

Genomic signatures of habitat isolation and paleo-climate unveil the “island-like” pattern in the glasshouse *Rheum nobile*

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Abstract

Background

The Himalaya-Hengduan Mountains (HHM), known as ‘Sky Islands’, present a unique opportunity for exploring the genomic differentiation of endemic plants, particularly in understanding their “island-like” genetic distinctness. Despite its ecological significance, the detailed population genomics and underlying mechanisms contributing to this distinctness remain underexplored. This gap limits our understanding of the impact of geological and climatic events on species evolution and diversification in these regions. Bridging the gap, the study focuses on *Rheum nobile*, a distinctive “glasshouse species” from the HHM’s alpine subnival summits, to unravel the genetic dynamics contributing to its “island-like” structure. Investigating this species provides crucial insights into genetic diversity formation, enhancing our knowledge of global biodiversity within these unique ecosystems.

Results

In-depth analysis of plastome and Internal Transcribed Spacer (ITS) sequences from 104 *R. nobile* samples revealed a distinct “island-like” structure, characterized by significant unique alleles and high genetic diversity across populations. This pattern aligned with three phylogroups, reflecting the floristic regionalization of HHM. The three main lineages emerged and diverged allopatrically approximately 7.72–4.18 million years ago (Mya). Notably, the species experienced a marked population bottleneck or decline, evident between 0.18–0.016 Mya (plastome) and 0.38–0.031 Mya (ITS). *Rheum nobile* showed no correlation between genetic patterns and geographic distance (Isolation by Distance) or environment (Isolation by Environment). Additionally, limited gene flow was observed among the discerned phylogroups. Ensemble Species Distribution Modeling (eSDM) suggested the existence of multiple refugia during/or even earlier of the Last Glacial Maximum, indicating a complex historical distribution.

Conclusion

Rheum nobile’s distinctive genetic patterns, marked by high population differentiation and restricted gene flow, are largely a result of the fragmented ‘Sky Island’ habitats and extended isolation periods during the Quaternary glaciation. This research underscores the intricate evolutionary trajectory of *R. nobile*, driven by the interplay of factors including the ‘Sky Island’ dynamic, adaptation to ecological niches, geological uplifts, climatic fluctuations, and habitat fragmentation. These factors collectively have shaped the current distribution and genetic makeup of *R. nobile*. Moreover, this understanding carries substantial ramifications for the conservation and management of *R. nobile* in its native environment.

Background

The ‘Third Pole’, comprising the Qinghai-Tibetan Plateau (QTP), the Himalayas, and the Hengduan Mountains, forms a vital biodiversity reservoir in the northern hemisphere, featuring biodiversity-rich hotspots, namely the Himalayas and the Mountains of Southwest China (*aka* Hengduan Mountains) [abbreviated as the HHM region; 1, 2]. The alpine subnival habitats in these regions feature unique ‘Sky Island’ ecological system of isolated ridges exceeding 4000 m in elevation separated by low-altitude forests [3–5], and host remarkable plant diversity [6, 7]. Such an intriguing landscape and biodiversity provides a fertile ground for researchers to delve into the influence of diverse habitats and complex geological changes on population genetics and the intricacies of speciation over time and space [8–10]. As a result, the HHM region has been a focal point for phylogeographers probing into “island-like” adaptive radiation and species lineage diversification [11–15].

Current genetic variations among populations might mirror the legacies of past geological phenomena, including mountain uplift, climatic change, Quaternary glaciation, and habitat fragmentation [16]. These events have created ecological niche disparity and landscape changes [17, 18], leading to the emergence of geographical barriers, resulting in genetic drift and natural selection [19–21]. While the conjectures of several studies link geological transitions and Quaternary climate changes to the origins of specific alpine subnival species within the ‘Sky Island’ ecological system in the HHM region [22–23], the precise mechanisms and their impacts on population divergence remain somewhat enigmatic, necessitating more in-depth investigations.

The study centers on comprehending the ecological and evolutionary dynamics within the summits of the ‘Sky Island’ ecological system to perform phylogeographical study on representative endemic species, *Rheum nobile* Hook f. & Thompson. It is a prominent alpine monocarpic herb native to the alpine subnival belt of the HHM region (4000 to 6000 m a.s.l.) [7, 24] and well-adapted to the harsh alpine environment. While there have been some ecological, molecular phylogenetic and genome inquiries [25–31], an exhaustive genetic exploration at the population-level remains uncharted, primarily due to sampling challenges resulting from their scattered smaller populations on the summits of the HHM region. Meanwhile, with the widespread utilization of advanced species distribution modeling (SDM) techniques, there’s now an avenue to discern the climatic niches of lesser-known and vulnerable species, precisely as exemplified by *R. nobile* [27]. Current climate models offer promising forecast on the impact of climatic shifts on species native to cold subnival belt [32], facilitating an in-depth analysis of climate change impacts on plant diversity, potential distribution based on ecological traits, and their sustainability in analogous environments like refugia [33, 34].

Our hypothesis posits that species like *R. nobile* exhibit “island-like” radiation differentiation due to habitat fragmentation/isolation in conjunction with tectonic movements, climatic fluctuations, and ecological niche diversity, leading to species divergence, lineage differentiation, and the emergence of refugial populations. Therefore, we focused on 1) elucidating the genetic patterns and differentiation resulting from habitat isolation, 2) discerning the origin and divergence trajectories grounded on the geological historical events, and 3) locating putative refugia during the glacial period and trace postglacial demographic changes in such refugial populations of *R. nobile*. To achieve these aims, we

conducted a detailed analyses of population structure, demography, and evolutionary history among populations of *R. nobile* using plastome-wide variation and Internal transcribed spacer (ITS). Despite the caveats of a smaller sample size in this mountaintop species, our study reaffirmed the existence of geographically isolated islands as locations to test the “island-like” genetic pattern and historical gene flow between populations.

Results

Genomic libraries, plastid haplotype diversity, and differentiation

The sequenced plastome length span from 159,933 bp to 161,706 bp, comprising 127 genes: 82 protein-coding genes (CDS), 35 tRNAs, and eight rRNAs. These plastomes of 104 individuals exhibited 170 SNPs after filtering and cleaning. Comprehensive plastome analyses identified 20 haplotypes (without indels), and most of them were unique (85%), with the exception of H3, H10 and H11 shared by two populations (Table 1 & Fig. 1). Among all the populations, DC and ZD manifested the highest variance with four haplotypes, followed by ML with three haplotypes. For ITS, we discerned 11 ribotypes (R1-R11; Table 1, Fig. 1 & Fig. 2d), primarily shared among the populations from the same phylogroup.

Table 1
Genetic and nucleotide diversity within the populations of *Rheum nobile* based on plastome and ITS datasets.

Population Code	n	Plastome			ITS		
		Haplotypes (No.)	H_d	$\pi (\times 10^{-3})$	Ribotypes (No.)	H_d	$\pi (\times 10^{-3})$
NHQ							
DR	11	H1(9), H2(2)	0.327	0.0041	R1(2), R5(9)	0.327	0.42
SHB	10	H3(10)	0	0	R1(10)	0	0
SHA	8	H3(7), H4(1)	0.250	0.0062	R1(8)	0	0
EHQ							
YMA	12	H5(12)	0	0	R8(8), R9(2), R10(2)	0.545	0.78
YMB	12	H6(9), H7(3)	0.409	0.0100	R8(10), R10(2)	0.303	0.39
ZR	12	H8(11), H9(1)	0.318	0.0031	R8(8), R9(3), R11(1)	0.530	0.84
LZ	10	H10(8), H11(2)	0.356	0.0088	R2(5), R3(5)	0.556	0.72
ML	10	H10(2), H11(6), H12(2)	0.622	0.0088	R3(10)	0	0
SHM							
DC	12	H13(6), H14(3), H15(2), H16(1)	0.712	0.0600	R4(6), R6(5), R7(1)	0.621	1.38
ZD	7	H17(1), H18(4), H19(1), H20(1)	0.714	0.0071	R4(7)	0	0
Overall	104		0.919	3.86		0.856	7.58
n = Sample size; No. = number of individuals; H_d = haplotype diversity; π = nucleotide diversity.							

The haplotype diversity (H_d)/nucleotide diversity (π) for the overall population was 0.919/3.86 $\times 10^{-3}$ (plastome) and 0.856/7.58 $\times 10^{-3}$ (ITS). Haplotype frequencies, diversity, and their distributions in populations were listed and shown in Table 1 and Fig. 1. The total genetic diversity (H_T) was high (plastome, 0.974; ITS, 0.911), whereas the average within-population diversity (H_S) was moderate (plastome, 0.356; ITS, 0.288), indicating high genetic differentiation among populations. We also discovered that the genetic differentiation N_{ST} (plastome/ITS, 0.998/0.947) was significantly greater

than G_{ST} (plastome/ITS, 0.635/0.684; $U > 1.96$, $p < 0.05$), indicating the existence of clear phylogeographic structure in *R. nobile*. The AMOVA results (Table 2) indicated considerable genetic differentiation among three distinct phylogroups in both datasets (91.25% and 89.26%; Table 2 & Fig. S1).

Table 2

Results of Analysis of molecular variance (AMOVA) of *Rheum nobile* for plastome and ITS dataset.

Sequence type	Source of Variation	d.f.	SS	VC	Variation %	$F_{ST}/F_{SC}/F_{CT}$
Plastome	Among populations	9	31569.19	338.18	99.74	0.99*
	Within populations	94	82.15	0.874	0.26	
	Total	103	31651.35	339.054		
	Among groups	2	28529.89	444.21	91.25	0.99*/0.98*/0.91*
	Among populations within groups	7	3039.29	41.71	8.57	
	Within populations	94	82.15	0.87	0.18	
	Total	103	31651.35	486.79		
ITS	Among populations	9	284.59	3.03	93.86	0.94*
	Within populations	94	18.64	0.20	6.14	
	Total	103	303.23	3.23		
	Among groups	2	261.76	4.10	89.26	0.59*/0.96*/0.89*
	Among populations within groups	7	22.82	0.30	6.42	
	Within populations	94	18.64	0.20	4.32	
	Total	103	303.22	4.60		
d.f. = Degrees of freedom; SS = Sum of squares; VC = Variance components; $F_{ST}/F_{SC}/F_{CT}$ = Fixation indices; NS = Not significant; NC = Not calculated.						
*P < 0.001.						

The genetic differentiation patterns among populations were further determined using IBD and IBE under the Mantel and partial Mantel tests, respectively. Both tests for *R. nobile* using plastome ($r = 0.0568/0.2719$) and ITS ($r = 0.0979/0.0227$) datasets were non-significant ($p > 0.05$; Fig. 3a–d), indicating no linear relation between the genetic structure and measured distances.

Population structure determination

Using Bayesian clustering via STRUCTURE for plastome dataset, we discerned an optimal subdivision into three distinct clusters (NHQ, EHQ, and SHM) with a maximum model value of ΔK (Fig. 2a, b). The NHQ cluster (including populations SHB, SHA, and DR), resides within the confines of Eastern Nepal Himalaya and the Upper & Middle Yarlung-Zangbo subregions. In contrast, the EHQ cluster (populations LZ, ML, YMA, YMB, and ZR) and SHM cluster (populations ZD and DC) are primarily located in the SE Xizang subregion (Eastern Himalayas) and south Hengduan Mountains, respectively. SAMOVA indicated a clear phylogeographic structure when $K = 3$, with the F_{CT} value reaching a peak based on the ITS dataset (Fig. S2). Three specified phylogroups from STRUCTURE and SAMOVA were also further verified by the SPLITSTREE network (Fig. 2c, d) based on both datasets. The plastome SNPs for the PCA [PC1/PC2, 63.52/11.53%; Fig. 2e] biplots, cluster factor map [Dim1/Dim2: 85.6/10.3%], and hierarchical cluster dendrogram (Fig. S3) supported the phylogeographical grouping of STRUCTURE and SPLITSTREE network. The subsequent analysis using the ITS dataset (Fig. 2f & Fig. S4) entirely corroborates the findings from the plastome dataset.

Phylogenetic inference, divergence time, and ancestral area estimation

The tree topologies constructed based on the CDS of plastome as well as ITS region inferred a monophyletic clade of *R. nobile* with high posterior probability (PP) values and likelihood scores, respectively (Fig. S5). These trees clustered 104 individuals from 10 populations into three lineages (NHQ, EHQ, and SHM), which is consistent with phylogeographical groups revealed by STRUCTURE/SAMOVA/network relationships and their geographic range.

The BEAST calibrated molecular dating revealed that the origin of *R. nobile* was c. 7.72 Mya; 95% highest posterior density [HPD]: 3.58–12.19 Mya (Node IV: stem age), and the onset of diversification started at c. 5.7 Mya; 95% HPD: 2.58–9.29 Mya (crown age: node V; Fig. 4a) in the late Miocene epoch. This crown age demarcates the initial divergence period between NHQ lineage and the common ancestor of EHQ and SHM lineages. The subsequent divergence between the EHQ and SHM lineages is estimated to the early Pliocene, i.e., c. 4.18 Mya (95% HPD: 1.72–6.99 Mya). Additionally, the onset of diversification for sub-lineages within each lineage occurred around the early Pleistocene, i.e., NHQ: c. 2.87 Mya, EHQ: c. 2.79 Mya, and SHM: c. 1.83 Mya (Fig. 4a), followed by population differentiation throughout the whole Quaternary period.

Biogeographically, BBM and DEC-Lagrange analyses reconstructed the NHQ (Upper and Middle Yarlung-Zangbo subregion) as the most likely ancestral area for *R. nobile* (Fig. 4a). According to our inference, *R.*

nobile diverged from its closest relatives *R. rhomboideum*/*R. spiciforme*, during historical dispersal events at node IV. Additionally, from the late Miocene to the early Pliocene, a series of vicariance and subsequent dispersal events at nodes V and VI caused the genetic divergence of three lineages (Fig. 4a).

Demographic history and gene flow

The mismatch distributions for both of pure population growth and spatial expansion models were all multimodal (Fig. 5a–d), and the statistics i.e., SSD and H_{Rag} presented an excellent statistical fit to both models ($p > 0.05$; Table 3) based on plastome and ITS datasets. Tajima’s D and Fu’s F_s of neutrality tests were positive but not statistically significant (Table 3, $p > 0.05$). For overall populations, BSP (Fig. 5e, f) depicted a population bottleneck or decline dynamic between 0.18–0.016 Mya (plastome) and 0.38–0.031 Mya (ITS). When examining the gene flow ($4Nm$) among the identified groups (NHQ, EHQ, and SHM, Fig. 4b), there appeared to be minimal exchange. The EHQ has a comparatively large effective population size ($\Theta = 0.00008/0.00121$). The gene flow value is highest between SHM and EHQ for both datasets (Fig. 4b).

Table 3

Results showing mismatch distribution analysis under models of (a) pure population growth and (b) spatial expansion, and neutrality test for the plastome and ITS sequences of *Rheum nobile*; tests were conducted in ARLEQUIN with the sum of squared deviations (SSD) and Harpending’s (1994) raggedness index (H_{Rag}).

Sequence type	Model	SSD	p -value	H_{Rag}	p -value	Tajima’s D test		Fu’s F_s test	
						D	p -value	F_s	p -value
Plastome	a	0.081	0.017	0.117	0.002	3.425	0.986	3.403	1.0
	b	0.055	0.220	0.117	0.425				
ITS	a	0.045	0.035	0.035	0.175	1.763	0.902	4.368	0.976
	b	0.032	0.248	0.035	0.851				

Niche dynamics

The species distribution model (SDM) was fine-tuned using eight algorithms (with indices ≥ 0.8) and employed seven predictive bioclimatic factors across LGM and present climate scenarios (Fig. 6a–d). This calibration produced a consensus habitat suitability and accurately depicted the actual distribution and ideal habitats that concord with the current location of the species (Fig. 1 & Fig. 6a, c). Compared to the current distribution range, the predicted paleo-distributional suitability (during LGM) is relatively low, with patchy or disjunct suitability throughout the current distribution range (Fig. 6b, d). Additionally, substantial parts of the Hengduan Mountains and eastern Himalayas are expected to be the most suitable habitat for *R. nobile* (High: $>75\%$; Fig. 6c, d).

Climatically, the habitat suitability of *R. nobile*'s population is predominantly governed by precipitation over the temperature (Table S1 & Fig. S6), showing that the former was the major driving force in differentiating the NHQ, EHQ, and SHM. As demonstrated in the overlaid consensus map for two scenarios (LGM and current), the predicted suitability range of three phylogroups is more or less isolated (Fig. 6e, f). In contrast to the overall occurrence points, the suitability prediction for each phylogroup (under their territories) resulted in a concise projection of less suitability.

Furthermore, the niche identity test (Schoener's D and Hellinger's-based I) revealed a pattern of low overlap for geographically isolated phylogroups (Fig. 6g–i). The test used all parameters to reveal significant environmental divergence in three phylogroups pair comparisons, demonstrating that the niches of NHQ vs EHQ, NHQ vs SHM, and EHQ vs SHM are distinct (Fig. 6g–i), and the observed differences were driven by ecological selection.

Discussion

Habitat isolation governs the “island-like” genetic pattern

The alpine plants are believed to be remnants of mountaintop refugia during the ice age and/or are thought to have evolved from cold-tolerant plants that migrated upward as a result of postglacial temperature rise [35]. Several endemic species found in the high alpine habitats of the HHM region and adjacent mountains are isolated by low-elevation barriers and unsuitable ecosystems [5]. As a result, they have experienced habitat fragmentation and isolation over a long period, leading to a high level of genetic differentiation compared to non-isolated populations. This can be attributed to the substantial influence of genetic drift and the sporadic alteration of gene ratios in small, segregated populations. The “island-like” genetic pattern observed in these plant species is characterized by significant private alleles in intraspecific genetic clusters and high genetic differentiation, which is primarily due to limited gene flow. Lower and inconsistent historical gene flow patterns (Fig. 4b) among *R. nobile* populations support the idea of habitat isolation commonly found in ‘Sky Island’ species. Similar genetic patterns have been observed in other alpine species with comparable traits, distributions, and evolutionary histories [11, 13–15], and it is believed that these patterns are driven by the isolated, heterogeneous habitats found on the top of the ‘Sky Island’ ecological systems.

From the perspective of the distinct physio-morphological traits of *R. nobile*, those features, such as a prolonged life cycle relying on insects (*Bradysia* sp.) for pollination [36], and limited seed dispersal capacity [primarily within 0.5 m distance of the mother plant; 26], jointly enhance its high genetic differentiation. Such traits curb long-distance gene flow and increase the impact of genetic drift on smaller and isolated populations. The shared genetic component exclusively in adjacent populations (such as SHA vs. SHB, ML vs. LZ, and YMB vs. ZR) (Table 1, Fig. 1 & Fig. 4a) suggests that gene flow between the distant populations is stifled due to restricted dispersal distances and limited insect-mediated pollination. In addition, the lack of IBD and non-significant IBE (Fig. 3) manifests that the genetic pattern of *R. nobile* was not associated with the geographical distance and environmental

difference (temperature and precipitation). Each population occupies such a similar ecological niche in the alpine subnival belt, but considerable genetic differentiation between each other (even within two adjacent ones) was identified (Fig. S1). Furthermore, small population sizes and distinct phylogroups of *R. nobile* have accelerated genetic drift, resulting in high genetic differentiation among populations. Therefore, enduring habitat isolation, primarily induced by the low-elevation barriers and harsh ecosystems surrounding high alpine habitats, has been instrumental in the diversification and differentiation of *R. nobile* and other 'Sky Island' species in the HHM region [37–39].

Intraspecific divergence in response to historical orogeny of the HHM region

This study illustrates how past geological events and climatic changes have shaped the diversification of native alpine species in the eastern Himalayas and the Hengduan Mountains during the Pliocene and Pleistocene epochs. The late Miocene origin of *R. nobile* coincides with the origin of many plant species in the HHM region [e.g., 12, 15, 40–42]. The rise of the QTP is an intricate geological development process with several orogenic stages that represented the progressive and heterogeneous rise from south to north [19, 43–45], and is believed to have had a significant impact on the topography of mountains in the south-eastern part of the QTP [46]. Many of phylogeographical studies have shown a correlation between this uplift and intra/interspecific divergence time [e.g., 47–51] in/and around the region. *R. nobile* advocates the same estimated time with the primary lineages divergence occurred between c. 5.7–4.18 Mya. Significantly, the habitat for *R. nobile* atop the mountains might have become fragmented as a result of the orographic movements of the HHM region in combination with the associated climate changes [52]. Climatic events, such as cooling and Asian monsoon intensification, might have influenced the phylogroups/intraspecific divergence of *R. nobile* around the late Miocene to early Pliocene [17, 52]. However, most of the terminal lineages underwent diversification in or close to the Pleistocene (Fig. 4a), before the most severe glacial period on the QTP reached its peak (the second glacial period in Quaternary, between 0.8 and 0.6 Mya) [53]. The spatio-temporal divergence patterns suggests that *R. nobile* underwent allopatric divergence in several regions of its distribution during its evolutionary history, and the phylogroups have not shared any haplotypes among them. The rugged terrain and highly fragmented habitat of the alpine subnival belt in the HHM region, along with the Quaternary glaciation, likely exacerbated the isolation of populations within each phylogroup. In conclusion, the findings evidenced that persistent habitat isolation and fluctuations in ecological factors have resulted in significant intraspecific divergence in *R. nobile*.

Multiple refugia in demographic history leading to current expansion

The demographic history observed in *R. nobile* during or after the Pleistocene (Fig. 4a) can likely be attributed to the Quaternary glaciation. Although the multimodal mismatch distribution (Table 3 & Fig. 5a–d) manifests there was no recent population size and spatial expansion in *R. nobile*. While, BSP inference (Fig. 5e–f), as well as palaeodistribution modeling (Fig. 6b, d), provide evidence that *R. nobile*

has experienced past demographic events. In particular, palaeodistribution modelling during LGM and long-term (from early Pliocene) distinct allopatric divergence of three lineages, jointly indicate the presence of multiple refugia in *R. nobile*, solely representing the current phylogeographical pattern with three regions [Nepal East Himalaya, eastern Himalayas (Shannan region of Xizang, China and Bhutan), and South Hengduan Mountains]. Several researches delving into species distribution and phylogeography [54–60] corroborates the hypothesis that refugia occurred along the south-eastern margin of the QTP, as well as in areas further west and north. This hypothesis aligns well with geological evidence indicating that ice-free regions were common throughout the Quaternary in the QTP and adjacent mountains [61]. Rana et al. [27] also marked South Hengduan Mountains as a potential refugial area for *R. nobile*. In the past, multiple refugia were commonly recorded in the cold-tolerant species e.g., *Juniperus tibetica* in forests [56]; *Potentilla glabra* in forests and alpine scrub [62]; *Aconitum gymnandrum* in alpine grasslands [54]; and *Rhodiola alsia* on alpine gravel slopes [63].

Mostly, during cold glaciation, plant species contracted to refugial areas and expanded their ranges during warm interglacial periods [16, 64]. For alpine plants, however, the past demographic dynamics may be reversed. The population bottleneck or minimum effective population size was supposed to occur during c. 0.18–0.016 Mya (plastome)/0.38–0.031 Mya (ITS), corresponding to the time from the interglacial period between the second and third glacial periods in Quaternary to the LGM (c. 22 Kya), suggests that *R. nobile* underwent interglacial/postglacial contraction, which is consistent with the idea that refugia play a crucial role in maintaining genetic diversity and providing sources of admixture during glacial cycles [65, 66]. The cooling climate of the Quaternary might have prompted three lineages of *R. nobile* to grow sympatrically and retreat into lowlands. The historical gene flow (highest value) estimated between EHQ and SHM favors the hypothesis of lowland sympatricity of adjacent populations/lineages during glacial period in *R. nobile*. Such a typical response was seen in many alpine plant species [15, 67]. Later, as the climate warmed up again, the parental clade moved back to the high-altitude “mountaintop” and became more segregated, leading to the evolution of the current lineages. The admixture among nearby populations respectively found in NHQ and EHQ phylogroups further supports the notion of populations colonization and isolation after the LGM period. By identifying these refugial populations, researchers can better understand how species responded to past climate changes and make predictions about how they may reciprocate to future climate change [68, 69].

The identification of refugial region is a critical yet challenging aspect of evolutionary and conservation biology [70]. Species respond differently to climatic changes based on their distinct biological traits and historical distribution patterns [71]. Typically, refugial populations tend to have greater genetic diversity than their descendants. This diversity is influenced by a species’ dispersal capabilities, making species with limited dispersal potential, like *R. nobile*, key indicators for inferring refugia in restricted regions [72]. A critical application of this knowledge is identifying regions with unique genealogies and biodiversity ‘hotspots’. Identifying these hotspots can serve to prioritize conservation efforts in regions vulnerable to climate shifts [73], thereby ensuring the species’ survival.

Conclusions

The comprehensive study of *R. nobile*, a unique alpine ‘Sky Island’ species in the HHM region, reveals a complex interplay of evolutionary forces shaped by geological, climatic, and ecological factors. The pronounced genetic differentiation and diversification leading to “island-like” genetic pattern of this species are largely attributed to long-term habitat isolation, driven by low-elevation barriers, challenging ecosystems, and associated restricted gene flow and intensified genetic drift. This isolation has been further compounded by the species’ specific traits, such as limited seed dispersal and specialized pollination strategies. Moreover, the study highlights the significant influence of the uplift of the Qinghai-Tibetan Plateau and corresponding climatic events during the Pliocene and Pleistocene epochs on the phylogeographical divergence within *R. nobile*. The adaptation of this species to these dynamic environmental conditions is evident in its allopatric divergence and the unique genetic patterns of its phylogroups. Additionally, the species’ demographic history, particularly in response to Quaternary glaciation events, underscores the importance of refugia in its survival and genetic diversity maintenance. These refugia have enabled *R. nobile* to withstand climatic oscillations, leading to a demographic contraction during warmer periods and expansion during cooler ones. Collectively, these insights provide a deeper understanding of the evolutionary trajectory of high-altitude plant species, specifically the ‘Sky Island’ species and their resilience to past climate changes, offering valuable predictions for their future adaptations the rapid environmental shifts.

Materials and methods

Sequencing, assembly, annotation, and SNP calling

We sampled 104 individuals from the entire geographic range of *Rheum nobile* (Table S2). Leaf samples were collected and dried in silica gel for DNA extraction, and vouchers were identified by Rita Chhetri (Research officer, National Herbarium and Plant Laboratories, KATH) for Nepalese sample and by Dr. Bo Xu (Assistant Professor, College of Forestry, Southwest Forestry University, Kunming, China) for Chinese samples. The identified samples were then deposited at National Herbarium and Plant Laboratories (KATH), Nepal and Herbarium of the Kunming Institute of Botany (KUN), China. For complete plastome sequencing, genomic DNA was extracted from 20 to 30 mg of silica-dried leaves using the cetyltrimethylammonium bromide (CTAB) method [74]. Following the manufacturer’s instructions, a 500 bp DNA TrueSeq Illumina (Illumina Inc., San Diego, CA, USA) sequencing pair-end libraries were constructed using 3–5 µg sonicated DNA. The libraries were pair-end sequenced on the Illumina HiSeq 2000 platform. Raw sequence data were subsequently filtered to remove the low-quality reads and adaptors using the NGS QC Toolkit [75], setting the cut-off value for percentage read length to 80 and the Phred quality score to 30. The remaining clean pair-end (PE) high-quality reads were assembled through *de novo* assembly using GetOrganelle v.1.7.0 [76] with K-mer values 21, 45, 65, 85, and 105 calling SPAdes v.3.10 [77]. We used Bandage v.0.80 [78] to visualize and filter the assembled contigs. The newly assembled plastome sequences were annotated using Plastid Genome Annotator [79] and manually verified against the reference chloroplast genome i.e., *Rheum nobile* (NC_046506 in Genbank) using Geneious v.8.0.2 [80]. Geneious was used to manually correct the consistency of start/stop codons and

intron/exon boundaries, and tRNAscanSE web service [81] was used to confirm the tRNA genes. Furthermore, the command line -k 35,85,115 -F embplant_nr was used to call the 3S region (ITS included), and the resulting sequences were aligned and annotated against the reference ITS sequence of *R. nobile* (KF258715 in Genbank).

The PE clean reads used in the *de novo* assembly were mapped onto the reference chloroplast genome of '*Rheum nobile*' [NC_046506; 82] using Burrows-Wheeler Aligner (BWA) v.0.5.9 with -M -B settings [83]. SAMtools v.1.3.1 [84] was then used to convert mapping results from a Sequence Alignment Map (SAM) file to a binary SAM (BAM) file, deduplicate, and sort. The sorted BAM files for each of the 104 accessions were used as input to call raw SNPs and InDels utilizing a combination of BCFtools v.1.8 [85] mpileup command. Subsequently, low-quality SNPs and InDels (QUAL values < 20, depth < 10) were filtered out, and the remaining high-quality SNPs and InDels were stored in a Variant Call Format (VCF) file. SNPs were separated from InDels, and heterozygous SNPs were eliminated manually. Furthermore, the VCF file containing the high-quality set of SNPs for the 104 sequenced accessions was converted into a concatenated sequence alignment of variable positions in fasta format using PGDSpider v.2.1.1.5 [86]. The resulting concatenated sequence alignment was used for population structure analysis.

Haplotype diversity, distribution, and population differentiation

We used DnaSP v.5.10.01 [87] to infer plastome haplotypes/ITS ribotypes for every individual (excluding sites with gaps/missing data). The number of haplo/ribotypes was computed, and the genetic relatedness between them was visualized via SPLITSTREE v.4.14.5 [88] using Neighbor-Net analysis to build the network with a Kimura-2-parameter distance model. The method combines reticulation events into a visualized interface.

The nucleotide diversity (π) and the haplotype diversity (H_d) for each population and overall populations were estimated using DnaSP. We further used HAPLONST [89] to estimate the genetic differentiation indices between populations [89]: G_{ST} , based on the identity of haplotypes, and N_{ST} , based on the pairwise nucleotide diversity matrices between haplo/ribotypes. We then tested for the presence of a phylogeographic signal (i.e., when $N_{ST} > G_{ST}$) indicating distinct haplo/ribotypes within a population are more related on average than haplotypes sampled between populations [89]. Furthermore, the total genetic variance (F_{ST}) among the populations was inferred through ARLEQUIN v.3.5 [90] and graphically visualized with circos plots using the '*chorddiag*' R-package [91].

We performed Mantel's [92] test using the *mantel* function for "Isolation by Distance" (IBD) based on Spearman's rank correlation and the partial Mantel test using *mantel.partial* function for "Isolation by Environment" (IBE) based on Pearson's correlation with 9,999 permutations to provide insight into the effects of geographic distance and environmental differences on the genetic structure, as well as net nucleotide divergence between populations of *R. nobile*. We analyzed the test in '*vegan*' R-package [93]. The necessary distance matrices, including pairwise genetic distances (F_{ST}) among populations,

Euclidean distances based on geographic coordinates, and environmental distances (Canberra) calculated from the selected predictor variables in ensemble species distribution modeling, were obtained using ARLEQUIN, GenALEx v.6.5 [94], and the 'vegan' package, respectively. For the analysis of IBD, genetic and geographic distances were employed as response and predictor factors, respectively, while for the analysis of IBE, genetic, environmental, and geographic distances served as response, predictor, and conditioner factors, respectively. In addition, we visualized the plots for IBD and IBE using 'ggplot' fitted with the 'lm' model in R.

Population structure

Population genetic structure analysis was conducted using high-quality SNPs of plastome datasets that were filtered by VCFtools. The model-based Bayesian clustering analysis was conducted using STRUCTURE v.2.2.4 [95] with 100,000 chains as burn-in and 500,000 Markov chain Monte Carlo chains and 20 independent runs for the whole dataset ($K = 2-10$). We determined the significant number of genetic clusters (K) based on the second-order rate of change in the log probability of the mean LogLikelihood (ΔK), as implemented in Evanno's method [96]. The ΔK values were calculated using STRUCTURE HARVESTER v.0.6.94 [97]. The coefficient for cluster membership of individuals was averaged across the ten independent runs using Clumpp v.1.1.2 [98] and plotted using Distruct v.1.1 [99]. The genetic structure of ITS was performed by using spatial analysis of molecular variance (SAMOVA) [100]. We also performed principal component analysis (PCA) to identify genetic clusters by observing the distribution of individuals and populations along PC scores of genetic variations based on both datasets. The resulting PC scores were visualized using the *ggplot2* R-package. This analysis was performed with prior grouping information on individuals and populations. Further, hierarchical clustering and cluster factor map based on the plastome SNPs and ITS variation were computed using the *factoextra* R-package to define the phylogroups within *R. nobile*.

Phylogenetic analysis, divergence time estimation, and ancestral range reconstruction

We implemented Maximum likelihood (ML) and Bayesian inference (BI) methods to reconstruct the phylogenetic trees using the concatenated 82 protein-coding genes (CDS) of plastome and ITS alignment, respectively. All the 104 accessions of *Rheum nobile* and outgroups (*R. rhomboideum*, *R. spiciforme*, *R. acuminatum*, *Oxyria sinensis*, *O. digyna*, and *Rumex acetosa* for plastome and *R. acuminatum*, *O. sinensis*, *O. digyna*, and *R. acetosa* for ITS) were aligned using MAFFT v.7 [101]. The appropriate nucleotide substitution model was then selected based on the Akaike Information Criterion (AIC) using Modeltest [102]. The BI tree was computed using MrBayes v.3.2.6 [103] sampled for 5×10^8 generations at every 10,000 generations with two runs and four chains until the average standard deviation of split frequencies was less than 0.01. On the other hand, the ML tree was constructed using the RAxML-HPC [104], applying the GTRGAMMAI model with a bootstrap value of 1,000 replicates. Tree visualization and editing were performed on Figtree v.1.4.3 [105].

The divergence time estimation was computed using the CDS dataset in BEAST v.2.6.3 [106]. We considered an uncorrelated relaxed clock model with a coalescent prior tree of constant effective size under the GTR + G + I site model determined by Modeltest. Using the relaxed clock model, we set the divergence time between *Rumex* and *Oxyria* (node I) as 31.81 ± 7.6 Mya, *Oxyria* and *Rheum* (node II) as 27.73 ± 7.0 Mya, *R. acuminatum* and rest of the *Rheum* species (node III) as 13.51 ± 4.8 Mya, and finally *R. nobile* stem age (the divergence time between *R. nobile* and *R. rhomboideum* + *R. spiciforme*, node IV) as 8.40 ± 4.1 Mya [30] using a normal tree prior settings. The MCMC analyses were run for up to 5×10^8 generations, sampling trees every 10,000 generations. BEAST log output files were examined using Tracer v.1.7.2 [107] to evaluate convergence and ensure sufficient effective sample sizes (ESS values) for all parameters (ESS > 200). Maximum clade credibility (MCC) tree with posterior node age distributions was obtained using TreeAnnotator v.1.10.4 and represented using FigTree v.1.4.3 [105].

Based on the floristic division of East Asia [108], three geographic regions were defined to determine the ancestral areas of *R. nobile*. NHQ (Upper and middle Yarlung-Zangbo along with adjoining eastern Nepal Himalaya) belongs to IIF17a; EHQ (the SE Xizang subregion along with adjoining eastern Himalaya) belongs to IIIE15a; and SHM (the south Hengduan Mountains) belongs to III14c. The Bayesian binary method (BBM) and dispersal–extinction–cladogenesis (DEC-Lagrange) model were implemented in RASP v.3.2 [Reconstruct Ancestral State in Phylogenies; 109]. The analysis used 100,000 trees from the BEAST MCMC of plastome (CDS regions) datasets together with *R. acuminatum*, which is sister to *R. nobile* and has almost overlapping distribution regions.

Historical demography and gene flow estimation

Historical demographic patterns of *R. nobile* were investigated using two different datasets-plastome and ITS. Mismatch distribution analysis (MDA) [110] was conducted separately for each dataset to detect recent population expansion by comparing the observed pairwise differences between haplotypes with theoretical distributions expected under the ‘pure population growth’ and ‘spatial expansion’ model. The sum of squared deviations (SSD) and Harpending’s [111] raggedness index (H_{Rag}) ‘goodness-of-fit’ was tested using 1,000 parametric bootstrap replicates. Neutrality tests using Tajima’s [112] D and Fu’s [113] F_S were also performed with 1,000 simulated samples in ARLEQUIN.

Bayesian skyline plot (BSP) [114] generated in BEAST was used to reconstruct the effective population size fluctuations since the time of the most recent common ancestor using plastome and ITS datasets, respectively. The MCMC chains (nchains = 4) were run for 2×10^8 generations sampling every 4,000 generations, with an effective sample size (ESS) greater than 200. We used the same settings for three independent runs to ensure the consistency of the results. The demographic history through time was reconstructed using TRACER v.1.7.2 [107].

To determine the extent of gene flow between three phylogroups, we used the coalescence theory-based maximum likelihood estimation of migration rates with Migrate v.3.6.11 [115, 116] on both plastome and ITS datasets separately. The equation for the number of migrants per generation is $4Nm = \Theta x My \rightarrow x$, where $\Theta = 4Ne$ (effective population size) $\times \mu$ (mutation rate per site per generation), and $My \rightarrow x = m/\mu$

(immigration rate divided by mutation rate per site per generation). The MCMC algorithm used ten short chains (10^4 gene trees), three long chains (10^5 gene trees), and a burn-in of 10^4 initial trees. All individuals were included, and parameters were estimated using the default settings.

Ensemble species distribution modeling and niche comparison

The compilation of occurrence points for *R. nobile* includes field visits, herbarium specimen (KATH and KUN), online databases [PE, E, K, BM, TI; acronyms following 117] along with CVH (www.cvh.ac.cn) and Global Biodiversity Information Facility (GBIF) database [118]. Unknown coordinates were georeferenced carefully, followed by rarefaction of duplicate points in different spatial grids to prevent geo-coding errors and spatial autocorrelation [119–121]. Finally, the rarefied 59 occurrence points at a 5-min grid [122, 123] and random background points within an area of a 50 km radius from the point of occurrence [124] were used for the model building.

Of the 19 worldclim bioclimatic variables (Bio1–Bio19; 2.5 arc-min) [125; www.worldclim.org], we selected predictive explanatory variables based on Variance Inflation Factor [VIF; 126] and Pearson correlation (r) (Table S1). Similar to Rana et al. [122] and Rana et al. [123, 27], we used R-programming language [127] to select a subset of the least correlated predictor variables (Table S1 & Fig. S7) for distribution modeling of *R. nobile* and its lineages. To model the distribution of *R. nobile* and its lineages, we evaluated ten simulated algorithms (refer to *Biomod2* package for algorithms) with default parameters and selected the ones that had model calibration statistics of TSS/KAPPA/AUC greater than 0.8 (see Table S3). For MaxEnt, we used the same regularization parameter as Rana et al. [27, 123]. Finally, the selected algorithms (Table S3) were employed for consensus mapping, and the contribution of each algorithm to the final ensemble model was proportional to their goodness-of-fit statistics.

The ensemble species distribution modeling (eSDM) of *R. nobile* was carried out using the '*Biomod2*' R-package [128]. Fifty-nine spatially rarefied occurrence points, seven bioclimatic variables, and eight algorithms in combination executed to project the potential distribution into the past (LGM ~ 22 Kya) scenario in response to current (1990–2000) climatic conditions. The paleoclimatic projections were derived using the multi-model median (MMM) approach of GCMs as in Rana et al. [123]. The final consensus ensemble model was prepared in ArcGIS v.10.4.1 [129]. Additionally, response curves for the selected four top models with KAPPA/TSS/AUC > 0.89 (representing selected variables) were plotted (Fig. S6a–g).

The niche comparison and range projection for the lineages, i.e., NHQ, EHQ, and SHM were performed based on the predicted LGM and current scenarios, particularly within the extent of occurrence [following method for overall occurrences and 130]. The obtained raster layers were then overlaid to unveil the niche differentiation in two scenarios [130]. Response curves for the selected four top models with KAPPA/TSS/AUC values > 0.89 were plotted for three lineages and total populations (Fig. S6e–z2). We further quantified and validated the climatic niches by calculating niche identity using Schoener's D and Hellinger's-based J indices in R's Ecospat package based on the habitat suitability scores [131–132]. The

indices D (0 to 1) measure the suitable range based on the probability of occupied grid cells, while I (0 to 1) work similarly to D but without its assumption [93].

Abbreviations

AUC	Area under the curve
BBM	Bayesian binary method
BI	Bayesian inference
BSP	Bayesian skyline plot
CDS	Protein coding gene sequence
DEC	Dispersal–extinction–cladogenesis
eSDM	Ensemble species distribution modeling
ESS	Effective sample sizes
F_{CT}	variance among groups defined by SAMOVA
GBIF	Global biodiversity information facility
H_d	Haplotype diversity
H_{Rag}	Harpending's raggedness index
H_S	Average within-population diversity
H_T	Total genetic diversity
IBD	Isolation by distance
IBE	Isolation by environment
ITS	Internal transcribed spacer
KATH	National Herbarium and Plant Laboratories, Nepal
KUN	Herbarium of the Kunming Institute of Botany, China
LGM	Last glacial maximum
m a.s.l.	Meter above sea level

MCC	Maximum clade credibility
MCMC	Markov chain monte carlo
MDA	Mismatch distribution analysis
ML	Maximum likelihood
Mya	Million years ago
PCA	Principal component analysis
rRNA	ribosomal Ribonucleic acid
SAMOVA	Spatial analysis of molecular variance
SSD	Sum of squared deviations
tRNA	transfer Ribonucleic acid
TSS	True skill statistic
VIF	Variance inflation factor
π	nucleotide diversity

Declarations

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Authors' contributions

HKR collected samples, conducted laboratory work, formal analysis, and wrote the original draft. SKR collected samples, assisted in the analysis, and reviewed/improved the manuscript. HS conceptualized the project, provided supervision, and reviewed/improved the manuscript. DL conceptualized the project, collected samples, contributed to the investigation, and reviewed/improved the manuscript.

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Data Availability

All annotated complete chloroplast sequence data (20 haplotypes) and ITS sequence data (11 ribotypes) used in this study are available at the National Genomics Data Center (<https://ngdc.cncb.ac.cn/>) under the accession number C_AA056721- C_AA056751. For any inquiries regarding the data, please contact Hum Kala Rana at humkalarana@gmail.com.

Ethics approval and consent to participate

This study did not involve any human tissue materials or animal tissue materials. It did not require ethical approval. We declare that all plant materials used in this study were collected in accordance with relevant institutional, national, and international guidelines and legislation. Relevant permits, and permissions were obtained prior to the collection and use of plant materials. All steps in the study were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interest

We declare the work described has not been published previously and is not under consideration for publication elsewhere. Further, they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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Figures

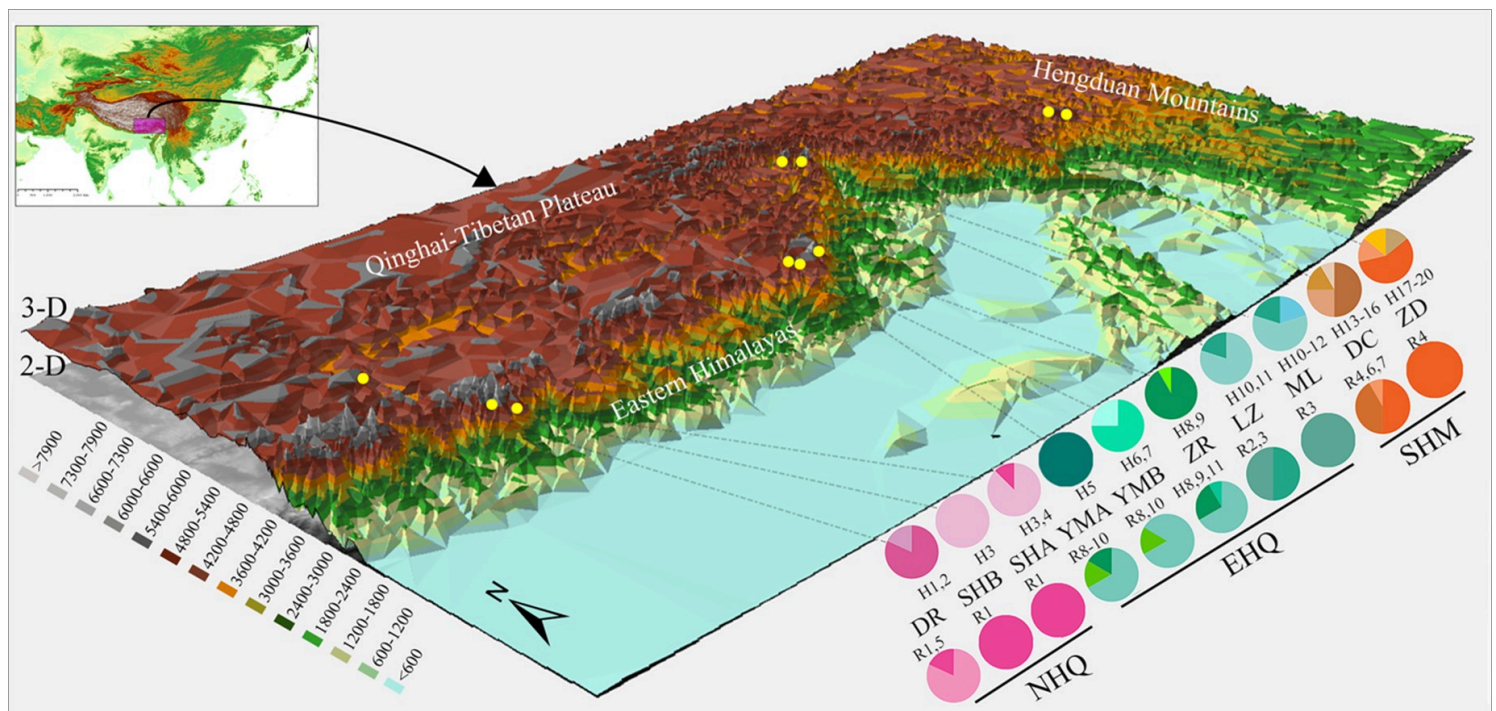


Figure 1

The 3-D representation of the geographic locations of *Rheum nobile* population. The figure also presents the distribution of haplotypes (H1–H20) extracted from plastome and ribotypes (R1–R11) from ITS datasets in pie charts (see Table S1 for population codes), where divisions correspond to haplo/ribotypes with the number of individuals.

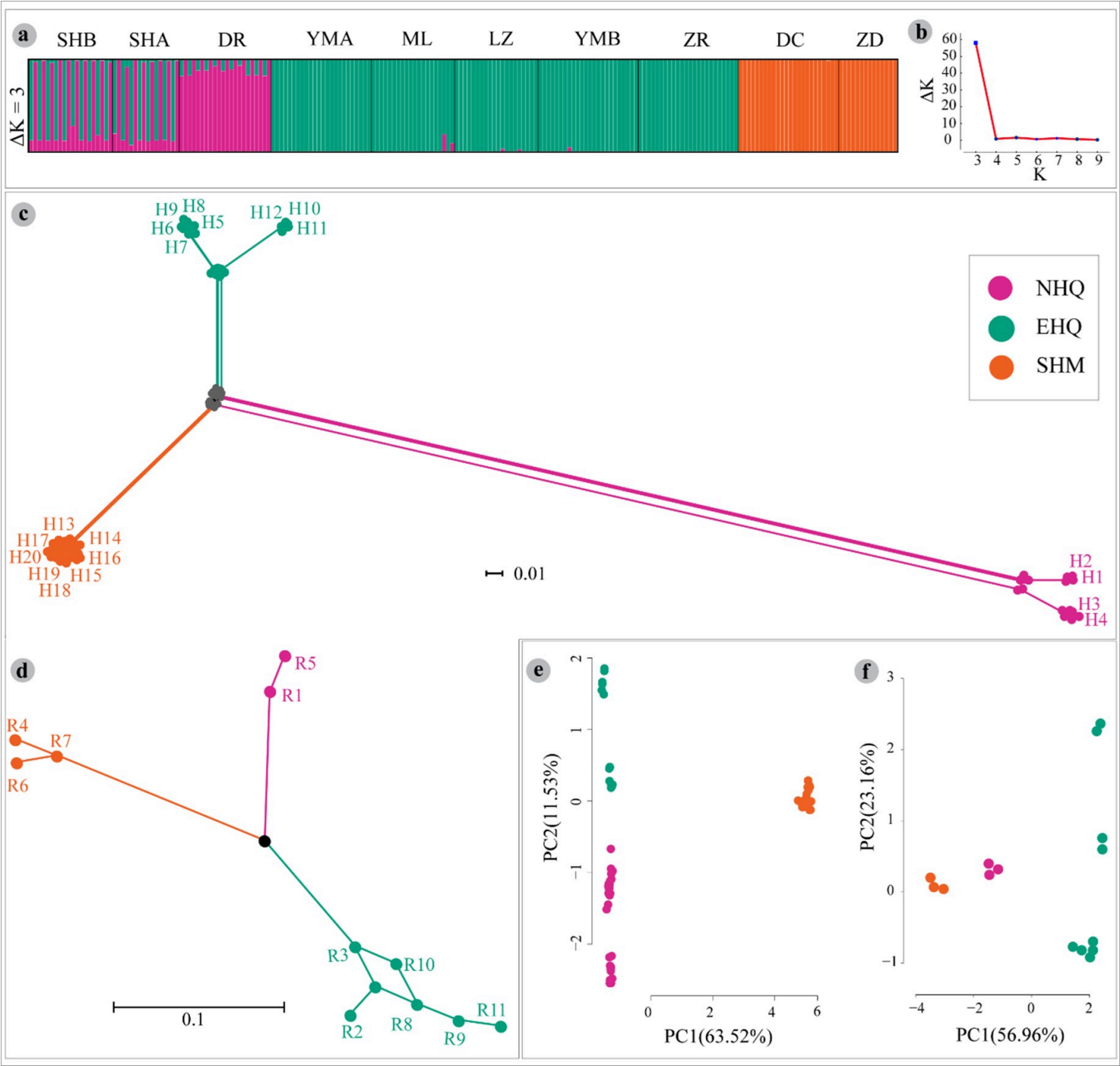


Figure 2

Individual based-clustering inferred by (a) STRUCTURE under the best cluster $\Delta K = 3$; (b) Delta K values identified using STRUCTURE HARVESTER; (c, d) SPLITSTREE network by the method neighbor-net, based on plastome haplotypes (H1–H20) and ITS ribotypes (R1–R11) for *Rheum nobile*. Circles represent the nodes of a network, and each colour corresponds to the phylogroups. Distribution of individuals among

groups, based on (e) plastome SNPs and (f) ITS datasets, analysis using *ggplot2* including PC scores of genetic variations. Circles represent individuals and individuals are colour-coded according to their group identities.

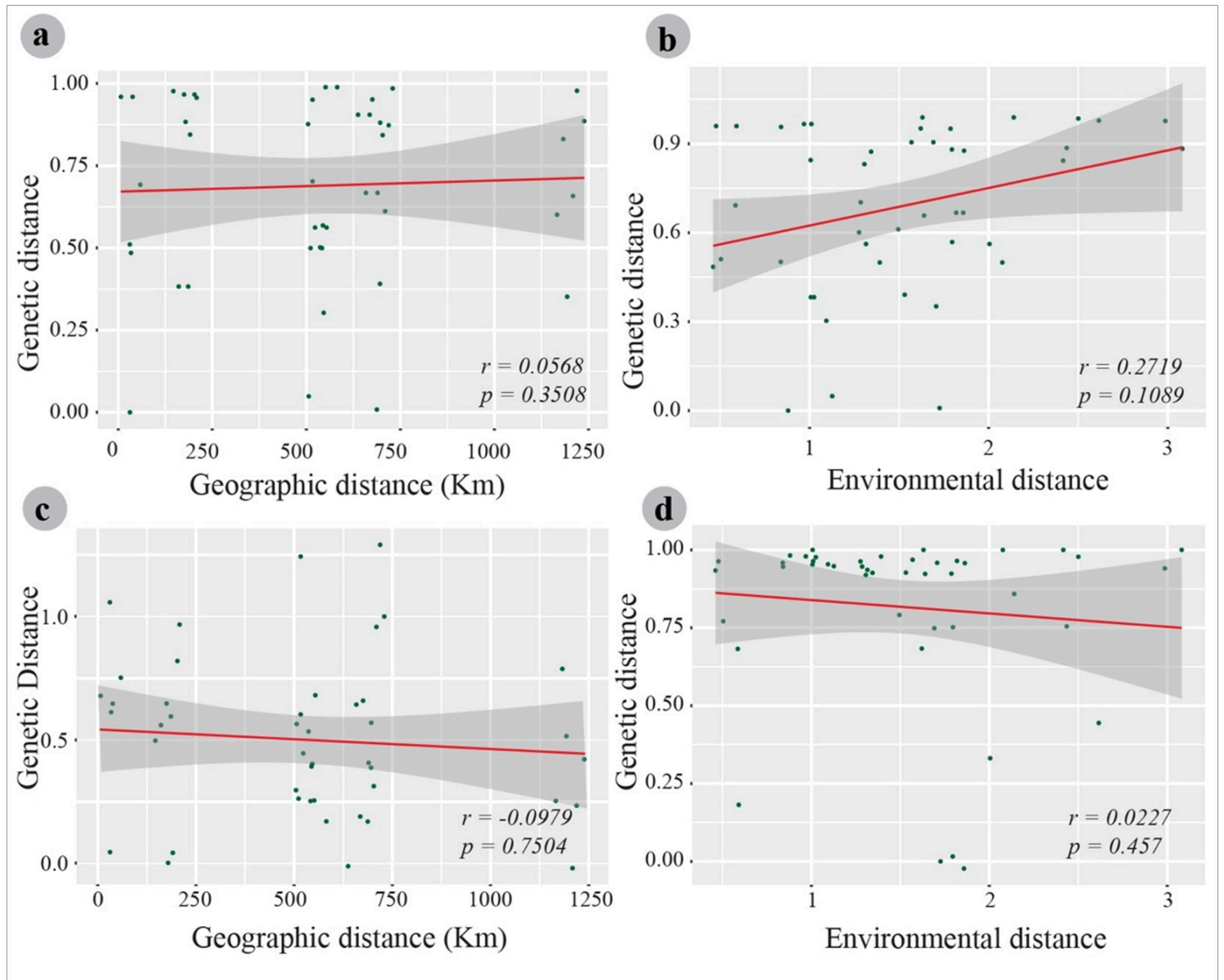


Figure 3

Linear regression lines showing the correlations between (a, c) genetic vs geographic (“Isolation by Distance” IBD), and (b, d) genetic vs environmental (“Isolation by Environment” IBE) for *Rheum nobile* based on (a, b) plastome-SNPs / (c, d) ITS dataset and bioclimatic variables. A Mantel/partial Mantel correlation coefficient (r) with significance at $p < 0.05$ is also shown in the figure.

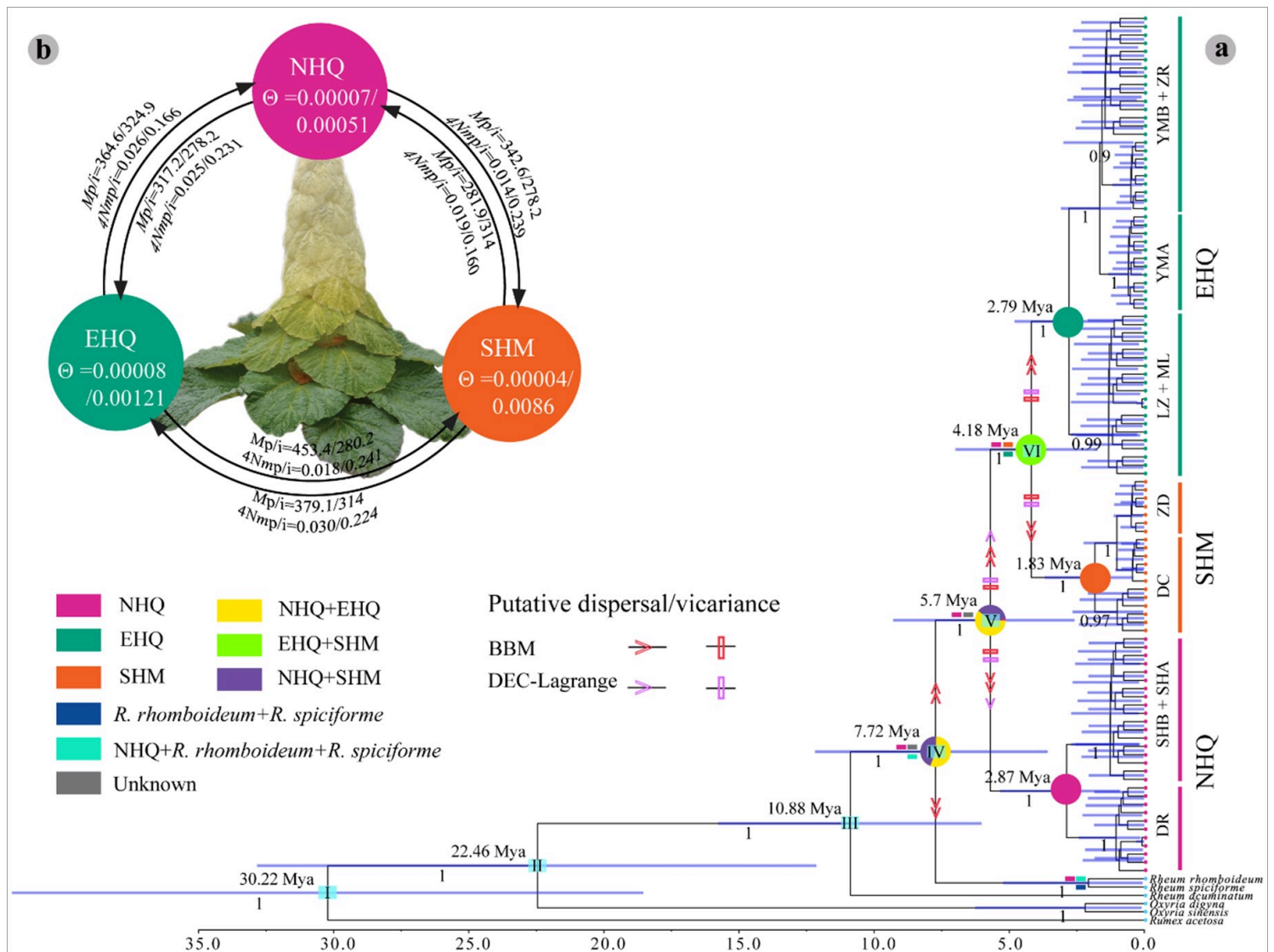


Figure 4

(a) Maximum credibility clade (MCC) tree with estimated divergence times (Mya; Million Years ago) of *Rheum nobile* estimated using BEAST and ancestral area reconstruction based on the Bayesian binary method (BBM) and dispersal–extinction–cladogenesis (DEC-Lagrange) method implemented in RASP. Blue bars represent the 95% highest posterior density intervals for node ages. Coloured charts at major nodes represent ancestral areas shown by both analyses. Pie charts for each node (i.e., IV–VI) are inferred by DEC-Lagrange and coloured rectangular boxes above and below the branch from BBM illustrate the likelihood proportions and probabilities of each ancestral range. Possible dispersal and vicariance events inferred by two different methods are indicated by coloured arrows and bars, respectively. Posterior probabilities based on BEAST are placed above the branches (shown for major nodes only). Node (I–IV) ages were calibrated from Zhang et al., 2022 [30]. (b) Gene flow estimation among three spatially structured populations of *Rheum nobile* based on plastome SNPs and ITS dataset. The mutation-scaled effective population size (Θ) is represented for each group (plastome/ITS), the mutation-scaled immigration rates is represented as M indicated by letters read as Mp/i , gene flow from

one group to another, and p for plastome and i for ITS. Similarly, gene flow values from one group to other were represented by $4Nmp/i$.

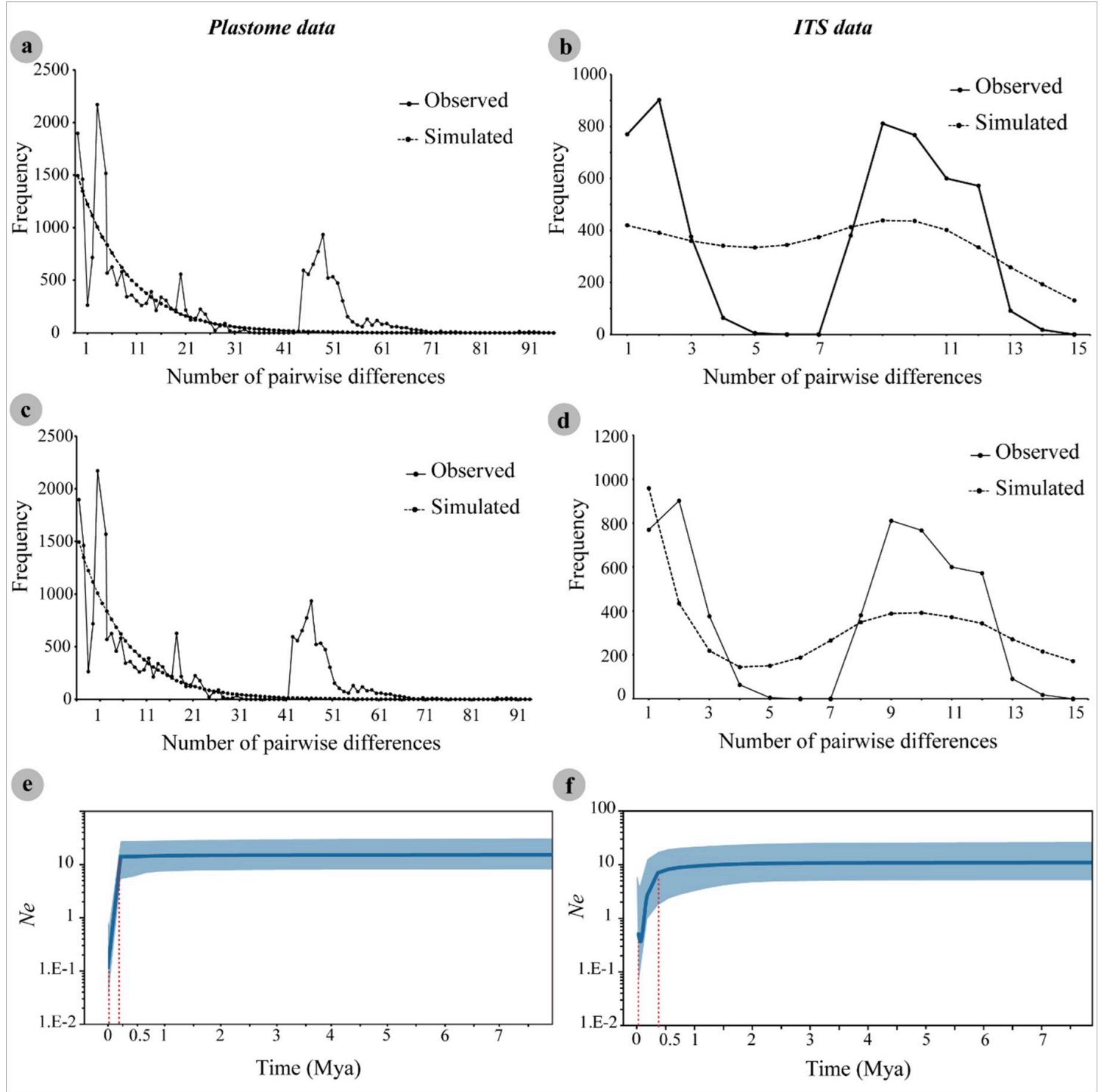


Figure 5

Mismatch distribution of *Rheum nobile*, under models of (a, b) demographic expansion and (c, d) spatial expansion [118], where the continuous lines with circle represent observed distributions, whereas dotted lines with circle represent simulated distributions. (e, f) Historical demographic trends of effective population sizes (N_e) constructed using the Bayesian skyline plot (BSP) approach. The x-axis in the plot

represents the timescale before the present, and the y-axis represents the estimated effective population size. Estimates of means are joined by a solid line, and the shaded range delineates the 95% HPD limits. Red dashed lines represent the time during which changes in effective population sizes took place.

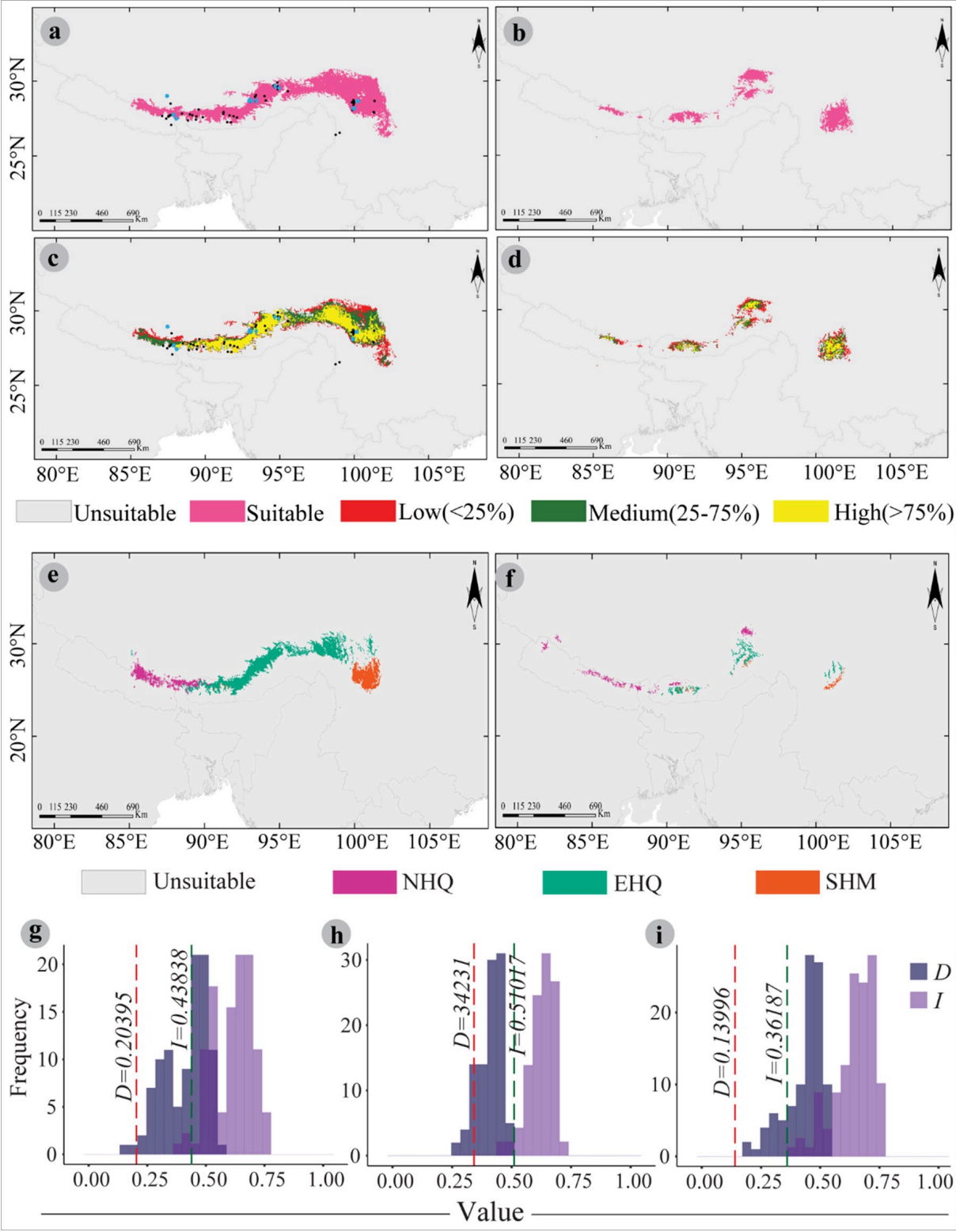


Figure 6

Habitat suitability projection of *Rheum nobile*, predicted by ensemble species distribution modeling (eSDM) under the assumption of the four climate scenarios: (a, c) Current (1990–2000), and (b, d) LGM

(c. 22 Kya). Black dots in a map (a) and (c) correspond to all available occurrence locations used in species distribution modeling and blue-coloured round, rhomboid, and square points (corresponding to NHQ, EHQ, SHM, respectively) represents populations under study. Three phylogroups based on ecological niche differentiation under three different scenarios: (e) current, and (f) LGM. Pairwise niche identity test between the phylogroups; (j) NHQ vs EHQ; (k) EHQ vs SHM; and (l) NHQ vs SHM, calculated by comparing the estimates of habitat suitability from *Ecospat* in R.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterial1.docx](#)
- [SupplementaryMaterial2.docx](#)