninsula may have migrated into the region that is now Kyushu when land bridges formed during glacial periods. Subsequent climatic oscillations during the Pleistocene may have facilitated repeated range shifts within the archipelago, creating opportunities for secondary contact and hybridization among diverging populations. Fig. 6 illustrates a hypothetical scenario inferred from the integration of phylogenetic relationships, divergence time estimates, and patterns of introgression, consistent with recent diversification and reticulate evolution. In this scenario, descendants of ancestral Kyushu populations expanded northward during favorable periods and diversified across Honshu and Shikoku, whereas relict populations persisted in the mountainous refugia of Kyushu. Because intrinsic reproductive isolation is nearly absent among these species (Sheng et al., 2011; Suzuki, 1983), admixture may have repeatedly occurred when populations came into secondary contact within the complex topography of the Japanese archipelago. Signals of multiple introgression events detected by TreeMix and *f*-branch statistics are consistent with such repeated contacts, although the precise direction and timing of gene flow remain uncertain under complex demographic histories (Fitak, 2021; Molloy et al., 2021; Pickrell and Pritchard, 2012). Several alternative processes may also contribute to the phylogenetic discordance observed in this study, including incomplete lineage sorting, potential taxonomic over-splitting, and operational grouping effects (i.e., lineage groups). These factors may obscure species relationships and contribute to the observed reticulate phylogeny.

Taken together, our genome-wide analyses revealed a complex evolutionary history of *Epimedium* sect. *Macroceras* in Japan, characterized by geographic structuring, reticulate relationships among lineages, and relatively recent diversification during the Pleistocene. Although morphologically defined taxa often correspond to genetic clusters, repeated secondary contact and hybridization among geographically structured populations appear to be associated with reticulate phylogenetic patterns and morphological polymorphism. The timing of diversification broadly coincides with intensified glacial–interglacial cycles around the Mid-Pleistocene Transition, suggesting that climatic oscillations may have contributed to range shifts and recurrent population contacts.

4.2. Complexity of taxonomic status in sect. Macroceras

Molecular phylogenetic analyses revealed discordance with morphology-based taxonomy in Japanese *Epimedium*. For example, *E. grandiflorum*, which is widely distributed in Japan, Korea, and China (Zhang et al., 2022), was found to be polyphyletic within Japan. Despite their morphological similarity, the three recognized varieties did not form a monophyletic group but instead clustered into geographically structured clades, often grouping with nearby species rather than with conspecific varieties. This pattern may reflect either multiple independent origins with phenotypic convergence or a single origin, followed by local hybridization and subsequent backcrossing. Given the multiple instances of gene flow detected in this study, the latter scenario likely contributed to the observed phylogenetic complexity, although phenotypic convergence remains to be tested. In addition, morphological variation within species may further complicate taxonomic identification in this genus (Xu et al., 2019). For example, distinguishing between *E. grandiflorum* and *E. koreanum* is taxonomically challenging (Zhang et al., 2022). In Japan, the major morphological difference between *E. grandiflorum* and *E. koreanum* is flower color, but substantial intraspecific variation in flower color has been reported in several *Epimedium* species (Xu et al., 2019).

Our molecular results are consistent with the morphology-based hypothesis that *E.* × *setosum* represents hybrid populations (Horie et al., 2012; Kurosaki, 1981). This taxon exhibited a reticulated and unstable phylogenetic position between southern *E. sempervirens* and *E. diphyllum*, consistent with a hybrid origin. Similarly, *E. trifoliolatobinatum* has previously been suggested to represent a hybrid between *E. diphyllum* and *E. grandiflorum*, based on its intermediate morphology (Maekawa, 1955; Suzuki, 1978). In our analyses, *E. trifoliolatobinatum* clustered with *E. grandiflorum* var. *grandiflorum*, whereas TreeMix and *D* and *f*-branch statistics suggested introgression involving *E. diphyllum* and the lineage leading to these taxa. In addition, *E. trifoliolatobinatum* subsp. *maritimum* showed signals of admixture in

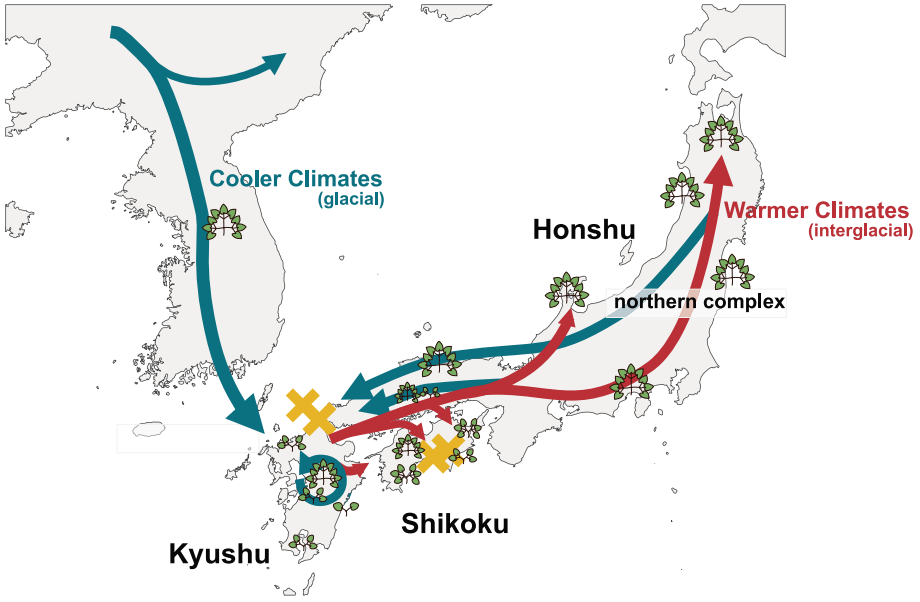

Fig. 6. A hypothetical migration scenario inferred from phylogenetic relationships, divergence time estimates, and introgression analyses. Blue arrows indicate potential southward dispersal directions during glacial periods, whereas red arrows indicate northward dispersal directions during warmer (interglacial or post-glacial) periods, suggesting repeated range shifts associated with climatic oscillations. Crosses indicate potential introgression among lineages. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the *D* and *f*-branch statistics and substantial residuals with Kyushu lineages in TreeMix. Although these signals do not directly demonstrate a hybrid origin, they are consistent with past gene flow between these lineages.

Hybridization may have also contributed to ecological differentiation. *Epimedium* × *setosum* and *E. trifoliolatobinatum* var. *trifoliolatobinatum* are mainly distributed on limestone and serpentine soils, respectively, and preliminary observations suggest possible salt tolerance in *E. trifoliolatobinatum* subsp. *maritimum* (Mimura et al., unpublished data). These patterns are consistent with a scenario in which hybridization may have contributed to diversification by increasing standing variation upon which selection can act (Stull et al., 2023). Together with the geographic structuring revealed here, these results imply that isolation and hybridization associated with Pleistocene climate oscillations across heterogeneous landscapes may have played a role in the divergence of *Epimedium* species.

5. Conclusion

Our study revealed reticulate phylogenetic relationships consistent with a history of introgressive hybridization within *Epimedium* sect. *Macroceras*, a recently diverged group occupying diverse environments in Japan. Rapid diversification in this lineage may have increased opportunities for hybridization, which may in turn have contributed to phenotypic diversification among ecologically differentiated species distributed across contrasting soil types, saline habitats, and other environmental gradients. Together, these findings highlight the potential importance of climatic fluctuations, complex island geography, and weak reproductive isolation in shaping reticulate evolutionary patterns in recently diverged plant lineages. These phylogenomic results provide a foundation for future studies testing ecological divergence and the role of hybridization in diversification.

CRediT authorship contribution statement

Emi Kusatake: Writing – original draft, Formal analysis, Data curation. **Momoka Konishi:** Writing – original draft, Formal analysis, Data curation. **Shuto Tomokuni:** Writing – original draft, Formal analysis, Data curation. **Yosuke Yanagi:** Writing – original draft, Formal analysis, Data curation. **Shungo Kariyama:** Resources. **Takehito Itoh:** Data curation. **Atsushi Toyoda:** Data curation. **Seung-Chul Kim:** Writing – review & editing, Resources. **Makiko Mimura:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2026.108646.

Data availability

Genomic sequencing data used for the draft genome assembly have been deposited in the DDBJ Sequence Read Archive under BioProject ID PRJDB16981 (Illumina: DRR546227–DRR546229; PacBio: DRR546230–DRR546235). The resulting genome assembly is available in the DDBJ/ENA/GenBank databases under accession BAAIJZ010000001–BAAIJZ010000780. GRAS-Di sequencing reads used for polymorphism analyses have been deposited in the DDBJ Sequence Read Archive under accession numbers DRR798433–DRR798490.

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