BMC Plant Biology

RESEARCH Open Access

Cryptic divergence in and evolutionary dynamics of endangered hybrid *Picea brachytyla* sensu stricto in the Qinghai-Tibet Plateau

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Abstract

Background The visual similarities observed across various plant groups often conceal underlying genetic distinctions. This occurrence, known as cryptic diversity, underscores the key importance of identifying and understanding cryptic intraspecific evolutionary lineages in evolutionary ecology and conservation biology.

Results In this study, we conducted transcriptome analysis of 81 individuals from 18 natural populations of a northern lineage of *Picea brachytyla* sensu stricto that is endemic to the Qinghai-Tibet Plateau. Our analysis revealed the presence of two distinct local lineages, emerging approximately 444.8 thousand years ago (kya), within this endangered species. The divergence event aligns well with the geographic and climatic oscillations that occurred across the distributional range during the Mid-Pleistocene epoch. Additionally, we identified numerous environmentally correlated gene variants, as well as many other genes showing signals of positive selection across the genome. These factors likely contributed to the persistence and adaptation of the two distinct local lineages.

Conclusions Our findings shed light on the highly dynamic evolutionary processes underlying the remarkably similar phenotypes of the two lineages of this endangered species. Importantly, these results enhance our understanding of the evolutionary past for this and for other endangered species with similar histories, and also provide guidance for the development of conservation plans.

Keywords Cryptic diversity, *Picea brachytyla*, Mid-pleistocene, Positive selection, Conservation

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Introduction

The worldwide decline in biodiversity poses a critical threat to the stability of ecosystems and the well-being of human populations. As such, researchers must deepen their understanding of biodiversity to include the often overlooked concept of cryptic diversity in which morphologically similar species are, in actuality, genetically distinct [1, 2]. Cryptic lineages, which often go undetected due to their subtle morphological distinctions, can offer insight into the evolutionary processes that contribute to the richness of life on Earth. This concept challenges the conventional emphasis on morphologically discernible diversity, a perspective which risks overlooking the vital genetic and phylogenetic intricacies crucial to ecosystem health and resilience [3–5]. Such oversight contributes to the "Darwinian shortfall", a phenomenon where the depth of biodiversity is underappreciated, and may potentially lead to the compromising of conservation efforts and of ecosystem stability [6]. The reclassification of the flatwoods salamander into two distinct species serves as a prime example of the importance of recognizing and accurately categorizing cryptic lineages as a precursor to implementing appropriate conservation strategies [7]. This revelation is crucial to acknowledge in conservation biology and equips managers and policymakers with a clearer understanding of the boundaries of population units for endangered species so as to refine conservation strategies accordingly [8, 9].

The advancement of molecular techniques has been instrumental in uncovering these hidden complexities within biodiversity and has thus led to a more refined understanding of species diversity [10–13]. These technological strides have enabled the detection of cryptic speciation across a wide array of organisms including birds [14], fish [15], plants [16] and primates [17], which goes to illustrate the profound impact that geographical and climatic changes have on species evolution [12, 18, 19]. Of special urgency, alpine flora have been significantly affected by historical climate fluctuations and continue to face challenges from ongoing global warming [20–22]. These climatic changes likely promote isolation and subsequent reconnection among neighboring populations, thus leading to habitat fragmentation, range shifts, and increased speciation due to new interactions and hybridization [23, 24]. The dynamic nature of biodiversity and the variability in ecological responses to global warming further highlight the complex interplay between environmental factors and genetic diversity [25]. An enhanced understanding of biodiversity that incorporates cryptic species will be critical to adopt in the context of global climate change and habitat loss, which are both expected to negatively impact the majority of current biodiversity throughout the 21st century [26].

Against this backdrop, the surge in accessible whole-genome data and high-quality reference genomes has transformed the study of biodiversity, enabling a deeper understanding of the genetic underpinnings of species [27, 28]. These genomic tools have brought about a new level of precision in identifying distinct populations and forming conservation units, thereby significantly enhancing conservation planning efforts [9, 29]. Furthermore, the application of genotype-environment association (GEA) approaches has revealed the genetic bases that play roles in unique adaptations and responses to environmental stresses. This enhanced understanding is particularly crucial for developing effective conservation strategies. For species facing unique environmental challenges, as is the case with cryptic lineages, it is essential to design conservation strategies that specifically address these unique conditions [30–33]. Such detailed analyses are invaluable, especially for rare or endangered species where comprehensive sampling may not be feasible. Even limited whole-genome sequence data can offer essential insight into demographics, phylogenetics, and potential hybridization [29].

Picea brachytyla, an endangered species of significant ecological and economic importance, is endemic to the Qinghai-Tibet Plateau (QTP), a region known for its biodiversity hotspots, cold temperatures and strong ultraviolet (UV) radiation. Alpine environments, such as the QTP, are characterized by narrow climatic niches and the limited dispersal ability of many cold-adapted species, which makes these species particularly vulnerable to the rapid changes brought about by climate change [34, 35]. This species presents a remarkable case of polyphyly within its genetic lineage [36]. It has generally been divided into two genetically distinct lineages, with the southern lineage aligning closely with the *Picea likiangensis* species complex (PLSC), and the northern lineage's unique lineage, referred to as *P. brachytyla* sensu stricto (s.s.), predominantly found in the mountains of Southwest China. *P. brachytyla* s.s. is believed to have originated from homoploid hybrid speciation events between the ancestor of the pre-diversification PLSC and *P. wilsonii* [37]. Research has predominantly focused on clarifying the origins of *P. brachytyla* s.s. via this speciation process, and its subsequent evolutionary developments have been less examined. The biodiversity in the mountains of Southwest China lends itself to cryptic speciation [38, 39], and identifying distinct lineages within *P. brachytyla* s.s. would be important for optimizing conservation efforts.

In this study, we conducted a transcriptomic analysis on 81 individuals derived from 18 populations of *P. brachytyla* s.s. spread across its known natural distribution area in Southwest China with the aim of investigating potential cryptic divergence within this endangered

species. We identified two cryptic lineages geographically separated by the mountains of Southwest China, and we further investigated genes associated with local adaptation. Additionally, we assessed the role of isolation by distance (IBD) and isolation by environment (IBE) in the formation of these lineages. By revealing hidden genetic diversity within the *P. brachytyla* s.s. species, our research highlights the importance of integrating genomic data into biodiversity assessments, which have traditionally relied heavily on morphological traits. Such assessments will be essential for the development of effective conservation strategies that accurately reflect the true complexity of local biodiversity. This approach helps to preserve the genetic diversity that supports the stability of ecosystems and enhances the ability of natural habitats to adapt to environmental change. In summary, our study of *P. brachytyla* s.s. serves as a poignant reminder of the rich genetic tapestry that underlies biodiversity and of the critical role that advanced molecular techniques play in unraveling this complexity for the benefit of conservation and ecosystem management.

Materials and methods

Data preparation

In this study, most of the raw RNA-seq *P. brachytyla* s.s. data was obtained from NCBI (NCBI BioProject Accession ID PRJNA846694, PRJNA401149). Additionally, 24 newly generated individuals were deposited into the National Genomics Data Center (NGDC, BioProject Accession ID PRJCA028370) (Tables S1, S2), bringing the total to 81 individuals from 18 populations. In addition, two individuals of *P. wilsonii* were downloaded from NCBI (NCBI BioProject Accession ID PRJNA401149) for use as an outgroup. For transcriptome sequencing, total RNA was extracted using TRIzol® Reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and was subsequently treated with DNase using the TURBO DNA-free™ Kit (Life Technologies, Thermo Fisher Scientific). The RNA was quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Sequencing libraries for each individual were prepared using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) and examined according to standard RNA-seq methodology [40–43]. The Illumina HiSeq 2500 platform was employed to generate 150-bp paired-end raw reads.

The geographical locations are shown in Fig. 1 and Table S1. For convenience, and based on geographical locations as well as the findings from Wang et al. [37], the populations were categorized into two lineages: braE and braW. The braE lineage includes the following populations: 06-WDL-17, 12-WDL-17, 14-WDL-17, 15-WDL-17, 21-WDL-17, WDL-17-CQ, and P_bra_QJB01, which is seven populations in total. The braW lineage comprises the populations 23-WDL-17, 26-WDL-17, 28-WDL-17, W17-01, W17-02, W17-03, WDL-17-HLG, P_bra_HST01, P_bra_HST06, WDL-17-LC-4, and YS-1, which is 11 populations in total.

Read mapping and variant calling

The raw sequencing data was processed using fastp v0.20.0 [44] with the following parameters: -f 10 -F 10 -x -g -c -q 15 -u 40 -n 5. The resulting paired-end reads were then aligned to the *Picea abies* reference transcriptome using BWA-MEM v0.7.10 [45] with default parameters. This reference transcriptome was an updated version with fungal transcripts removed [46, 47]. The Binary Alignment/Map files were sorted using SAMtools v0.1.19 [48] and duplicate reads were identified and marked using Picard v2.18.11 (http://broadinstitute.github.io/picard/), before being excluded from downstream analyses. Local re-alignment around INDELs was performed using GATK v3.8 [49].

Single nucleotide polymorphism (SNP) calling was implemented in SAMtools v0.1.19 (mpileup and BCFtools v0.1.19) [48]. The SNP dataset was then filtered using the following criteria: (i) only genotypes with a Phred-scaled likelihood of ≥20 were retained (those with low-quality scores were treated as missing values) to maintain a genotyping accuracy rate of at least 99%; (ii) only biallelic loci were retained for later population-level analyses; (iii) SNPs located in INDELs (including a 5-bp buffer) were discarded to reduce the false positive rate; (iv) SNPs with a non-reference allele frequency of <1% were removed; and (v) sites with depth (DP) < 10 per individual were considered missing, and the missing rate within each species was <50%. After filtering, a subset of high-quality, eligible SNPs was retained for analysis. For convenience, we refer to this filtered dataset as the D-ALL dataset.

It is noteworthy that four of the 18 populations consisted of only a single individual (P_bra_QJB01, P_bra_HST01, P_bra_HST06, and WDL-17-LC-4). To improve the robustness of our analyses, these single-individual populations were excluded from the D-ALL dataset, resulting in the creation of a subdataset referred to as D-SUB.

Population structure

The population structure was assessed using ADMIXTURE analysis (K=1–4), PCA, and a maximum-likelihood (ML) tree, based on both the D-ALL and D-SUB datasets. A model–based genetic clustering analysis was conducted using ADMIXTURE v1.30 [50] to identify genetic clusters. The cluster numbers (K) were set from 1 to 4. The optimal number of clusters was determined based on the cross-validation error (CV error) estimated for each cluster. A curve showing the change in CV

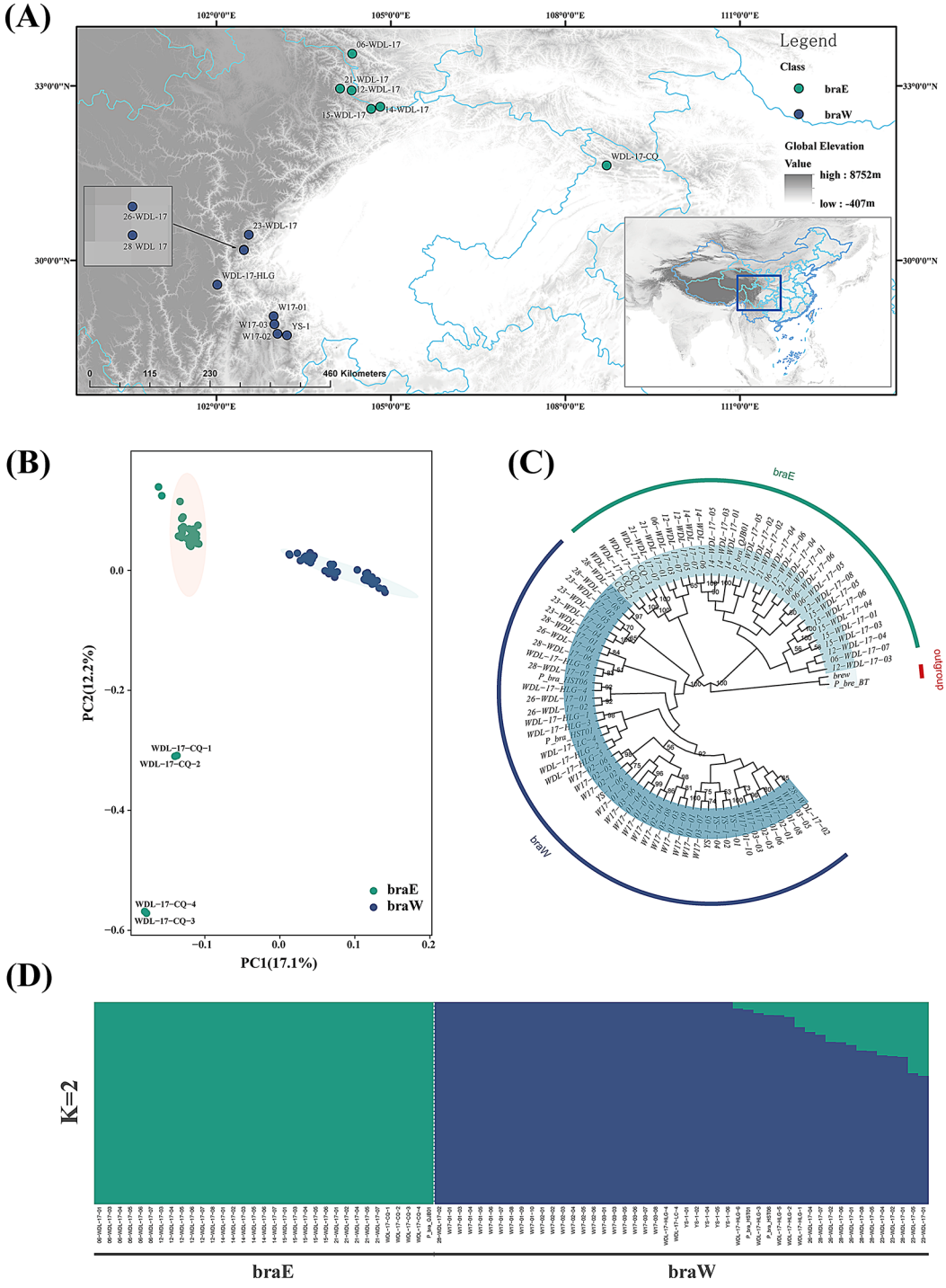

Fig. 1 Genetic distribution of and relationships between *P. brachytyla* s.s. samples. (**A**) Geographic distribution of *P. brachytyla* s.s. samples used in the analyses. (**B**) Principal component analysis (PCA) plots showing the first two principal components. (**C**) Maximum likelihood tree based on all 83 transcriptome sequences, with *P. breweriana* as the outgroup. (**D**) Bar plots indicating assignment probabilities from ADMIXTURE analysis of 81 transcriptomes, assuming two clusters (K = 2)

error across varying K-values was created (Fig. S1), and then the K-value with the lowest CV error was selected. Meanwhile, PLINK v1.90 was employed to conduct the PCA analysis [51]. To minimize linkage effects, the PLINK command "--indep-pairwise 50 5 0.2" was applied prior to the ADMIXTURE and PCA analysis. An ML phylogeny with 1,000 bootstraps was constructed using RAxML v8.1.20 in a GTR+GAMMA model [52] with *P. breweriana* serving as the outgroup. Finally, we visualized the phylogenetic tree using R package ggtree v3.6.2 [53].

Demographic history

We used all individuals to reconstruct the demographic history by applying two different methods. Firstly, we employed Stairway Plot v2.1.1 [54, 55] to deduce historical fluctuations in effective population size (Ne). This methodology leverages information from the site frequency spectrum (SFS) to infer past demographic dynamics. The folded SFS was derived using the realSFS function within ANGSD v0.941-8-gcc85d8e [56], based on fourfold degenerate SNP frequency spectra for each lineage (braE and braW lineages, respectively). For the analysis, default training utilised 67% of sites, with median estimates and 95% pseudo-confidence intervals (CI) derived from 200 replicates. The following assumptions were set for both lineages: a mutation rate of 4.01×10^{-8} mutations per site per generation and a consistent generation time of 50 years [57].

Secondly, the fastsimcoal2 v2.8.0.0 [58] approach was employed to estimate the divergence time and gene flow between the two lineages of *P. brachytyla* s.s. Only SNPs at fourfold degenerate sites were used to generate 2D-SFS using the realSFS function within ANGSD v0.941-8-gcc85d8e [56]. Six different demographic scenarios, varying in the presence or absence of migration, were evaluated (Fig. S2). For each scenario, 50 independent simulations were executed, with 100,000 coalescent simulations utilised for each likelihood computation. Additionally, 50 iterations of the likelihood maximisation procedure were performed to ensure accurate maximum likelihood (ML) estimates. To assess the degree of correspondence between the models and the actual data, we employed Akaike's Information Criterion (AIC) and Akaike's weight. The scenario that received the highest Akaike's weight was therefore considered to be the most appropriate. The calculations incorporated the assumptions of (i) a mutation rate of 4.01×10^{-8} per base per generation and (ii) a generational interval of 50 years [57]. To establish 95% confidence intervals, a parametric bootstrapping method was employed, and 100 separate trials were run.

Environmental variable extraction

Both to investigate the influence of the isolation by environment (IBE) and to perform the genotype-environment association analyses (GEAs), environmental variables were extracted according to the geographic coordinates. Four *P. brachytyla* s.s. populations downloaded from online sources were excluded from the environmental analysis due to the lack of geocoordinates and the presence of only one individual per population (Table S1). The 19 bio-climatic variables we collected were sourced from Worldclim v2.1 (https://www.worldclim.org), at a spatial resolution of 30 arc-seconds, according to the geocoordinates of the other 14 populations.

Effects of isolation by distance (IBD) and IBE on genetic structure

The influence of both IBD and IBE on the genetic structure of the remaining 14 populations of *P. brachytyla* s.s. (D-SUB dataset) was evaluated using two distinct analytical approaches: the partial Mantel test [59–61] and partial redundancy analysis (RDA) [62, 63].

The first approach involved the use of the partial Mantel test to identify the significance of geographic and environmental distances relative to genetic distances. This was conducted using R package Vegan v2.6-2 [64] with 9999 permutations. All distances were presented in the form of pairwise comparison matrices. The calculation of geographic distance was based on the Vincenty algorithm, which is used to compute the distance between two points on a great circle using their longitudinal and latitudinal coordinates and accounts for the ellipsoidal shape and irregularities of the Earth [65]. The environmental distance matrix was constructed based on the Euclidean distance metric. Prior to the construction of the environmental distance matrix, we calculated the Pearson correlation coefficients among 19 climate variables (Fig. S3). We removed highly collinear climate factors using an $r=0.7$ threshold, and the remaining climate factors (bio1, bio2, bio12 and bio15) were retained as inputs for the matrix. Furthermore, we employed the VCFtools v0.1.14 [66] to calculate the genetic distance among populations.

In the second approach, we employed a partial RDA to ascertain the impacts of geography and the environment on genetic structure. The R package vegan provided the *rda* function that was employed in this study.

RDA

Given the critical importance of environmental variables in explaining genetic variation (see results), we identified only those SNPs associated with environmental factors. Using the R package Vegan v2.6-2 [64], we conducted RDA to identify candidate SNPs associated with local adaptations in response to the multivariate environment. Before conducting RDA, we transformed the longitudinal and latitudinal coordinates of individuals in the D-SUB dataset into Moran's eigenvector maps (dbMEM1 to dbMEM6) to represent geographical factors. We adopted an RDA model recommended by Thibaut Capblancq [67], calculated eigenvalues for each constrained axis, and selected the first two axes for further investigation due to their significant contribution to genetic variation. Subsequently, we performed gene ontology (GO) enrichment analysis containing candidate SNPs. GO annotations for the corresponding protein sequences were identified using the EGGNOG-MAPPER tool (http://eggnogmapper.embl.de), followed by enrichment analysis using TBtools v2.088 [68], with an 'evalue' threshold of 1×10^{-5}.

Detection of the selective signals

We used the D-SUB dataset to detect selective signals. A sliding-window methodology was employed, with 10-kb windows and a 1-kb step-size, to quantify polymorphism levels (θπ, pairwise nucleotide variation) within both the braE and braW lineages. Genetic differentiation (F_{ST}) between lineages was also assessed, with both analyses conducted using VCFtools v0.1.14 [66]. For enhanced interpretability, we $\log_2$-transformed the nucleotide diversity ratios (θπ ratios, θπbraE/θπbraW). We empirically identified regions with strong selective sweep signals by selecting windows with both the highest and lowest log2(θπ ratios) (top 10%: log2(θπbraE/θπbraW) ≥ 0.695652891 or log2(θπbraE/θπbraW) ≤ -0.5275865) and high F_{ST} values (top 10%: F_{ST} ≥ 0.0400967) [69]. Subsequently, candidate genes were identified for each region, and these underwent a GO enrichment analysis to obtain functional annotations. Additionally, these candidate genes were aligned with the *Arabidopsis thaliana* proteome using the Basic Local Alignment Search Tool (BLASTX).

Results

SNP calling and population structure

Our *P. brachytyla* s.s transcriptome data includes 81 individuals, with a total size of 521.41 Gb and an average size of 6.437 Gb per individual (Table S2). We used the *P. abies* transcriptome [36, 46] as a reference genome for SNP identification and ultimately identified a total of 295,656 SNPs for D-ALL and 240,780 for D-SUB.

Phylogenetic analysis of the SNP loci showed that there were two lineages of *P. brachytyla* s.s., corresponding to the pre-categorized lineages of braE and braW (Table S1; Fig. 1). At the root of the phylogenetic tree was braE lineage, followed by braW lineage (Fig. 1C). However, two lineages of *P. brachytyla* s.s. (braE and braW) did not form monophyletic clades, and the bootstrap values at the internal nodes are relatively low. In addition, clustering analysis software ADMIXTURE was utilized to analyze the population structure of *P. brachytyla* s.s. The CV error was at its lowest when the clustering number was K=1 (Fig. S1), which means that all the populations were confirmed to belong to one species. Nevertheless, upon contemplation of K=2, a discernible population structure aligning with the phylogenetic tree emerged, specifically signifying the segregation of the braE and braW lineages (Fig. 1D). In the PCA results, the first principal component (PC1) explained 17.1% of the genetic variance, and the second principal component (PC2) explained 12.2%, which separated 48 individuals in braW lineage from the rest of the individuals (Fig. 2B). Accordingly, the results of the ADMIXTURE, phylogenetic analysis and PCA jointly revealed the differentiation between braE and braW lineages at the molecular level. Notably, the findings from the population structure analysis aligned with the geographic distribution of the sampling sites.

Considering that four of the 18 populations consisted of only one sample (P_bra_QJB01, P_bra_HST01, P_bra_HST06, and WDL-17-LC-4), we reanalyzed the genetic structure using D-SUB dataset. This subsequent analysis confirmed that the species has diverged into two distinct lineages (Fig. S4).

Demographic history

Our Stairway Plot analysis showed that, throughout their histories, neither of the two lineages underwent significant population expansion. However, the effective population size (Ne) of braW lineage had decreased by approximately two-thirds from past levels to the present. Simulations indicated that the quantity of the two lineages split at approximately 800 thousand years ago (kya) (Fig. 2A). The braE lineage showed an initial decline in population size, followed by an increase, peaking around 400 kya before stabilizing. In contrast, while the braE lineage's Ne remained relatively stable in recent times, the braW lineage experienced a continuous decline starting around 5 kya, and only restabilized around 2 kya.

The demographic model with the best fit, as determined by fastsimcoal2, was model 5 (Table S3; Fig. S2). This model estimated an ancestral population size of 28,846 (95% CI: ~28,277−29,518), with the braE and braW lineages diverging at about 444,800 years ago (95% CI: ~432,152–504,349). The current Ne was estimated to be 21,222 (95% CI: ~20,074−22,220) for the braW lineage and 41,667 (95% CI: ~40,528−43,565) for the braE lineage. Gene flow from the braE lineage to the braW lineage was higher than that in the opposite direction (5.39E-04 vs. 4.87E-04). The simulation also suggests that gene flow between the two lineages commenced at 83.5 kya (95% CI: ~66.0-118.3 kya) and concluded at 8.4 kya (95% CI: ~2.6–12.2 kya) (Table S4; Fig. 2B).

Effects of IBD and IBE on genetic structure

After removing highly collinear climate factors, four variables were retained for the Mantel test: BIO1 (Annual Mean Temperature), BIO2 (Mean Diurnal Range (Mean of monthly (max temp - min temp)), BIO12 (Annual Precipitation), and BIO15 (Precipitation Seasonality). The absolute values of the Pearson correlation coefficients between these four retained environmental factors were all below 0.7 when paired with each other (Fig. S5). The Mantel test results indicate that both geographic and environmental distances were significantly correlated with genetic distance (Table S5). However, geographic distance (Spearman's r=0.7847) had a stronger influence on genetic structure than environmental distance (Spearman's r=0.6952). Given the high degree of interaction between geography and environment (Fig. 2C),

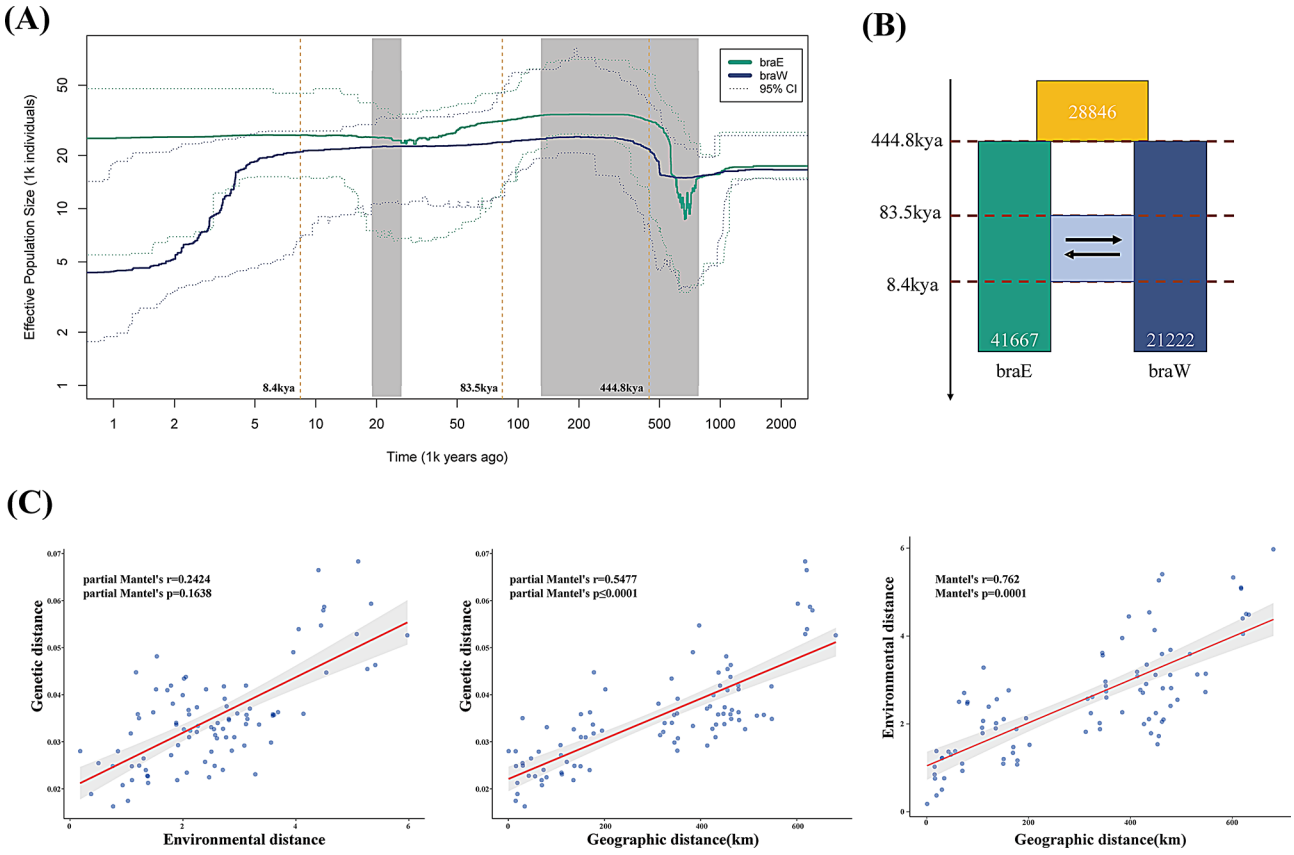

Fig. 2 Demographic history and relationships between genetic, geographic and environmental distance of *P. brachytyla* s.s. (**A**) Changes in effective population size (Ne) over time in braE and braW lineages inferred according to the Stairway Plot method. (**B**) Schematic of demographic scenario modeled in fastsimcoal2. (**C**) Relationships from left to right: genetic distance vs. environmental distance, genetic distance vs. geographic distance, and environmental distance vs. geographic distance

we subsequently employed partial Mantel test to mitigate any potential interaction. The partial Mantel test results indicate that genetic distance exhibited a significant relationship with geographical distance (Spearman's r=0.5477, $p \leq 0.0001$), but not with environmental distance (Spearman's r=0.2424, p=0.1638) (Fig. 2C; Table S5).

In order to circumvent the limitations of a particular model, we further investigated the independent contributions of environmental and geographic variables to genetic variation using partial RDA. As shown in Table S6, when controlling for environmental variables (partial model: gen. ~ geo. | env.), geographical variables had a significant effect on genetic variation and explained 1.32% of genetic variation on their own (adjusted R^2=0.0132, $p \leq 0.001$). Similarly, when controlling for geographical variables (partial model: gen. ~ env. | geo.), environmental variables significantly influenced genetic variation, explaining 1.27% of genetic variation alone (adjusted R^2=0.0127, $p \leq 0.001$) (Table S6). The joint geographical and environmental variables (confounded) explained 2.64% of the genetic variation. The sum of genetic variation explained by geographic and environmental variables together (model: gen. ~ geo.+env.) was 5.23% (Table S6). It can thus be deduced that, in alignment with the outcomes of the partial Mantel test, geography had a greater impact on genetics than did the environment.

As the two aforementioned models demonstrate, both geographical and environmental variables exerted a notable influence on the genetic differentiation of *P. brachytyla* s.s. lineages. However, geographical variables had a more pronounced impact than environmental variables.

Identification of genetic variants with local adaptation using RDA

To further investigate the association between genotype and the environment, we employed RDA to examine the SNPs linked to environmental variables. The environmental data remained consistent with the previously selected four environmental factors (Fig. S5). The results of the RDA analysis indicate that, with the exception of the geographic impact on the environment, genetic variation was explained by RDA axes 1, 2, and 3 at rates of 28.93%, 25.95%, and 22.81%, respectively (Fig. S6A) ($p \leq 0.001$). Bio1 and Bio2 exhibited higher loads on RDA axis 1, while Bio12 and Bio15 had significant loads on

RDA axis 2 (Fig. 3A). Further, we examined the separation of these populations under the model and found that, overall, the two lineages were not significantly distinguished by the environment (Fig. 3B).

Through RDA, we identified 13,069 SNPs in total that were distributed across 4,818 genes (Table S7; Fig. 3A). These 4818 genes include one gene, comp96487_c6_seq1, that was found in a previous article and is associated with water deprivation [37]. In addition, we designated genes with an occurrence frequency exceeding twice that of the other lineage (braE or braW lineage) as prominent genes. In total, 923 genes were filtered, comprising 237 prominent genes in the braE lineage and 686 prominent genes in the braW lineage (Table S7). We performed GO enrichment analysis on this gene subset and found that some belonged to function categories associated with adaption to the environment, including "regulation of response to stimulus", "response to cold", "response to water", "response to salt", "regulation of root development", "response to temperature stimulus" and so on (Fig. S7; Tables S8, S9).

Notably, we employed an additional RDA model to preserve the influence of geographical factors on the environment and to observe whether the divergence of these populations became distinct. In this additional model, RDA axis 1 accounted for a significantly prominent share of the explanatory power (41.95%, Fig. S6B) and roughly segregated the 14 *P. brachytyla* s.s. populations into either the braE or the braW lineage (Fig. 3C). In contrast to the initial RDA model, which excluded geographic impact, the clear differentiation in lineage observed in the additional RDA model provides great insight into the divergence process of this species. The additional model indicates that IBD played a primary role, while also highlighting the complex interplay between environmental and geographical factors. This finding is consistent with our deduction in the context of IBD/IBE.

Positively selected genes in *P. Brachytyla* s.s

Selective sweep regions were identified from *P. brachytyla* s.s. SNPs (D-SUB) by combining π and F_{ST} information and selecting outliers for both metrics. A total of 547 genes were detected after annotating these regions, with 180 belonging to braE lineage and the rest belonging to braW lineage (Table S7; Fig. 4A). GO functional enrichment analysis of these 547 genes revealed 111 significantly enriched categories (Tables S10, S11), including "seed development," "fruit development," "regulation of photoperiodism, flowering," "regulation of developmental process," "response to stimulus," "seed germination," "regulation of reproductive process," "seedling development" and "photoperiodism". These nine categories related to plant growth and development directly (Fig. 4B and C). Research indicates that the GO terms related to plant growth and development often intersect with terms relevant to environmental adaptation [70, 71], highlighting the potential importance of studying how *P. brachytyla* s.s. adapts to climatic changes.

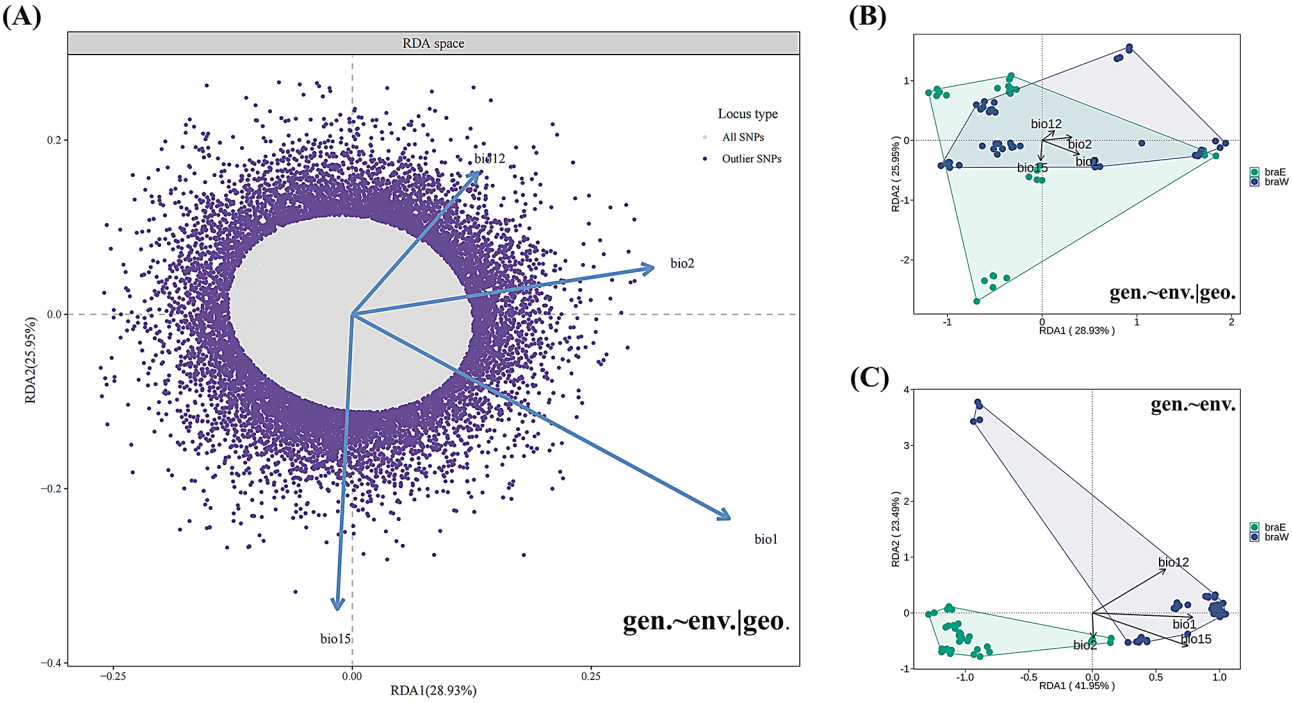

Fig. 3 Redundancy analysis (RDA) plot for *P. brachytyla* s.s. (**A**) RDA plot for SNPs based on the first two RDA axes. (**B**) RDA plot for 81 *P. brachytyla* s.s. individuals based on the first two RDA axes, controlling for geographic variables and (**C**) without controlling for geographic variables

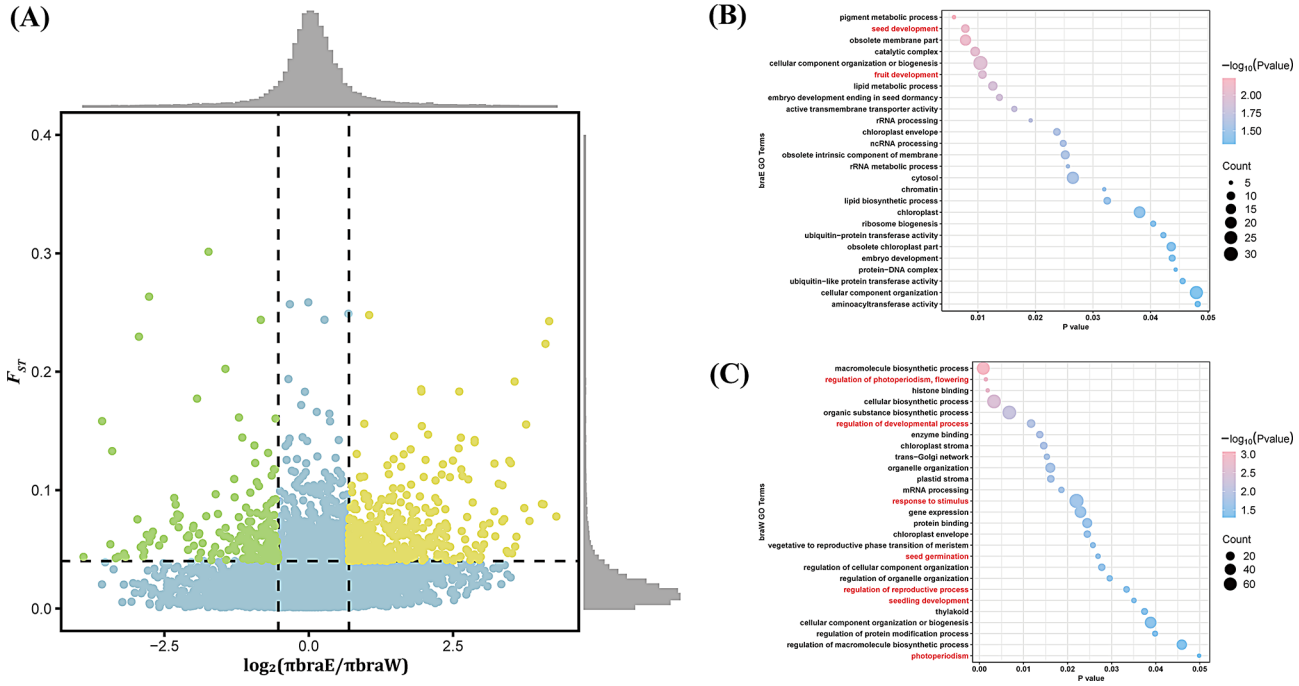

Fig. 4 Genomic regions with strong selective sweep signals in the two lineages of *P. brachytyla* s.s. (**A**) Genomic regions selected in braE lineage (green dots) and braW lineage (orange dots). Black dashed lines represent the top 5% thresholds. (**B**) GO enrichment results for positively selected genes in braE lineage and (**C**) in braW lineage

To identify the proteins with the highest similarity scores, the *A. thaliana* proteome was searched using BLASX. We inspected the 15 genes with the highest similarity scores and found that four of them were related to plant growth and development (Table S12). Specifically, for photosynthesis, we found that comp49356_c0_seq1 and comp93775_c1_seq1 showed significant similarity to known protein sequences involved in photosystem I and photosystem II, respectively. This suggests that these two genes may encode proteins closely related to the function of photosynthesis [72, 73]. In addition, comp80667_c0_seq2 was annotated as encoding a myosin that is primarily expressed during reproductive development. It is a member of a subclass of actins composed of ACT2 and ACT8, and its mRNA is strongly expressed in leaves, roots, stems, flowers, pollen, and siliques [73]. Further, after alignment, comp41064_c0_seq1 was considered a member of the TOPLESS gene family, which plays a crucial role in the regulation of plant development and hormone signaling pathways [74]. The results of the BLASTX analysis further indicated the potential significance of environmental adaptation during the differentiation of this species.

Discussion

The present study aimed to investigate the genetic divergence within *P. brachytyla* s.s., an endangered plant species. This was achieved by analyzing the whole transcriptome dataset spanning the species' distribution. The results revealed the existence of two cryptic local lineages. The divergence observed was influenced by geographic and environmental isolation during the Mid-Pleistocene epoch, which then led to the different evolutionary trajectories within the species. The braW lineage has notably declined over the last 8,000 years, while the braE lineage has remained stable. A multitude of gene variants associated with environmental adaptation and signals of positive evolution were identified, thus indicating evidence of dynamic evolution despite phenotypic similarities. These findings are pivotal for developing targeted conservation strategies to ensure the protection of this endangered species and for advancing our understanding of its adaptive mechanisms.

Cryptic divergence and demographic history after homoploid hybrid speciation

The results of our study, which employed the ADMIXTURE, PCA and phylogenetic tree analyses, indicate that *P. brachytyla s.s.* comprises two distinct genetic clusters (Fig. 1). However, despite this genetic differentiation, a relatively low divergence between the clusters (F_{ST} = 0.01594) was evident in the phylogenetic tree, where braE lineage and braW lineage did not form two distinct monophyletic clades and node supports were low. The phylogeny pattern mirrors that of *P. crassifolia* [12], and may be attributed to potential gene flow, incomplete lineage sorting, or historical introgression, phenomena commonly observed in *Picea* species [75, 76].

These factors are critical to consider when interpreting phylogenetic results, especially in species with complex evolutionary histories, such as *P. brachytyla* s.s., which originated through homoploid hybrid speciation [37]. Similarly, in the PCA, individuals such as WDL-17-CQ-1, WDL-17-CQ-2, WDL-17-CQ-3, and WDL-17-CQ-4 did not cluster tightly within the braE lineage. This is consistent with their geographical distribution, as the WDL-17-CQ population is located at a considerable distance from the rest of the braE lineage (Fig. 1A). These observations further underscore the complexity of genetic structuring within these lineages and highlight the possible role of historical demographic events or genetic exchange in shaping the current genetic landscape of *P. brachytyla* s.s [37]. This conclusion is further corroborated by the results of IBD/IBE and RDA (Figs. 2 and 3; Table S5). Such high genetic diversity within each lineage enhances the species' adaptive potential for responding to environmental changes because diverse genetic resources are critical for adjusting to new environmental challenges [77, 78].

During the Pleistocene period, around 800 kya, the eastern QTP witnessed significant climatic shifts due to the largest glaciation of the era [79]. This major glaciation led to a decline in population size among the two genetic lineages. As the glaciation retreated, environmental conditions ameliorated, resulting in a subsequent increase in population size in these two lineages (Fig. 2A). The population size of the braE lineage experienced a sharp decline followed by a rapid expansion during this period. In contrast, the population size variation in the braW lineage, which is predominantly located in the Hengduan Mountains (HM), was more moderate. This is consistent with the limited glacial extensions in the HM between 460 and 710 kya [80, 81]. By approximately 440 kya (95% CI, ~432–504 kya; Table S4), during the Marine Isotope Stage (MIS) 12 (~478–424 kya) glacial [82], the two lineages began to diverge. This stage represents one of the most significant glacial intervals in the Quaternary period, characterised by the largest ice volumes observed throughout this geological epoch [83, 84]. It also saw significant vegetational shifts. For example, the cold and arid conditions of MIS 12 led to the retraction of temperate forests and the expansion of steppe vegetation [85], which likely promoted species diversification via allopatric speciation that was facilitated by geographically isolated mountains [38, 39, 86, 87]. The alteration of topography and the climatic changes associated with mountain uplifts can fragment species distributions, thus leading to reduced gene flow between isolated populations. This process initiates an allopatric divergence that can ultimately drive populations towards speciation [88, 89]. It is noteworthy that the divergence time within the *P. brachytyla* s.s. species predates that of *P. crassifolia* and *P. asperata*, thereby suggesting a more ancient evolutionary split [12, 90]. However, despite this ancient divergence, the relatively narrow distribution of *P. brachytyla* s.s. and the limited barriers to its gene flow have resulted in a lower level of genetic differentiation compared to that observed in *P. crassifolia* or *P. asperata*.

The temperatures in the QTP remained low until the late Ionian stage, approximately 300–126 kya [79]. Despite this, multiple minor glaciations occurred from 170 kya onwards, notably during the intervals of 130−90 kya, 75−60 kya, and 50−30 kya [91–93]. As temperatures increased, the impact of glaciations on the two genetic lineages was minimal, although the braE lineage was more adversely affected than the braW lineage. Notably, the smaller glaciers during the 75−60 kya and 50−30 kya periods appeared to have little to no impact on the lineages located along the eastern edge of the QTP and adjacent areas. It can, therefore, be reasonably concluded that the conditions on the QTP improved significantly following the glacial retreat during the 130−90 kya period. This environmental change facilitated a second contact between the genetic lineages and supported gene flow. Our results confirm the above assumption, that isolation between these lineages persisted until 83.5 kya, at which point gene flow commenced (Table S4; Fig. 2B). This gene flow ceased around 8.4 kya, coinciding with the arrival of hunter-gatherer groups in the high Tibetan Plateau and the onset of agriculture around 5.2–10 kya [94, 95]. This shift led to a significant decline in the braW lineage. In contrast, the braE lineage, deeply entrenched in the mountains, remained relatively unaffected by human activity. This observation is consistent with the results of the IBD and IBE analyses, which indicate that geography played a significant role in the genetic differentiation between the two lineages (Fig. 2C; Table S1).

The biodiversity of the Pleistocene epoch, which had long been relatively understudied due to cryptic diversity, is now being illuminated in recent studies. These studies reveal that significant environmental changes, such as those on "sky islands" [96, 97], and interactions with pollinators and herbivores [98, 99], have likely driven cryptic speciation processes [100, 101]. Our research underscores the significant role of allopatric speciation, as demonstrated by the two distinct metapopulation lineages in *P. brachytyla* s.s. (Figures 1 and 2). Additionally, our findings highlight the impact of recent Pleistocene glaciation as a substantial evolutionary force. The geographic separation and recent divergence of these lineages suggest the possibility for cryptic speciation, thus posing challenges for classification under the traditional biological species concept that emphasizes reproductive isolation and distinct morphological traits [4]. This scenario emphasizes the necessity to recognize and conserve emerging species to maintain biodiversity [102],

especially in the face of ongoing global climate changes that threaten numerous species [103]. This comprehensive approach not only advances our understanding of Pleistocene biodiversity but also enhances our ability to protect genetic treasures in a rapidly changing world.

Evidence for polygenic adaptation to climate

Geographic and environmental isolation are critical in driving genetic divergence as populations adapt to specific ecological niches [104, 105]. The genetic diversity within a species, sculpted by selection pressures and its demographic history, underlies its capacity to withstand environmental change. Investigating the interplay between demography, geography, and selection illuminates the distribution of genetic variation across landscapes and the evolutionary potential of species in the context of climate change [106–109]. In the case of *P. brachytyla* s.s., climatic oscillations during the Quaternary likely enhanced allopatric divergence and local adaptation [39, 109–112], with populations undergoing cycles of alternating between distribution retreat and postglacial expansion [113–115]. These demographic shifts have impacted genetic variation patterns within and among populations [116].

Although the partial IBD analysis indicates that geographic factors significantly influenced the within species divergence, the initial partial IBE analysis suggests that the divergence between the two lineages was minimally impacted by environmental factors (Fig. 2C). However, further RDA analysis revealed that certain environmental variables accounted for 1.27% of the observed genetic variation, which was slightly less than the 1.32% accounted for by geographic variables (Fig. 3; Table S6). This suggests a subtle yet significant environmental influence on genetic diversity and highlights the nuanced roles that both geographic and environmental factors play in shaping the genetic landscape of this species.

In line with similar studies in other species [12, 117–120], local adaptation in *P. brachytyla* s.s. appears to be highly polygenic, where multiple genes contribute to a species' adaptation to its local environment. The Mid-Pleistocene epoch was characterized by significant environmental upheavals, which, as evidenced in our study, have left a lasting imprint on the genetic makeup of species. For instance, we identified allelic variations in 4,818 genes that were correlated with environmental factors within the RDA (Table S7). The functional enrichment of these genes revealed involvement in processes related to development, catabolic, metabolic process and stress response (Tables S8, S9).

Variations in geographic and environmental selection can promote local adaptation, thus leading to specific genomic regions showing elevated F_{ST} and reduced π [121–123]. In braE lineage, our analysis highlighted positive selection in genes critical for lipid biosynthesis, pigment metabolism, and various stages of reproductive development (e.g. embryo development, fruit development and seed development) (Table S10). In contrast, braW lineage exhibited clear selection signatures in genes involved in stress response, photoperiodism, flowering, immune regulation, and other stimulus responses (Table S11). These genetic variations have driven local adaptation and divergence among lineages, thereby enhancing the species' ability to thrive under diverse environmental conditions and leading to distinct evolutionary paths for each lineage. This study emphasized the intricate relationship between genetic diversity and environmental pressures, underscoring the necessity to conserve such diversity to maintain adaptive capacity in a changing climate.

Threats of climate change to this species and conservation implications

P. brachytyla s.s. exhibits cryptic diversity [37, 124] (Fig. 1), making it crucial to recognize and preserve these lineages as they reflect the species' intricate genetic responses to environmental changes. However, the existence of such cryptic diversity also presents challenges for conservation. It has been demonstrated that species which have undergone unrecognized evolutionary changes are particularly vulnerable [125]. This is evident from the sharp decline of the western lineage, while the eastern lineage has remained stable (Fig. 2A). The polygenic nature of local adaptation in *P. brachytyla* s.s. suggests the potential for an adaptive lag due to the long generation times typical of forest trees. This lag may impede the ability of these trees to adapt to rapid climate change and suggests that current adaptive strategies may be inadequate for coping with ongoing environmental shifts [126, 127]. This scenario highlights the necessity for conservation strategies that not only preserve genetic diversity but also enhance the adaptive potential of populations in the context of accelerated climate change.

In light of these challenges, it is imperative that conservation strategies for *P. brachytyla* s.s. be proactive and multifaceted. First and foremost, the preservation of genetic diversity must be at the foundation of any plan. Protecting existing habitats of both lineages of *P. brachytyla* s.s. can achieve this goal [128, 129]. The selection of these areas should be based on ecological criteria that account for future climate scenarios, thereby ensuring that the habitats will remain suitable as the climate changes. Secondly, connectivity between fragmented populations must be enhanced. Creating new habitat corridors to facilitate gene flow can assist in maintaining genetic diversity and enhancing the species' overall adaptability [130, 131]. This strategy is of particular importance in enabling the dissemination of adaptive

traits that may emerge in response to changing environmental conditions [132]. However, it is essential to carefully monitor gene flow to ensure that local adaptations, which may be vital for each lineage's survival in its specific environment, are not compromised [133].

In addition to in situ conservation, ex situ strategies such as the creation of germplasm banks and seed repositories are critical [134]. These banks can preserve genetic material from different populations, thus providing a resource for the restoration and reinforcement of populations suffering under climate change [135, 136]. Cultivating plants from these banks in diverse geographic settings may also help researchers understand how different populations respond to varying climatic conditions, which would provide invaluable information for future conservation efforts [137]. Furthermore, it is essential to understand the ecological roles and interactions of *P. brachytyla* s.s., such as pollinator relationships and competition with other species. These interactions often influence reproductive success and survival, factors that are directly impacted by climate change [138]. It is therefore imperative that conservation plans account for potential shifts in ecological dynamics and are capable of responding to real-time changes in ecosystem conditions. It is also crucial to foster public awareness and engagement in conservation efforts. It is recommended that local communities be educated about the importance of *P. brachytyla* s.s. and its many ecological roles so as to foster support for conservation initiatives. Community-based monitoring programs may also enhance the effectiveness of management strategies by providing early detection of ecological changes that might affect the species.

The study also underscores the need for further investigation into the functionality of the adaptive genes identified. Understanding how these genes contributed to adaptation in different environmental contexts is crucial for uncovering the mechanisms that allow species to evolve and persist in changing environments. This knowledge could inform targeted conservation strategies aimed at boosting species' adaptive capacity [139, 140]. Additionally, exploring the genetic basis of adaptation in closely related species can reveal key evolutionary processes [141]. Comparative studies of related species facing similar environmental challenges can help identify shared and unique adaptive strategies. This comparative approach can provide a more comprehensive understanding of the genetic factors that drive species survival and evolution in dynamic ecosystems [142].

In conclusion, the conservation of *P. brachytyla* s.s. necessitates a comprehensive approach that integrates genetic, environmental, and ecological data. The recognition of cryptic species is of paramount importance for scientific advancement and the formulation of efficacious conservation strategies, particularly in biodiversity hotspots where the dynamics of cryptic speciation exert a significant influence [32, 97]. By implementing strategies that enhance the adaptive capacity of this species, conservationists will allow *P. brachytyla* s.s. to not only survive, but to thrive in the context of global climate change. This comprehensive approach to biodiversity conservation will serve as a model for the preservation of other species around the world within similarly affected mountainous regions.

It is important to note that the genetic differentiation, including all PSGs identified in this study, was based on transcriptomic data. While genetic variants in coding regions can directly influence protein function, research suggests that greater divergence often occurs in noncoding regions [143–145]. Exploring genetic divergence in these noncoding regions of *P. brachytyla* s.s. could provide valuable insights into adaptation. Additionally, our current analysis did not differentiate between neutral loci and potential adaptive SNPs, which limits our understanding of how natural selection versus neutral evolutionary processes contribute to speciation. Analyzing both neutral and adaptive SNPs, can reveal the genetic landscape of divergence and provide a more nuanced view of local adaptation [146, 147]. Incorporating adaptive SNPs would allow us to better assess the risk of nonadaptedness and predict the species' capacity to respond to environmental changes [143]. Future research should involve whole-genome sequencing, a more detailed analysis of adaptive SNPs, and functional validation of candidate alleles. Additionally, common garden experiments will be crucial to thoroughly characterize the relationship between local adaptation and genetic divergence in *P. brachytyla* s.s., offering deeper insights into the underlying mechanisms driving these evolutionary processes and helping to inform conservation strategies.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05851-6.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

We would like to thank Elizabeth Tokarz at Yale University for his assistance with English language and grammatical editing of the manuscript. We thank the comments on the manuscript from anonymous reviewers.

Author contributions

D.R. planned and designed the research. S.L., L.L., W.L., D.W., D.R., H.Z., Q.L., and L.M. conducted fieldwork, performed experiments and analysed data etc. D. R., S.L., and L.L. wrote the manuscript; and all authors revised and approved the final manuscript.

Funding information

National Natural Science Foundation of China (Grant Numbers: 32001085).

Data availability
Raw sequence reads have been deposited in the National Genomics Data Center, Beijing Institute of Genomics, Chinese Academy of Sciences, under BioProject accession number PRJCA028370.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 4 August 2024 / Accepted: 19 November 2024
Published online: 19 December 2024

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