Genomic insights on the contribution of balancing selection and local adaptation to the long-term survival of a widespread living fossil tree, *Cercidiphyllum japonicum*

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Summary

- 'Living fossils' are testimonies of long-term sustained ecological success, but how demographic history and natural selection contributed to their survival, resilience, and persistence in the face of Quaternary climate fluctuations remains unclear.
- To better understand the interplay between demographic history and selection in shaping genomic diversity and evolution of such organisms, we assembled the whole genome of *Cercidiphyllum japonicum*, a widespread East Asian Tertiary relict tree, and resequenced 99 individuals of *C. japonicum* and its sister species, *Cercidiphyllum magnificum* (Central Japan).
- We dated this speciation event to the mid-Miocene, and the intraspecific lineage divergence of *C. japonicum* (China vs Japan) to the Early Pliocene. Throughout climatic upheavals of the late Tertiary/Quaternary, population bottlenecks greatly reduced the genetic diversity of *C. japonicum*. However, this polymorphism loss was likely counteracted by, first, long-term balancing selection at multiple chromosomal and heterozygous gene regions, potentially reflecting overdominance, and, second, selective sweeps at stress response and growth-related genes likely involved in local adaptation.
- Our findings contribute to a better understanding of how living fossils have survived climatic upheaval and maintained an extensive geographic range; that is, both types of selection could be major factors contributing to the species' survival, resilience, and persistence.

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Introduction

Some species, for instance, coelacanths, horseshoe crabs, maiden-hair tree (*Ginkgo biloba*), or dawn red wood (*Metasequoia*), survived for millions of years with little morphological change. For those 'anomalous forms that endured to the present day', Darwin (1859) coined the term 'living fossils'. Living fossils are not easy to define unambiguously, but, apart from their morphological stasis and persistence over long periods of evolutionary time, they also tend to have distribution ranges that are currently more restricted than in the geologic past (Werth & Shear, 2014; Lidgard & Love, 2018). As already foreseen by Darwin, such organisms provide excellent materials to address questions on

species evolution. In particular, they can offer insights into a wide array of adjustments (e.g. morphological, physiological, genetic, demographic) that may have been instrumental in adaptation and survival through numerous episodes of environmental change (Werth & Shear, 2014). One can ask, for instance, whether and how living fossils responded to the climatic oscillations of the Quaternary (Grandcolas *et al.*, 2014). Did their effective population sizes contract and expand, as they did in many species, or did they remain rather stable through these periods of climatic upheaval, as reported for others (Kryvokhyzha *et al.*, 2019)? Furthermore, it would be interesting to know how much of their genome is directly or indirectly influenced by natural selection, or, more specifically, which selective forces are shaping genomic variation and driving local adaptation (Kern & Hahn, 2018).

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The greatest concentration of ‘living fossil’ plants presently occurs in the warm-temperate forests of East Asia (Axelrod, 1998; Manchester *et al.*, 2009). Here, most of these Tertiary relict trees (e.g. *G. biloba*, *Davidia involucrata*, *Emmenopterys henryi*, *Euptelea pleiosperma*, *Liriodendron chinense*, *Tetracentron sinense*) are confined to relatively narrow, discontinuous ranges in subtropical China, where they often occur in small, isolated mountain populations on unstable sites, such as scree slopes and eroded rocky stream banks (reviewed in Shang *et al.*, 2016). Earlier phylogeographic studies, primarily based on organellar markers, indicated that these Tertiary relict trees, indeed, experienced repeated range contractions and expansions associated with Quaternary glacial–interglacial cycles (Qiu *et al.*, 2011; Sun *et al.*, 2014; Ma *et al.*, 2015; Cao *et al.*, 2016; Zhang *et al.*, 2016; Shen *et al.*, 2019). However, with the exception of a couple of studies (Tsumura *et al.*, 2012; Zhang *et al.*, 2016; Zhao *et al.*, 2019), there have so far been limited attempts to link those demographic changes with adaptive responses, even though one could expect that both are associated (Shaw & Ertterson, 2012). As shown by a recent study in *G. biloba* (Zhao *et al.*, 2019), the advance of whole-genome sequencing technologies has dramatically increased our ability to understand the joint effects of demography and selection on the genetic polymorphisms of a Tertiary relict tree. By taking advantage of *Ginkgo*’s reference genome, these authors inferred multiple population declines but also recoveries of the species in China over late Tertiary/Quaternary timescales. Moreover, signatures of positive selection were detected at genes involved in abiotic (e.g. climate) and biotic (e.g. pathogen defence) stresses (Zhao *et al.*, 2019). Together, these results suggest that both demographic history and natural selection contributed to the species’ long-term persistence and resilience. However, it would be premature to generalize from a single study, considering the great variability of evolutionary responses to Quaternary environmental change across species (e.g. Petit *et al.*, 2003). Hence, further population genomics work is required to elucidate the interplay between historical demographic and selective factors in shaping the genetic diversity and evolution of East Asia’s Tertiary relict flora.

To fill this gap, we report here the genome draft and population genomic structure of *Cercidiphyllum japonicum* Sieb. et Zucc., which, much in contrast to *G. biloba*, is the epitome of a widespread ‘living fossil’ tree in East Asia (Qi *et al.*, 2012). This diploid ($2n=38$), dioecious, and wind-pollinated/dispersed tree is widely distributed across subtropical (Central/South/East) China all the way to north Japan (Hokkaido), and thus covers various climate and vegetation zones, including warm-temperate deciduous, cool-temperate, and subalpine forests (Fu & Endress, 2001; Isagi *et al.*, 2005; Krassilov, 2010). There is phylogeographic evidence to suggest that *C. japonicum* has survived in East Asia for at least 5 Myr, and experienced dramatic population fluctuations in both China and Japan during the Quaternary glacial–interglacial cycles (Qi *et al.*, 2012). Based on population genetics theory (Hedrick, 2006) and empirical data (Bernatchez, 2016; González-Martínez *et al.*, 2017; Lin *et al.*, 2017; Gossmann *et al.*, 2019), one might, therefore, expect these climate-induced fluctuations

in population size and migration rates to have been accompanied by bouts of natural selection.

In this study, we generated a chromosome-level *de novo* assembly of the whole genome of *C. japonicum* and resequenced 82 individuals, representing 24 sampled populations (localities) from China (eight) and Japan (16). For comparison, we also resequenced 17 individuals (four populations) of *C. magnificum* Nakai, which is the only other species of the genus and is today restricted to the cool-temperate/subalpine forests of Central Honshu/Japan (Qi *et al.*, 2012). Hence, we first inferred the demographic history of the two species and then tested for the presence of genome-wide selection in the Chinese vs Japanese lineages of *C. japonicum*. Overall, our study provides evidence that this exceptionally widespread ‘living fossil’ repeatedly recovered from demographic bottlenecks over late Tertiary/Quaternary periods of adverse climate conditions, and that its survival might have been facilitated through the maintenance of genetic diversity by genome-wide balancing selection and selective sweeps at particular genes involved in local adaptation.

Materials and Methods

Genome sequencing, assembly, and annotation

For whole-genome sequencing, we collected fresh young leaves of an adult *C. japonicum* tree from Tianmu Mountain, Zhejiang, China (30.326°N, 119.442°E). We sequenced and assembled the genome using a combination of PacBio single-molecule long reads and Illumina short reads and further improved the assembly by adding 10 × Genomics Linked-Reads. Then, a Hi-C library was sequenced to assist the chromosome-level assembly. The completeness of the genome assembly was assessed using both CEGMA (Parra *et al.*, 2007) and BUSCO (Simão *et al.*, 2015). Gene prediction was conducted through a combination of homology-based inference, *ab initio* prediction, and transcripts from RNA sequencing (RNA-Seq). We also used a combination of homology-based and *de novo* approaches to identify transposable elements (TEs) in the *C. japonicum* genome. For details, see Supporting Information Methods S1 and Tables S1–S10.

Genomic synteny and gene family analyses

MCSCANX (Wang *et al.*, 2012) was employed to identify syntenic blocks within and between the genomes of *C. japonicum*, *Vitis vinifera* and *Kalanchoe fedtschenkoi*. We also calculated the four-fold synonymous third-codon transversion (4DTv) rate for orthologous/paralogous genes in syntenic blocks to identify putative whole genome duplication events in *C. japonicum* (Maere *et al.*, 2005). ORTHOMCL v.2.0 (Li *et al.*, 2003) was used to identify paralogues and orthologues between *C. japonicum* and 17 other sequenced angiosperm species (see Table S11). The 413 single-copy orthologous genes of all 18 species (Fig. S1) were used to reconstruct a maximum likelihood (ML) phylogeny with RAXML v.8.0 (Stamatakis, 2014). Gene family expansion and contraction analysis was performed with CAFÉ v.3.1 (Han *et al.*, 2013).

Divergence time and mutation rate estimation

We derived 287 single-copy orthologous genes among 19 angiosperm species, including *C. japonicum* and *C. magnificum* (from transcriptome data), for phylogenetic analysis and molecular dating. For each species, the genes were concatenated into a ‘supergene’ and aligned using MUSCLE v.3.8.31 (Edgar, 2004). The alignment was curated with GBLOCKS v.1.0.4 (Castresana, 2000) and then used to perform phylogenetic analysis using RAXML v.8.0 with 1000 bootstrap replicates. We further estimated the divergence time for each ancestral node of these 19 species in BEAST v.1.8.1 (Drummond *et al.*, 2012), using four calibration times. The mutation rate was estimated as $\mu = D/2 \times T$, where D is the observed frequency of pairwise differences between *C. japonicum* and *C. magnificum*, and T is their estimated divergence time (for details, see Methods S1).

Resequencing, mapping, and genotyping

For whole-genome resequencing, individual leaf samples were collected from a total of 24 populations ($n=82$) of *C. japonicum* throughout its range in China ($n=31$; eight populations) and Japan ($n=51$; 16 populations) and four populations ($n=17$) of *C. magnificum* from its narrow range in Central Honshu/Japan (Fig. S2). Raw sequence reads of each of those 99 individuals were mapped to the reference genome of *C. japonicum* using BWA v.0.7.7 (Li & Durbin, 2010). SAMTOOLS v.0.1.19 (Li & Durbin, 2009) was used for sorting and removing PCR duplicates. Raw single nucleotide polymorphisms (SNPs) were called with SAMTOOLS, using the command ‘samtools mpileup q 1 C 50 S D m 2 F 0.002 u’. Three filtering steps were performed to reduce false positives from SNP and genotype calling: (1) removal of SNPs with quality score of <20 ; (2) removal of SNPs with more than two alleles; and (3) assignment of individual genotypes with reads less than one-third of mean depth as ‘missing’. We also applied the following criteria to obtain high-quality genotype calls. Only SNPs containing $\leq 20\%$ of missing data (missing ratio) and minor allele frequencies (MAF) ≥ 0.01 were retained for population genetic analyses in the combined data set of *C. japonicum* ($n=82$) and *C. magnificum* ($n=17$). To evaluate whether our inference of population structure is robust to the calling of rare variants due to unequal sample sizes of the two species, we also applied the aforementioned filtering criteria with the exception of MAF ≥ 0.05 to each species. We performed population structure analysis using both data sets.

Genetic relationship and population structure analyses

An approximately ML phylogenetic tree was constructed based on the total SNP data set of all 99 accessions of *C. japonicum*/*C. magnificum* using FASTTREE v.2 (Price *et al.*, 2010). Population structure was further assessed by Bayesian clustering in ADMIXTURE v.1.23 (Alexander *et al.*, 2009). The number of clusters K was set to vary from 2 to 6, and the best value was chosen based on the lowest cross-validation error. We also assembled complete chloroplast genome sequences from all whole-genome

re-sequenced *Cercidiphyllum* individuals ($n=99$), following procedures described in Huang *et al.* (2014) (for details see Methods S2). We also conducted phylogenetic analysis based on these 99 chloroplast genome sequences with four outgroups (*Bergenia scopulosa*, *Heuchera parviflora* var. *saurensis*, *Sedum oryzifolium* and *Sedum sarmentosum*) using RAXML. Considering coding sequence regions only, we identified a total of 30 chloroplast haplotypes for this dataset; genealogical relationships among these ‘chlorotypes’ were assessed through a 95% statistical parsimony network, using the program TCS, as implemented in POPART v.1.7 (Leigh & Bryant, 2015; for details, see Methods S2).

Reconstruction of demographic history

We used the pairwise sequentially Markovian coalescent (PSMC) method (Li & Durbin, 2011) to infer historical changes in effective population size N_e based on the reference genome of *C. japonicum* and 28 resequenced individuals from each locality of *C. japonicum* ($n=24$)/*C. magnificum* ($n=4$). We also used the sequentially Markovian method (SMC++; Terhorst *et al.*, 2017), which uses polymorphism data of multiple unphased genomes, to reconstruct the population size histories of Chinese and Japanese *C. japonicum* as well as *C. magnificum*. SMC++ is more powerful than PSMC in estimating changes of N_e over shallow timescales. Finally, we implemented coalescent simulations of six divergence models in FASTSIMCOAL2 (Excoffier *et al.*, 2013) to infer the past demographic histories of the two *Cercidiphyllum* species using the joint site frequency spectrum (SFS; Fig. S3; for details see Methods S2).

Genome-wide scanning for selection in *C. japonicum*

To identify genomic regions potentially under selection in *C. japonicum*, we calculated average values of π , Tajima’s D , F_{ST} , and D_{xy} across all populations per region (China vs Japan) using a genome-wide sliding window strategy (100 kb in length with steps of 10 kb) as implemented in VCFTOOLS v.0.1.15 (Danecek *et al.*, 2011). Windows with z -score-normalized $F_{ST} > 2$ (95% tail) were considered to be candidate regions likely driven by diversifying selection for local adaptation. Then we merged the neighboring windows into regions and filtered those regions with a cutoff of length >1 Mb and mean Tajima’s $D < -0.95$ (10% tail). The mean of Tajima’s D was calculated from all windows with more than 100 SNPs within the merged regions of the samples by averaging their D values. To identify candidate regions under selective sweeps, we first performed haplotype phasing in MACH v.1.0 (Li *et al.*, 2010) with parameters ‘states 100’ and ‘rounds 50’. Linkage disequilibrium between pairs of SNPs within 1 Mb was calculated by EMERALD (Quick *et al.*, 2018). The likelihood ratio test statistic T (Harris & DeGiorgio, 2019) was used to test for selective sweeps based on haplotype diversity decrease at surrounding sites. Putative selective sweep regions were selected based on $T > 51.9$ (99.5% tail).

We identified putative regions under balancing selection based on the distributions of Tajima’s D (95% tail, >1.3 and >1.56

for Chinese and Japanese populations, respectively), π (95% tail, >0.0024 and >0.0023 , respectively), and F_{ST} (5% tail, z -score <-0.92). Percentages of heterozygous sites were calculated for the four regions and the whole genome of each individual (see the Results section). Individuals were considered carriers of balancing polymorphism only if the percentage of heterozygous sites was $>30\%$. This threshold was determined as the values of other individuals dropped greatly from 30% to below 20%. We further validated signatures of long-term balancing selection using normalized β statistics, as calculated in BETASCAN (<https://github.com/ksiewert/BetaScan>). As shown by simulations (Siewert & Voight, 2017), this measure provides greater power than other summary statistics for balancing selection, especially in nonequilibrium populations.

Estimates of selective efficacy and distribution of fitness effects

For each protein-coding gene, we also calculated the nucleotide diversity π at nonsynonymous (zero-fold) and synonymous (four-fold) sites (i.e. π_N and π_S , respectively) for the Japanese and Chinese populations of *C. japonicum*. The ratio of the average π_N/π_S was then used as a measure of selection efficacy (James *et al.*, 2017). It was also used in connection with the analysis of the candidate region of selective sweeps, since π_S is affected by hitchhiking, with a stronger effect of hitchhiking in areas of low diversity (Castellano *et al.*, 2018). The distribution of fitness effects (DFE) of new mutations (Eyre-Walker & Keightley, 2007) was estimated by comparing the unfolded SFS between nonsynonymous and synonymous sites, using POLYDFE v.2 (Tataru & Bataillon, 2018). Likelihoods of fitted DFE parameters, either constrained to be shared (invariant) across data sets or fitted independently for each data set, were then compared to test for significant differences in selection between the Chinese and Japanese populations. For each group, we first tested a model with deleterious mutations following a gamma distribution and then a full DFE model with both deleterious mutations still distributed according to a gamma distribution and beneficial mutations distributed according to an exponential distribution. Parameter estimates for the proportion of beneficial mutations p_b , mean positive selective strength S_b , shape of the gamma distribution β , and mean negative selective strength S_d were taken by averaging across values of both DFE models as weighted by the model's Akaike information criterion value (Posada & Buckley, 2004).

Genotype–environmental association analyses

For the environmental data, we downloaded 19 bioclimatic variables for the period 1960–1990 from WorldClim v.1.4 (Hijmans *et al.*, 2005) for each location of the Chinese and Japanese populations of *C. japonicum*. We performed principal component (PC) analysis (PCA) in R (R Development Core Team, 2018) to remove redundancies among correlated variables and keep most of the variance. The first six PCs (explaining $>99\%$ of the total variance of the sampled populations) were then chosen to test for

the correlation between environmental factors and SNP frequencies (MAF ≥ 0.05 ; missing ratio ≤ 0.2) using a latent factor mixed model (LFMM) implemented in the R package LFMM (Caye *et al.*, 2019; see details in Methods S2). SNPs with correlated P -values (false discovery rate, FDR) <0.05 and effect scores >10 were regarded as significantly associated with environmental differentiation between China and Japan. In addition, we selected candidate genes harboring environmentally associated SNPs for Gene Ontology (GO) enrichment analysis using *Arabidopsis thaliana* orthologues at agriGO (Du *et al.*, 2010). To test the significance of GO terms, we applied a Bonferroni-adjusted P -value <0.05 as the threshold.

Results

Genome assembly and genome evolution of *C. japonicum*

In total, 299 Gb data (*c.* 400 $\times$) were assembled into a high-quality genome of 719 Mb for *C. japonicum*, with N50 of 494.88 kb (Tables S1–S3). About 90.18% (648 Mb) of the assembly was anchored to 19 pseudo-chromosomes (Figs 1, S4, S5). A total of 27 393 gene models were predicted, and 26 176 were verified by transcriptome sequences and existing homologous sequences (Tables S4–S10; Methods S1). Syntenic analysis revealed that *C. japonicum* experienced a whole-genome triplication event, referred to as gamma (γ) in core eudicots (Jaillon *et al.*, 2007; Figs 2a,b, S6; Methods S1). A total of 72 gene families in *C. japonicum* had undergone significant expansions ($P < 0.05$; Fig. S7). These gene families were mostly enriched with 'responding to stimulus' and 'nucleotide binding' functional subcategories, and associated with disease resistance genes (e.g. *R*-genes) encoding nucleotide-binding site–leucine-rich repeat proteins (Tables S11–S13). The ML tree inferred from 287 single-copy orthologues among 19 angiosperm species confirmed the monophyly of *Cercidiphyllum* as sister to a clade comprising the two other members of Saxifragales; that is, *K. fedtschenkoi* and *Rhodiola crenulata*. The crown age of *Cercidiphyllum* was estimated at 13.41 million years ago (Ma; 95% highest posterior density (HPD): 15.02–11.77 Ma; Fig. 2c). The estimated 4DTv rate of the *Cercidiphyllum* sequences was *c.* 5.35×10^{-10} per site per year (range: 4.77×10^{-10} – 6.09×10^{-10}), which is lower than that of other trees (e.g. 1.16×10^{-9} for *Populus trichocarpa* (Tuskan *et al.*, 2006); 1.09×10^{-9} for *Salix suchowensis* (Dai *et al.*, 2014); and 2.50×10^{-9} for *Juglans* species (Bai *et al.*, 2018)).

Population structure and admixture

Whole-genome resequencing resulted in a total of 10 148 438 142 paired-end reads for *C. japonicum* ($n=82$) and 2127 448 854 for *C. magnificum* ($n=17$). About 89% of *C. japonicum* sequence reads and 83% of *C. magnificum* sequence reads were accurately mapped to the reference genome. The mean sequencing depth was 22 $\times$ and 24 $\times$ in samples of *C. japonicum* and *C. magnificum*, respectively (Table S14). After filtering, we detected a total of 6923 574 SNPs across the 99 individuals

Fig. 1 Circos plot of chromosome-level assembly of the *Cercidiphyllum japonicum* genome. The circles, from outermost to innermost, show (a) gene density, (b) repeat element density, (c) single nucleotide polymorphism density for population genomic analysis, and (d) GC content. The four metrics are calculated in 1 Mb sliding windows. The central lines show the syntenic regions within the *C. japonicum* genome.

analyzed (Table S15). Not unexpectedly, among the 1080 097 rare variants (i.e. SNPs found in only one individual and as homozygotes, or in two individuals and as heterozygotes), the majority (957 859; 88.7%) were present as heterozygotes. When filtering SNPs for each species with $MAF \geq 0.05$ and missing ratio ≤ 0.2 separately, we identified 2251 212 and 590 523 SNPs across the 82 *C. japonicum* samples and 17 *C. magnificum* samples, respectively.

The ML tree based on the aforementioned two data sets consistently recovered two highly supported main clusters corresponding strictly to species taxonomy (Figs 3a, S8; Tables S14, S15). Within *C. japonicum*, most Chinese accessions formed a separate cluster, except for those of the most eastern population (CTT). The corresponding ADMIXTURE analyses at $K=3-5$ (with $K=4$ being the optimal cluster number; see Fig. 3b) revealed that CTT populations were genetically admixed with conspecific populations from south Japan (JSY, JSD, JST, JSE). At $K=5$, samples of Japanese *C. japonicum* were further subdivided into regional subclusters from south and north Japan, with central populations showing genetic admixture to some extent (Figs 3c, S8). By contrast, the ML phylogeny based on whole chloroplast genome sequences of all 99 accessions recovered two major clades: one composed of *C. japonicum* from China and south Japan, and the other containing *C. japonicum* from central-north Japan and *C. magnificum* (Fig. 3d). Similarly, a parsimony network of the 30 chloroplast haplotypes indicated a closer relationship of central-north Japanese *C. japonicum* to *C. magnificum* than to other conspecifics (Fig. 3e).

Reconstructing past population dynamics

Based on the PSMC analysis of the high-coverage reference genome of *C. japonicum*, the species' ancestral population started to expand from *c.* 10 Ma until 3–2 Ma, followed by a severe bottleneck that lasted until *c.* 0.5 Ma, during which N_e was reduced by *c.* 65% (Fig. S9). Notably, *C. magnificum* followed a similar demographic trajectory of population growth and subsequent decline over the same periods, except that the bottleneck lasted 0.5 Myr longer and was even more severe (Fig. 4a). Based on the SMC++ analysis of all 99 whole-genome resequenced individuals, population sizes of each species increased rapidly after *c.* 20 000 yr BP and then started to decline *c.* 3000–2000 yr BP (Fig. S10).

According to the best-supported model (Figs 4b, S11; Tables S16, S17), *C. japonicum* and *C. magnificum* diverged from their common ancestor at the mid-Miocene, *c.* 10.33 Ma (95% HPD: 10.96–6.73 Ma). The divergence of Chinese and Japanese *C. japonicum* occurred at the Early Pliocene, *c.* 4.17 Ma (95% HPD: 4.88–3.93 Ma). Estimates of bidirectional gene flow revealed low levels of ancient gene flow between the common ancestor of Chinese/Japanese lineages of *C. japonicum* and *C. magnificum* ($0.036/0.003$; Fig. 4b). By contrast, after the divergence of Chinese and Japanese *C. japonicum*, estimates of gene flow between the two species were extremely low ($9.02 \times 10^{-6}/7.53 \times 10^{-6}$), and the same was true for the latter two lineages ($1.55 \times 10^{-5}/1.29 \times 10^{-5}$; Fig. 4b). According to this best-fit FASTSIMCOAL2 model, all three lineages experienced a bottleneck event during the Early to Middle Pleistocene (*c.* 1.6–1.1 Ma), resulting in estimates of current N_e that were only slightly smaller in the narrow endemic *C. magnificum* relative to Japanese and, to a lesser extent, Chinese *C. japonicum* (Fig. 4b).

Selective sweeps increased genetic differentiation between Japanese and Chinese *C. japonicum*

Across their entire genomes, Chinese and Japanese populations of *C. japonicum* exhibited similar levels of nucleotide diversity (mean $\pi = 1.1 \times 10^{-3}$ and 9.9×10^{-4} , respectively), and low levels of inter-lineage differentiation ($F_{ST} = 0.07$, compared with 0.72 between *C. japonicum* and *C. magnificum*; Fig. S12). Average linkage disequilibrium was low (mean r^2 within 1 kb was 0.18 and 0.19 for Chinese and Japanese populations, respectively) and decreased by half within *c.* 2.5 kb (Fig. S13). When these two regional groups were subjected to an F_{ST} -based genome-wide sliding window scan (100 kb windows with steps of 10 kb), 2854 outlier regions were identified with z -score-normalized F_{ST} values > 2 (Fig. 5a). Of those potential selection regions, 19.7% (563) were located on Chr14, covering *c.* 19.3% of its total length, and thus significantly more than on other chromosomes (4.3% on average). Specifically, 86.5% of high F_{ST} windows were located in a region (5–14 Mb) in which Japanese populations had significantly lower diversity than Chinese ones (mean $\pi = 0.0008$ vs 0.0012, $P < 0.01$), as well as significantly lower negative values of Tajima's D (mean: -0.23 vs 0.21, $P < 0.01$). Notably, those differences were even more pronounced

Fig. 2 Evolution of the *Cercidiphyllum japonicum* genome and gene families. (a) Distribution of the four-fold synonymous third-codon transversion rate (4Dtv) for paralogous genes within the genomes of *C. japonicum*, *Vitis vinifera*, and *Kalanchoe fedtschenkoi* and orthologous genes between those of *C. japonicum* and *K. fedtschenkoi*. (b) Macrosynteny patterns showing that a typical ancestral region in *C. japonicum* can be tracked to up to one region in *V. vinifera* and to up to four regions in *K. fedtschenkoi*. (c) BEAST-derived chronogram of 19 angiosperm species, including *C. japonicum* and *Cercidiphyllum magnificum*, based on 287 single-copy orthologous genes. The number at each node represents the median divergence time (in million years ago, Ma), with blue bars representing the 95% highest posterior density interval. Numbers with circles represent calibration times of divergence between ① *Amborella trichopoda* and *Oryza sativa* (168–194 Ma), ② *O. sativa* and *Populus trichocarpa* (148–173 Ma), ③ *Coffea canephora* and *Theobroma cacao* (110–124 Ma), and ④ *Arabidopsis thaliana* and *P. trichocarpa* (97–109 Ma). Calibration times were obtained from the Timetree database (<http://www.timetree.org/>).

for the central region of Chr14 at 8.5–12 Mb (mean π : 0.0007 vs 0.0015, $P < 0.01$; Tajima's D : -0.97 vs -0.26 , $P < 0.01$) (see Fig. 5b). This region also had the highest estimate of Nei's genetic distance (D_{xy} = 0.0037 vs 5.29×10^{-4} for the whole genome) and contained all 189 fixed differences D_f between the Chinese and Japanese populations.

Taken together, these results suggest that selective sweeps took place in the Japanese populations but were absent or less pronounced in those from China. To ascertain these results, we performed two additional analyses. First, based on the T statistic, we identified a mixture of hard sweeps (number of sweeping haplotypes $m = 1$) and soft sweeps ($m = 3$ –7; Fig. 5b) in the region 8.5–12 Mb of Chr14 in Japanese populations. Second, since hitchhiking effects can elevate π_N/π_S in areas of low diversity (Castellano *et al.*, 2018), we compared π_N/π_S ratios for each gene and each regional lineage separately. On average, values of π_N/π_S across all chromosomes were rather low and similar (Japan: 0.29; China: 0.28). However, in the selective sweep region of Chr14 (8.5–12 Mb, including 41 genes; see details in

Table S18), this ratio was significantly larger for the Japanese than the Chinese populations (0.58 vs 0.48). Estimates of DFE further confirmed a small but still higher proportion of beneficial mutations p_b in Japan than in China (2.8×10^{-4} vs 2.4×10^{-5}). On Chr14, this difference was even two orders of magnitude higher (0.013 vs 1.3×10^{-4}). Moreover, mean positive selective strength S_b on Chr14 was higher in Japan (0.076 vs 0.019). Such selective sweeps also elevated F_{ST} between the Japanese populations of *C. japonicum* and *C. magnificum* (see Fig. 5b).

A patch-like pattern of heterozygous sites across the 3.5 Mb region of Chr14 (8.5–12 Mb) were detected in Japanese and Chinese *C. japonicum* (Fig. S14). For example, a majority of Japanese *C. japonicum* individuals shared a long stretch of homozygous alleles (type I) across the 'selective sweep' region of Chr14 (8.5–12 Mb; see an example of gene *VLN4* in Fig. 5c). By contrast, about half of the Chinese individuals shared a different set of homozygous alleles (type II) across this region, whereas the rest appeared to be heterozygous for both allele types at various sites

Fig. 3 Genetic relationships and population structure of *Cercidiphyllum japonicum* and *Cercidiphyllum magnificum* individuals based on whole-genome nuclear (nu) and chloroplast (cp) sequence data. (a) Phylogenetic relationships (maximum-likelihood tree) of all accessions ($n=99$) inferred from 6923 574 single nucleotide polymorphisms with 100% bootstrap support for almost all nodes. (b) Geographic distribution of ADMIXTURE clusters at $K=4$ (i.e. the optimal solution) within and among 24 populations of *C. japonicum* in China (eight) and Japan (16), and four populations of *C. magnificum* in Central Honshu/Japan (see inset). (c) Population structure of 99 *Cercidiphyllum* individuals based on ADMIXTURE analysis with $K=3, 4$ and 5 . (d) Maximum likelihood phylogeny of all accessions ($n=99$) based on whole-chloroplast genome sequences. Asterisks indicate nodes with $>90\%$ bootstrap support. (e) The 95% statistical parsimony (TCS) network of genealogical relationships between the 30 cp haplotypes identified by considering coding sequence regions only. The sizes of circles are approximately proportional to sample sizes n , with the smallest circles representing $n=1$ and the largest representing $n=10$.

(Fig. 5c). This could indicate that Japanese vs Chinese populations were initially fixed for different alleles (type *I* vs type *II*) due to strong selective sweeps and that a considerable number of Chinese individuals are recombinant hybrids due to introgression from Japanese *C. japonicum*.

Signatures of long-term balancing selection in *C. japonicum*

For *C. japonicum*, we identified regions putatively under balancing selection based on the 95% tail of Tajima's D ($D>1.3$ and

Fig. 4 Demographic history of the two *Cercidiphyllum* species. (a) Population size through time plot for 28 whole-genome resequenced individuals from each population of *Cercidiphyllum japonicum* (Japan: purple; China: green) and *Cercidiphyllum magnificum* (red) based on pairwise sequentially Markovian coalescent modeling. The atmospheric surface air temperature is given by the gray line. (b) Schematic of demographic scenarios modeled using FASTSIMCOAL2 with median split times in million years ago (Ma), estimates of effective population sizes N_e (in white within each segment), and migration rates (see Supporting Information Table S17 for 95% confidence intervals). Estimates of gene flow between species and lineages are given as the migration fraction per generation. Chinese *C. japonicum* (green), Japanese *C. japonicum* (purple), their common ancestor (gray), and *C. magnificum* (red). A generation time g of 10 yr (Wan & Zhang, 2002) and a mutation rate μ of 5.35×10^{-9} per site per generation were applied (see Methods S2 for details).

>1.56 for Chinese and Japanese populations, respectively; SNPs >100 per 100 kb), π (>0.0024 and >0.0023, respectively, 95% tail), and F_{ST} ($z < -0.92$, 5% tail). Interestingly, most of these regions were shared by both Chinese and Japanese populations, especially for the four longest stretches on Chr03 (29.11–29.32 Mb), Chr16 (6.47–6.70 Mb), Chr17 (20.78–21.01 Mb), and Chr19 (21.31–21.79 Mb) (Table S19). Using normalized β statistics, the presence of long-term balancing selection was confirmed in all four regions (Fig. 6b). We note that the mean read depths of all sites within those putative balancing selection regions (range: $19.78 \times$ – $25.55 \times$) was similar to that of the whole genome ($21.08 \times$; see Fig. S15). Hence, the signatures of balancing selection detected herein cannot be a false signal due to exceptionally elevated read depths. Moreover, within these balancing selection regions, only a small percentage of sites (range: 0.8–5%; see Fig. S16) were found to significantly deviate from Hardy–Weinberg equilibrium due to heterozygote excess (adjusted P -value (FDR) <0.05). After removing these sites, the percentages of heterozygous sites within these four regions increased to 35–51% compared with 14% across the whole genome (Fig. 6a). Balanced polymorphisms in these four major regions were distributed in all phylogenetic clusters of Chinese and Japanese populations of *C. japonicum*, suggesting that balancing selection traced back to their common ancestor (Fig. 6c). Genes under balancing selection were related to the growth and development of flowers, pollen, leaves, and meristems (*CLV2*, *PIN5*) – for details on SNP genotypes and genes in these regions, see Fig. S17 and Table S20. No evidence of balancing selection was found in the same chromosomal regions of *C. magnificum*, suggesting that

the balanced polymorphisms identified in *C. japonicum* are specific to this widespread Tertiary relict.

Signatures of local adaptation in *C. japonicum*

The PCA biplot of 19 bioclimatic variables completely separated Chinese and Japanese populations (Fig. S18; Table S21), whereby PC1 and PC2 (explaining 37.1% vs 32.1% of the total variance) were highly correlated with aspects of temperature (e.g. Bio1, Bio6, Bio11) and precipitation (e.g. Bio13, Bio16, Bio18), respectively (data not shown). Furthermore, the LFMM approach identified 5791 SNPs (representing 823 genes) showing significant genotype–climatic correlations based on FDR values <0.05 and LFMM scores >10. Of those 5791 SNPs, 1546 (representing 102 genes) were located in high F_{ST} (z -score >2) chromosomal regions, and 1297 (83.9%) were restricted to particular chromosomes (i.e. Chr02 (732 SNPs, seven genes), Chr14 (349 SNPs, eight genes), Chr07 (128 SNPs, eight genes), and Chr06 (88 SNPs, 10 genes)). Notably, on Chr14, the majority of SNPs (320/349; i.e. 91.7%) were located in the ‘selective sweep’ region at 8.5–12 Mb. These findings suggest that environmental differences could play a significant role in the selective sweep on Chr14 for different haplotypes in China and Japan. GO enrichment analyses of the 823 genes detected 109 significantly overrepresented GO terms (FDR <0.05; Table S22). These GO categories were associated with reproduction, cellular development and proliferation, auxin metabolism and stress response (Fig. S19). Among those genes, a few were enriched for different functions, such as pollen formation (*MGPI*) and root hair elongation (*VLN4*) (on Chr14 and

Fig. 5 Genomic differentiation and selection between Chinese and Japanese *Cercidiphyllum japonicum*. (a) F_{ST} values per chromosome (numbers 1–19) and chromosomal region between Chinese and Japanese *C. japonicum*. The dashed line marks the regions with z-score-normalized $F_{ST} > 2$. (b) Detection of putative selective sweep regions on chromosome (Chr)14 at 8.5–12 Mb (gray window). Estimates of nucleotide diversity π and Tajima's D for Chinese (CHN) and Japanese (JPN) *C. japonicum* are represented by green and purple lines, respectively. Estimates of F_{ST} between Chinese and Japanese *C. japonicum* are represented by red (z-score (F_{ST}) > 2) and black (z-score (F_{ST}) < 2) dots, respectively, and those between either group and *Cercidiphyllum magnificum* by yellow and blue lines, respectively. Also shown are results of the likelihood ratio T statistic (Harris & DeGiorgio, 2019) for detecting putative selective sweep regions on Chr14 based on $T > 51.9$ (99.5% tail; red dashed line). (c) Single nucleotide polymorphism (SNP) genotypes of gene *VLN4* (Chr14: 11.21–11.23 Mb) located within the putative selective sweep region of Chr14 (8.5–12 Mb; see Supporting Information Fig. S14) in Chinese (CHN) and Japanese (JPN) populations of *C. japonicum* and *C. magnificum*. The black, green, brown, and red grids indicate SNPs of no call, homozygote type I, homozygote type II, and heterozygote, respectively.

Chr07, respectively), the regulation of photosynthesis (*CGR3*, *KEA3*), and vegetative phase change (*CGR3*, *SQN* – on Chr06), or possibly the circadian-clock pathway (*PCL1*, *BOA* – on Chr02; for details see Table S23).

Discussion

Divergence and demographic history of *Cercidiphyllum*

Our previous fossil-calibrated chloroplast DNA (cpDNA) phylogeny of 'woody Saxifragales' (Qi *et al.*, 2012) suggested that the two extant species of *Cercidiphyllum* diverged at the Mio-Pliocene boundary, *c.* 6.53 Ma; however, this point estimate was afflicted with very large margins of error, amounting to many millions of years (95% HPD: 13.42–1.34 Ma). By contrast, we here dated the divergence of *C. japonicum* and *C. magnificum* to around the mid-Miocene, whether based on the fossil-calibrated 287 single-copy-gene phylogeny (*c.* 13.41 Ma (95% HPD: 15.02–11.77 Ma); Fig. 2c) or population genomic (FASTSIMCOAL2) analyses (*c.* 10.33 Ma (95% HPD: 10.96–6.73 Ma); Fig. 4b). The similarity of both latter estimates and their relatively narrow confidence limits suggest that the temporal scale of *Cercidiphyllum*

speciation inferred herein (mid-Miocene) may be more accurate than the one (Mio-Pliocene boundary) based on cpDNA only (Qi *et al.*, 2012). Our newly estimated divergence time coincides with the increasingly colder and arid climates following the mid-Miocene Climatic Optimum (MMCO, *c.* 17–15 Ma; Pound *et al.*, 2012; Lin *et al.*, 2016). Likewise, we here established with high confidence that the Chinese and Japanese lineages of *C. japonicum* split during the Early Pliocene, *c.* 4.17 Ma (95% HPD: 4.88–3.93 Ma; Fig. 4b); that is, shortly after the Japanese islands, for the first time, had completely separated from Eurasia *c.* 5 Ma (Kimura, 2003). Our PSMC modeling (Fig. 4a) further indicated that each splitting event was initially accompanied by a drastic reduction in effective population size N_e , resulting in increased genomic differentiation between the two *Cercidiphyllum* species and among the Chinese and Japanese lineages of *C. japonicum* (Qi *et al.*, 2014; Cao *et al.*, 2016). Taken together, these data support the notion that increasingly colder and arid climates following the MMCO not only caused contraction and fragmentation of numerous Tertiary relict trees in East Asia (Yabe *et al.*, 2018), but also fostered their speciation and lineage diversification (e.g. *Cercidiphyllum*, this study; *T. sinense*: Sun *et al.*, 2014; *Cyclocarya paliurus*: Kou *et al.*, 2016; *Juglans*

Fig. 6 Characterization of balancing selection in *Cercidiphyllum japonicum*. (a) Percentages of heterozygous sites in the whole genome and in the balancing selection regions on chromosome (Chr)03 (29.11–29.32 Mb), Chr16 (6.47–6.70 Mb), Chr17 (20.78–21.01 Mb), and Chr19 (21.31–21.79 Mb). The error bars indicate SE. (b) Balancing selection regions (gray windows) on Chr03 (29.11–29.32 Mb), Chr16 (6.47–6.70 Mb), Chr17 (20.78–21.01 Mb), and Chr19 (21.31–21.79 Mb) were also identified by normalized β statistics. (c) Single nucleotide polymorphism-derived maximum-likelihood tree of *Cercidiphyllum* (the same as in Fig. 3a) highlighting *C. japonicum* heterozygous individuals putatively under balancing selection on different chromosomes, as indicated by colored dots (red: Chr03; green: Chr16; blue: Chr17; yellow: Chr19). Individuals were considered carriers of balancing polymorphism only if the percentage of heterozygous sites within regions was > 30%.

spp.: Bai *et al.*, 2018). However, when combined, our PSMC (Fig. 4a) and FASTSIMCOAL2 (Fig. 4b) analyses suggest an overall demographic scenario in which each *Cercidiphyllum* species first experienced long-term population growth from shortly after its origin (*c.* 10 Ma) to the Plio-Pleistocene boundary (*c.* 3–2 Ma), followed by recurrent declines in N_e (and thus genetic diversity) but also recoveries during the climatic upheavals of the late Tertiary–Quaternary. Hence, the population sizes of these two relict species have not continuously decreased, as might be expected for living fossil taxa (Sakai, 1971); rather, they increased over much of their evolutionary history before starting to fluctuate in response to more recent environmental change. Evidently, such demographic insights could not have been deduced from the

fossil record alone, given its limitation in taxonomic resolution and spatial coverage (Mao & Liu, 2012). Therefore, our results challenge the oft-stated view of relative demographic stasis in ‘living fossils’ (e.g. Lidgard & Love, 2018). Moreover, in line with previous molecular phylogeographic data (Qi *et al.*, 2012), they indicate that the late Tertiary–Quaternary range history of *C. japonicum* was highly dynamic. As further discussed later herein, positive results from selective sweeps should therefore be expected, especially in this widespread species, not least because adaptations (and thus sweeps) often occur in response to environmental change; that is, when populations colonize new habitats and thereby experience reductions in effective population size (e.g. González-Martínez *et al.*, 2017; Stephan, 2019).

Genomic differentiation and signatures of local adaptation in *C. japonicum*

Fossil records provide convincing evidence that drastic climatic fluctuations since the Late Pliocene in the Japanese Islands, which continued into the Middle Pleistocene, caused extensive extinction of the Japanese Tertiary forest flora while restricting survivors to subtropical China (e.g. *G. biloba*, *D. involucrata*, *Metasequoia glyptostroboides*) (Manchester *et al.*, 2009; Yabe *et al.*, 2018). By contrast, both fossil data (e.g. Crane & Stockey, 1985; Onoe, 1989) and our population genomic data (Fig. 4a) indicate that *C. japonicum* survived in both subtropical China and the Japanese islands during the Pleistocene glacial–interglacial cycles. However, although Chinese and Japanese *C. japonicum* already diverged *c.* 4 Ma (Fig. 4b), our population genomic (FASTSIMCOAL2) analyses of nuclear SNP data recovered only very low levels of post-divergence gene flow between these two lineages ($1.55 \times 10^{-5}/1.29 \times 10^{-5}$; Fig. 4b). Such gene exchange might have been facilitated by the glacially exposed East China Sea land bridge (*c.* 2.0–1.3 Ma and 0.2–0.015 Ma; Kimura, 2003).

When combined with previous ecological niche models (Qi *et al.*, 2012), our coalescent-based demographic analyses using PSMC (Fig. 4a) suggest that both Chinese and Japanese *C. japonicum* experienced range contractions/expansions over the last glacial–interglacial cycles, implying that populations had to adapt to newly colonized habitats. In this study, our analysis revealed extensive signals of selective sweeps or positive selection across the genome of *C. japonicum*, particularly regarding signs of local adaptation to environmental differences between China and Japan at genes related to growth, development, and stress response. These loci under positive selection appear to be non-randomly distributed along chromosomes. For example, a large region of Chr14 (8.5–12.0 Mb) was shown to have experienced selective sweeps and to harbor genes with polymorphisms that are strongly correlated with environmental factors. Selective sweeps tend to reduce genetic diversity in a single population. However, in structured populations, where selective sweeps may lead to the fixation of different alleles in different parts of the range, this will increase overall genomic variability (Coop *et al.*, 2009). Lineage-specific selective sweeps usually lead to rapid coalescence within populations (Cruickshank & Hahn, 2014) and result in an excess of fixed polymorphisms (D_F). This agrees with our observation that all 189 fixed nucleotide site differences between Chinese and Japanese populations of *C. japonicum* were located in this selective sweep region of Chr14 (Fig. 5b). Interestingly, this particular sweep region also contained a higher proportion of beneficial mutations (in terms of DFE parameter p_b) and higher average strength of selection on positively selected mutations (S_b) in Japanese populations than in those from China. This could indicate that Japanese *C. japonicum* responded more strongly to selection than its mainland counterpart, possibly due to greater range fluctuations over the last glacial cycle(s), and especially while adapting to extreme environments outside the species' subtropical niche in the island's far north (i.e. northern Honshu/Hokkaido) after inter-/postglacial expansion from southern refugia (see also Qi *et al.*, 2012).

Balancing selection contributed to adaptation

Though selection usually results in a reduction of diversity at selected loci and linked gene regions, balancing selection usually maintains diversity at such loci, whereby various advantageous variants can be maintained over long time periods, if not across species boundaries (e.g. major histocompatibility complex in vertebrates; self-incompatibility loci in plants; mating-type loci in fungi; reviewed in Fijarczyk & Babik, 2015). Yet, when compared with positive selection, it is more difficult to detect genomic footprints of stable and pervasive balancing selection (DeGiorgio *et al.*, 2014; Fijarczyk & Babik, 2015), as older alleles had more time to recombine and may lead to narrow signatures around selected sites (Teixeira *et al.*, 2015). In our case, however, low overall genetic diversity and an excess of rare alleles caused by severe bottlenecks, as likely characteristic of 'living fossils' (Avice *et al.*, 1994), made it easier to detect loci under balancing selection (i.e. through signatures of high diversity and positive Tajima's D).

In *C. japonicum*, signatures of balancing selection are scattered across different chromosomes (on Chr03, Chr16, Chr17 and Chr19), with many regions characterized by multiple genes in heterozygote state; this pattern could potentially reflect overdominance (heterozygote fitness advantage; Delph & Kelly, 2014). Notably, some of the genes potentially under balancing selection in *C. japonicum*, such as CLAVATA2 (*CLV2*), are highly pleiotropic and could therefore have a major effect on fitness. In *A. thaliana*, the *CLV2* gene (along with several other receptors) regulates shoot/flower meristem and flower organ development (Kayes & Clark, 1998; Jeong *et al.*, 1999), and has also been found to be highly polymorphic, possibly due to balancing selection (Shepard & Purugganan, 2003). In fact, balancing selection has been shown to be responsible for the maintenance of advantageous genetic diversity, notably heterozygote advantage, in a variety of discrete plant traits, such as self-incompatibility, nuclear-cytoplasmic gynodioecy, flower color, heterostyly, and heterodichogamy (Delph & Kelly, 2014). Populations of *C. japonicum* predominantly occur in heterogeneous (i.e. riparian) habitats (Kubo *et al.*, 2010; Qi *et al.*, 2012), which could have provided selective pressure strong enough to maintain polymorphisms over long periods of time within each population. Moreover, balancing selection acting on *CLV2* might have increased the species' fitness under different circumstances; for instance, by enhancing or reducing the strength of signaling for different alleles involved in organ development (Shepard & Purugganan, 2003; Turelli & Barton, 2004). Our findings thus indicate that, in *C. japonicum*, genetic adaptation to, for example, disturbed or newly encountered environments may also involve ancestral polymorphisms, except for younger advantageous polymorphisms maintained by positive selection. Recent studies have demonstrated that balancing selection is playing crucial roles in adaptation and evolutionary processes driving genetic diversity and population differentiation (*A. thaliana*, *Capsella rubella*: Wu *et al.*, 2017; *Brachypodium distachyon*: Bourgeois *et al.*, 2018; *Boechera stricta*: Wang *et al.*, 2019). Nevertheless, these studies have generally focused on model species, including very few forest trees

(*Populus*: Wang *et al.*, 2020). Our genomic study thus provides the first evidence for balancing selection acting on ancestral polymorphisms in a 'living fossil' tree.

Conclusions

Understanding how genetic diversity is maintained or lost through selective and neutral evolutionary forces has important implications for explaining current patterns of biodiversity and predicting future adaptive potential (e.g. Wang, 2010; Leffler *et al.*, 2012). Until recently, however, this question has been largely restricted to model systems with abundant genomic resources, which may have biased our understanding of how the genomes of nonmodel species were shaped by evolutionary pressures (Bourgeois *et al.*, 2018). Our population genomic analyses in *Cercidiphyllum* inferred a mid-Miocene speciation event, whereas the Chinese and Japanese lineages of *C. japonicum* diverged at the Early Pliocene. Although population bottlenecks during the climatic upheavals of the late Tertiary–Quaternary (<3–2 Ma) greatly reduced the genetic diversity of *C. japonicum*, long-term balancing selection (most likely in the form of overdominance) and selective sweeps associated with local adaptation likely counteracted polymorphism loss. In fact, both types of selection could be major factors contributing to the species' long-term survival, resilience, and persistence in the face of past (and current) climate change. Overall, these results undercut the earlier assumption of relative demographic and evolutionary stasis in 'living fossils', in general (Sakai, 1971; Lidgard & Love, 2018), and in an exceptionally widespread Tertiary relict tree in particular.

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Author contributions

YQ planned and designed the research. SZ, PL, RL, CF, WX, YF, WY, SS and YI participated in various aspects of biological sample collection. SZ performed experiments. JC, SZ, JZ and XX analyzed data. JC, SZ, HPC and YQ wrote the manuscript. LL, ML, SS and YI gave insightful suggestions and comments on the manuscript. SZ, JC and JZ contributed equally to this work.

Data availability statement

The assembled genome of *C. japonicum* and all raw sequencing data have been deposited under NCBI BioProject PRJNA549370

with accession nos. SRR 9640518–SRR 9640530. The transcriptomic raw data have been deposited under NCBI BioProject PRJNA554204 with accession nos. SAMN12240873–SAMN12240879. The whole-genome resequencing raw data have been deposited under NCBI BioProject PRJN553317 with accession nos. SAMN12163473–SAMN12163572. The whole-genome SNP data sets and summary statistics have been deposited in GitHub (<https://github.com/Cercidiphyllum/population-genomics-analysis-of-Cercidiphyllum>).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Gene family classification for 18 species (including *C. japonicum*) in comparative genomic analysis.

Fig. S2 Geographic distribution of sampling locations for *C. japonicum* in China and Japan ($n = 24$) and *C. magnificum* in Central Honshu/Japan ($n = 4$).

Fig. S3 Schematic diagrams of six alternative divergence models with different gene flow events among the three lineages of *Cercidiphyllum* (*C. magnificum*, Japanese *C. japonicum*, Chinese *C. japonicum*, their common ancestor) using FASTSIMCOAL2.

Fig. S4 *K*-mer analysis for *C. japonicum* genome size estimation.

Fig. S5 Chromosomal Hi-C contact map of the *C. japonicum* genome.

Fig. S6 Syntenic depth patterns between *C. japonicum* vs *Vitis vinifera* and between *C. japonicum* vs *Kalanchoe fedtschenkoi*, respectively.

Fig. S7 The expansion and contraction of gene families in *C. japonicum* and 17 other species.

Fig. S8 Genetic relationships (ML tree) and population structure of *Cercidiphyllum japonicum* and *C. magnificum* individuals based on whole-genome nuclear dataset ($n = 99$; 2794 617 SNPs).

Fig. S9 Changes of effective population size (N_e) through time in *C. japonicum* estimated on its reference genome by using pairwise sequentially Markovian coalescent (PSMC) modelling.

Fig. S10 Changes in effective population size (N_e) through time in *C. japonicum* from China and Japan and *C. magnificum* using the SMC++ method.

Fig. S11 Schematic diagram of the best-fitting divergence model (no. 6) of *Cercidiphyllum* (*C. magnificum*, Japanese *C. japonicum*, Chinese *C. japonicum*, their common ancestor) as inferred by FASTSIMCOAL2.

Fig. S12 Genetic parameters for *Cercidiphyllum* species at the whole genome level.

Fig. S13 The linkage disequilibrium (LD) decay of *C. japonicum* all individuals, Chinese and Japanese *C. japonicum* individuals, respectively.

Fig. S14 SNP genotype variation of *C. japonicum* individuals from, respectively, China and Japan within the selective sweep region of chromosome 14 (Chr14) from 8.5 Mb to 12 Mb, as depicted for different length intervals.

Fig. S15 Frequency distribution of reads depth within balancing selection regions of Chr03 (29.11–29.32 Mb), Chr16 (6.47–6.70 Mb), Chr17 (20.78–21.01 Mb) and Chr19 (21.31–21.79 Mb).

Fig. S16 Hardy-Weinberg Equilibrium (HWE) test for all sites in the balancing selection regions of Chr03 (29.11–29.32 Mb), Chr16 (6.47–6.70 Mb), Chr17 (20.78–21.01 Mb) and Chr19 (21.31–21.79 Mb).

Fig. S17 SNP genotype variation of *C. japonicum* individuals from, respectively, China and Japan within the balancing selection regions of Chr 03 (29.11–29.32 Mb), Chr16 (6.47–6.70 Mb), Chr17 (20.78–21.01 Mb), and Chr19 (21.31–21.79 Mb).

Fig. S18 Principal component analysis (PCA) based on 19 bioclimatic variables of altogether 24 populations of *C. japonicum* from China ($n = 8$) and Japan ($n = 16$).

Fig. S19 The Gene Ontology (GO) terms with enrichment ratio more than 1 for genes significantly correlated with environmental variables in the genome of *C. japonicum*.

Methods S1 Genome assembly and genome evolution of *Cercidiphyllum japonicum*.

Methods S2 Population genomics analysis.

Table S1 Whole-genome sequencing data statistics of *C. japonicum*.

Table S2 Genome sizes estimated for *C. japonicum* based on 17-mer statistics.

Table S3 Characteristics of DNA base composition within the genome of *C. japonicum*.

Table S4 Validation of the completeness of the *C. japonicum* genome, using the Core Eukaryotic Genes Mapping Approach (CEGMA) based on 248 ultra-conserved core eukaryotic genes (CEGs).

Table S5 Validation of the completeness of the *C. japonicum* genome, using the Benchmarking Universal Single Copy Orthologs (BUSCO) approach.

Table S6 Validation of the genome assembly of *C. japonicum* using reads mapping and transcript alignment, respectively.

Table S7 Statistics for RNA-seq data from five tissues of *C. japonicum*.

Table S8 Prediction of protein-coding gene models in the genome of *C. japonicum*.

Table S9 Functional annotation of the predicted genes within the genome of *C. japonicum*.

Table S10 Classification of transposable elements (TEs) identified in the *C. japonicum* genome, including their length (in bp), the percent of total repeats and the percent of genome.

Table S11 Genomic comparison of gene families across 18 plant species, including *C. japonicum*.

Table S12 Gene ontology (GO) enrichment analysis of the expanded gene families in *C. japonicum*.

Table S13 PFAM functional enrichment analysis of the expanded gene families in *C. japonicum*.

Table S14 Sample information and sequencing statistics for population genomics of *C. japonicum* and *C. magnificum*.

Table S15 Annotation of single nucleotide polymorphism (SNP) variants across the 99 re-sequenced individuals of *Cercidiphyllum*, using ANNOVAR (Wang *et al.*, 2010) and with different filtering criteria.

Table S16 Comparison of six divergence models analysed with FASTSIMCOAL2.

Table S17 Parameter estimates with 95% highest posterior density (HPD) intervals for the best divergence model (no. 6) with parameter tags corresponding to Fig. S11.

Table S18 The 41 protein coding genes located in a region stretching from 5 Mb to 14 Mb of Chromosome 14 with statistics of nonsynonymous and synonymous sites in Chinese and Japanese *C. japonicum*.

Table S19 Putative regions under balancing selection in Chinese and Japanese populations of *C. japonicum*.

Table S20 Genes under balancing selection shared by both Chinese and Japanese populations of *C. japonicum*.

Table S21 Nineteen bioclimatic variables downloaded from WORLDCLIM.

Table S22 Gene Ontology (GO) enrichment analysis of the genes associated with environmental factors in *C. japonicum* using LFMM.

Table S23 The genes are correlated with environmental factors and located within high F_{ST} (z score > 2) regions in *C. japonicum*.

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