Genomic analyses provide new insights into the evolutionary history and reproduction of the Paleogene relictual *Kingdonia* (Circaeasteraceae, Ranunculales)

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Abstract

Asexual lineages are perceived to be short-lived on evolutionary timescales due to accumulation of deleterious mutations. Hence reports for exceptional cases of putative 'ancient asexual' species usually raise doubts about whether such species are recently derived or engage in some undocumented sexual reproduction. However, there have been few studies to solve the mystery. The monotypic *Kingdonia* dating to the early Eocene, contains only *K. uniflora* that has no known definitive evidence for sexual reproduction nor records for having closely related congeneric sexual species, seeming to have persisted under strict asexuality for long periods of time. In this study, we use population genomic analyses to test for reproduction mode and infer the evolutionary process and mechanisms facilitating the survival of the species. Our results indicate the presence of three differentiating genetic lineages within the species and support that asexual reproduction in *K. uniflora* indicated by high allelic heterozygosity had occurred prior to the lineage differentiation (~0.5 mya). We also detected DNA recombination events in some populations, in line with occurrence of unseen and unevenly distributed sexual reproduction among populations. However the observation of high linkage disequilibrium, relatively high ratio of π_N/π_S (nonsynonymous versus synonymous nucleotide diversity), together with high allelic heterozygosity suggest the sexual reproduction is infrequent. Furthermore, we found that genes containing SNPs with elevated *F*st values are significantly enriched in functions associated with seed development, suggesting differentiation in genes regulating seed development is likely to be the key reason of the uneven distribution

of sexual reproduction in *K. uniflora*. Evidence from our study supports predominate asexual reproduction in *K. uniflora*, but unseen sexual reproduction must have played a key role to ensure the long-term survival of the lineage in general. Uneven distribution of sexual reproduction in the species may be a key factor underlying the observed genetic differentiation between populations. This study provides novel insights into the reproduction and evolution of *Kingdonia*, a relict lineage evolved in the Paleogene and known for asexual reproduction, and demonstrate the power of data from population genome sequencing in resolving long-standing evolutionary questions.

Key words: asexual reproduction, allelic heterozygosity, DNA recombination, nonsynonymous nucleotide diversity, seed development.

Introduction

Sexual and asexual reproduction are the two basic methods of plant reproduction. Although sexual reproduction is the predominant mode in vascular plants, asexual reproduction occurs in many taxonomic groups (Mogie 1992). Compared to sexual reproduction, asexual reproduction is often described as “a short cut” and “cost effective”, requiring no waiting time and resources for fertilization to occur, resulting in production of more offspring in less time (Corley et al. 2001). Evolutionary theory predicts that asexual reproduction is not a successful long-term strategy. In the absence of sexual reproduction, accumulation of deleterious mutations, e.g., elevated

ratio of non-synonymous (selected) to synonymous (neutral) polymorphisms (π_N/π_S) is expected due to relaxed purifying selection in asexual lineages (Normark and Moran 2000; Ament-Velásquez et al. 2016). One consequence is the eventual extinction of asexual lineages once the accumulation reaches a high load of deleterious mutations (Muller's ratchet; Muller 1964; Felsenstein 1974). Hence, asexual lineages are traditionally perceived as evolutionary dead-ends (Maynard-Smith 1978; Zimmer 2009). However, a number of exceptions, the so-called 'ancient asexual' species, e.g., darwinulid ostracods and parthenogenetic oribatid mites, have been reported (Heethoff et al. 2007; Schoen et al. 2009; Brandt et al. 2021). These lineages were suggested to have persisted under obligate asexuality over millions of years, which violates the expectation that sexual reproduction and recombination are necessary for long-term survival (Schurko et al. 2009). A long standing hypothesis is that such 'ancient asexual' lineages should have special adaptive mechanisms, e.g., an efficient DNA repair system, to cope with the accumulation of deleterious mutations (e.g., Birky et al. 2005; Gladyshev and Meselson 2008). Alternatively, such lineages may not be true 'ancient asexuals', and several species (including the famous Bdelloid rotifers) previously believed to be long-term asexuals were indeed later shown to be either recently derived or to engage in some unseen sexual reproduction (Lunt 2008; Schurko et al. 2009; Signorovitch et al. 2015; Schwander 2016; Laine 2020; Brandt et al. 2021).

In the literature, the determination of obligate asexual reproduction in such 'ancient asexual' lineages has usually relied on negative evidence, such as the failure to find

individuals of the opposite sex for mating or failure to detect a recent sexual ancestor (Neiman et al. 2009). Such evidence is not conclusive regarding sexual reproduction is absent due to the possibility that unseen sexual reproduction may exist, and the apparent "obligate" asexuality might reflect our inability to observe sexual reproduction, making dominantly asexual lineages appear strictly asexual (Schurko et al. 2009). Hence, assessing the true mode of reproduction in putative 'obligate asexual' species requires more reliable methods. In contrast to previous organismal-based methods, molecular approaches provide an effective way to distinguish obligate from facultative asexual lineages. There are a number of expected genetic consequences under obligate asexual reproduction. For example, in asexual diploid species, alleles are expected to highly divergent due to the independent accumulation of mutations in the absence of segregation and genetic exchange, the well-known Meselson effect (Birky 1996; Mark Welch and Meselson 2000). Highly divergent alleles are also expected in recently derived homoploid hybrid species due to the divergence of gene copies derived from different species (Beck et al. 2012; Jaron et al. 2021). Correspondingly, a negative value of the F_{IS} index, which measures the level of within-individual heterozygosity, is expected in these lineages, as observed in obligate asexuals (Balloux et al. 2003; Ament-Velásquez et al. 2016). Another well-acknowledged signature of asexual reproduction is the generation of non-random associations between loci, i.e., linkage disequilibrium (LD), which is often used for estimating the amount of asexual reproduction. Therefore, genome-wide linkage disequilibrium is expected in obligate asexuality (de Meeûsa and Balloux 2004;

Henry et al. 2012; Lovell et al. 2014). Additionally, in a strictly asexual lineage, where mutation is the only source of genetic novelty and all loci show complete linkage, the genealogy of genes and genomes of the lineage are expected to be a strictly branching tree rather than a network (Normark et al. 2003). Although some of these genetic consequences related to asexual reproduction have been assessed in animals, investigating all of these consequences in a single study has been rare, and largely lacking in plant lineages appears to be asexual only. .

Kingdonia, represented by a single species *K. uniflora*, is one of two monotypic genera (*Kingdonia* and *Circaeaster*) in the family Circaeasteraceae (Ranunculales; Angiosperm Phylogeny Group, 2016). The genus represents an ancient lineage estimated to have diverged from its sister *Circaeaster agrestis* Maxim. during the early Eocene based on molecular dating using the DNA sequences of three chloroplast spacers (52 mya; 95% HPD=27-75 mya), 497 single-copy genes (51.8 mya; 95% HPD=31-76 mya) and whole plastome sequences (52.2 mya; 95% HPD=26-83 mya), respectively (Ruiz-Sanchez et al. 2012; Sun et al. 2020; Zhang et al. 2020). Unique among angiosperms, both *K. uniflora* and *C. agrestis* possess an unusual dichotomous leaf venation similar to that of ferns and *Ginkgo*, indicating a reversal to an ancestral venation type of vascular plants. *Kingdonia uniflora* (diploid, 2n = 18) is a herbaceous species with a genome size of ~ 1 Gb, endemic to alpine regions of southwest China and grows in cold and humid habitats with deep humus (Sun et al. 2020). The species has a very narrow distribution being restricted to the Qinling Mountains, Minshan Mountains and Daxue-Qionglai Mountains (Fig. 1a). Notably, *K. uniflora* is

well-known to produce new "individuals" by means of rhizome rupture, which occurs on rhizomes more than three years old (Lei et al. 2000; Li et al. 2003; Supplemental Fig. S1). Although production of seeds was observed occasionally, but different from its sexual sister species *C. agrestis*, the seed embryo of *K. uniflora* can only develop to the torpedo stage (Ren et al. 1998). Efforts to germinate *K. uniflora* seeds in the natural habitat and lab had a zero rate of success (Li et al. 2004). So far, field investigations throughout the species' range found no seedlings of the species in natural populations (Li et al. 2003; Xu 2015). Available evidence suggests that the species may have evolved without sexual reproduction for long periods of time, representing a putative 'ancient asexual' lineage. Here we characterize genome-wide genetic variation across nearly all known *K. uniflora* populations. Specifically, we aim to determine (1) if the species is indeed an obligate asexual lineage or cryptic sexual reproduction is present, and (2) when asexual reproduction has evolved in the species. To answer these questions and better understand the evolutionary mechanisms of the species, we conducted various analyses to (a) characterize the genetic variation and population structure, (b) examine the genomic signatures of reproductive strategies, (c) reconstruct the demographic and evolution histories, and (d) identify and annotate outlier genetic variants.

Results

Sequence data processing

We produced 1601 Gb of data containing 2,356,880,312 raw reads for 60 individuals of *K. uniflora*, and 2,342,304,596 clean reads (SRA------) after filtering. The depth of coverage for the 60 samples ranged from 21.6× (WZ4) to 34.8× (TN1), with a mean coverage of 26.6x (Supplemental Table S1). Using the *K. uniflora* reference genome (GenBank: PRJNA587615, Sun et al. 2020), we obtained 9,349,354 raw SNPs, and 8,678,779 high quality SNPs after primary standard strict filtering. Additionally, by applying deep filtering standards, we obtained 114,746 SNPs located in 960 contigs (Supplemental Table S2).

Genetic diversity and population structure

Our LEA (Landscape and Ecological Association) analysis with the cross-entropy method failed to identify an ideal best-fit number of *K* (Supplemental Fig. S2). However, our phylogenetic analysis using the SVDQuartets method (Chifman and Kubatko 2014) revealed three clusters (supported by three quarters (361494/487635) of all the quartets), corresponding to the QL (Qingling Mountains), MS (Minshan Mountains) and DQ (Daxue-Qionglai Mountains) groups (Fig. 1c). Therefore, we repeated the LEA analysis (Fig. 1b; Supplemental Fig. S3) by setting *K*=3. The result recognized the same three groups identified by SVDQuartets. Additionally, two of the three populations from the QL group, three of the five populations from the MS group, and two of the four populations from the DQ group exhibit different levels of genetic admixture with the other groups (Fig. 1a,1b).

Nucleotide diversity (π) (the average π value) or per-site heterozygosity analysis

177 showed that the three groups have similar genetic diversity, ranging from 1.359×10^{-3}
178 to 1.497×10^{-3} (Table 1; Fig. 1d). The genetic differentiation statistics (fixation index;
179 F_{ST}) among the three groups estimated from VCFtools v0.1.16 (Danecek et al. 2011)
180 was 0.136 between the non-adjacent QL and the DQ groups (highest), 0.086 between
181 the adjacent MS group and DQ group (lowest), and 0.0104 between the adjacent
182 groups MS and QL (Fig. 1d), indicating low genetic differentiation among the three
183 groups. The Mantel test showed significant isolation by distance (IBD) ($r = 0.468$, $P =$
184 0.0002) (Fig. 1e). Results from the AMOVA showed small genetic variation among
185 the three groups (5.6%; Table 2). The results also showed no significant amount of
186 variation among individuals within populations (with a negative component value of
187 -48%; Table 2). And most of the genetic variation in *K. uniflora* was explained by
188 variation within individuals (with a component value of 123.83% within individuals)
189 (Table 2).
190

191 **Table 1.** Summary of genetic diversity of *K. uniflora* calculated using 114,746 variant positions.

Group IDs	Population IDs	*n*	π (10^{-3})
QL		**15**	**1.359**
	FP	5	1.592
	TB	5	1.466
	TN	5	1.696
MS			**1.457**
	AX	5	1.771

	HS	5	1.648
	JZG	5	1.602
	WZ	5	1.870
	ZQ	5	1.715
DQ			**1.497**
	EM	5	1.867
	HLG	5	1.755
	MB	5	1.686
	WL	5	1.625

192 *n*, number of individuals sampled; π, average number of pairwise nucleotide differences per site.

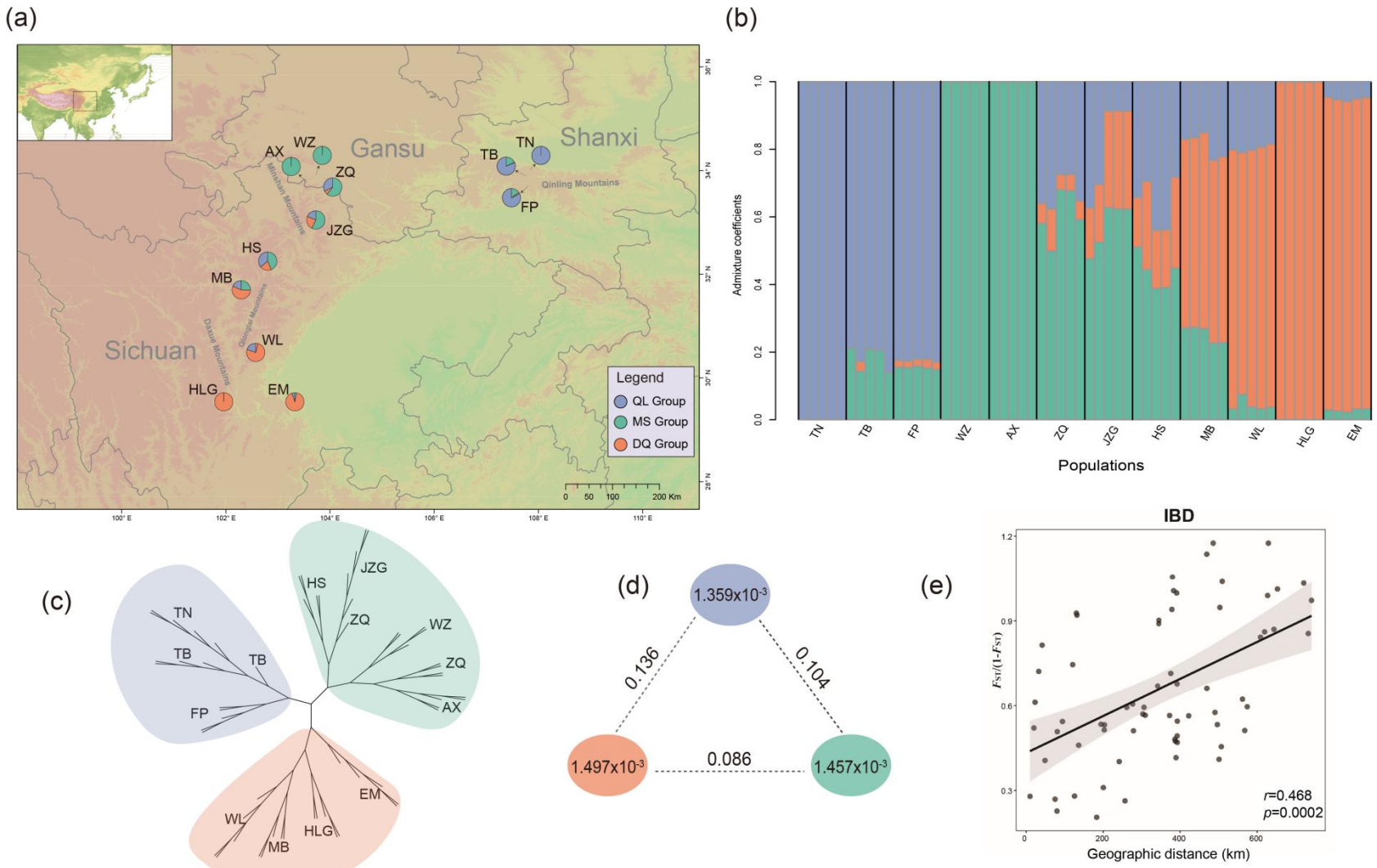
193 The statistics of three defined groups are given in bold.

194

195 **Table 2.** Details on the results of the analysis of the molecular variance (AMOVA).

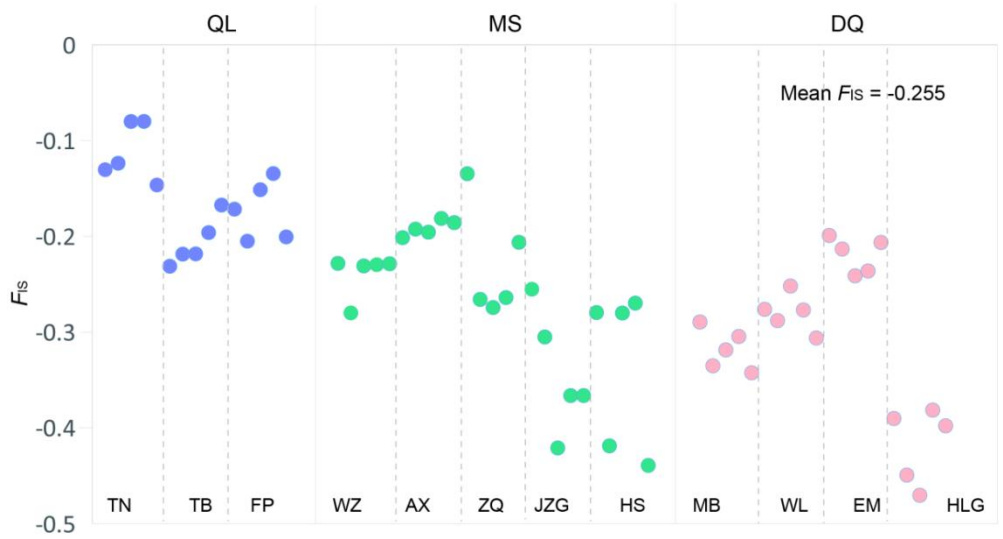
Source of variation	**Sum of squares**	**Variance components**	**Percentage variation**	**Fixation indices**	***P*-values**
Among groups	166030.43	993.35	5.06	0.05	**0.00**
Among populations within groups	396979.34	3879.21	19.76	0.21	**0.00**
Among individuals within populations	255202.00	-9443.63	-48.10	-0.64	1.00
Within individuals	1452238.00	24203.97	123.28	-0.23	1.00

196 Significant *P*-values are in bold.


(a)
Gansu
Shanxi
Sichuan
Minshan Mountains
Qinling Mountains
Daxue Mountains
Qionglai Mountains
AX
WZ
ZQ
JZG
TN
TB
FP
HS
MB
WL
HLG
EM
Legend
QL Group
MS Group
DQ Group
0 50 100 200 Km
100° E
102° E
104° E
106° E
108° E
110° E
38° N
34° N
32° N
30° N
28° N
(b)
Admixture coefficients
0.0
0.2
0.4
0.6
0.8
1.0
TN
TB
FP
WZ
AX
ZQ
JZG
HS
MB
WL
HLG
EM
Populations
(c)
TN
TB
TB
FP
HS
JZG
ZQ
WZ
ZQ
AX
EM
WL
MB
HLG
(d)
1.359x10-3
0.136
0.104
1.497x10-3
0.086
1.457x10-3
(e)
IBD
Fst/(1-Fst)
0.3
0.6
0.9
1.2
0
200
400
600
Geographic distance (km)
r=0.468
p=0.0002

197

Fig. 1 Genetic structure and diversity within *K. uniflora*. (a) Sampling sites and genetic structure detected by LEA analysis (K = 3 populations) mapped using ArcGIS v10.3. The genetic QL group is shown in blue, the MS group is shown in green, and the DQ group is shown in orange. (b) Genetic groups of *K. uniflora* inferred by the LEA analysis when setting K=3. (c) A phylogeny derived from SVDQuartets using 114,746 SNPs. (d) Nucleotide diversity (π) and population divergence (F_{ST}) across three genetic groups. The value in each circle represents a measure of nucleotide diversity for this group, and the value on each line indicates divergence between groups. (e) Correlation between genetic distance and geographical distance (isolation by distance, IBD), as tested by Mantel test.



205 Fig. 2 Distribution of values of inbreeding coeffificient (F_{IS}). Under Hardy-Weinberg equilibrium F_{IS} is 0, negative values of F_{IS} indicate excess of individual

206 heterozygosity.

207 **Genomic signatures and signs of sexual and asexual reproduction**

208 The calculation of F_{IS} using VCFtools v0.1.16 (Danecek et al. 2011) revealed negative
209 F_{IS} values (ranging from -0.07 to -0.47) for all 60 *K. uniflora* individuals (mean
210 F_{IS}=-0.26) (Fig. 2), indicating greater observed individual heterozygosity
211 (Supplemental Table S3) than expected from random mating, a sign of asexual
212 reproduction. The site frequency spectrum (SFS) across all 12 populations showed
213 sites with heterozygous SNPs shared among three lineages/all 12 populations were
214 most abundant (12,367 sites), *c.* 250 times more frequent than expected under
215 Hardy-Weinberg equilibrium (HWE) (purple bar in Fig. 3), suggesting a large
216 proportion of the observed excess individual heterozygosity in the 12 populations
217 were attributed to accumulation by asexuality prior to the evolutionary divergence of
218 the three genetic lineages (QL, MS and DQ).
219

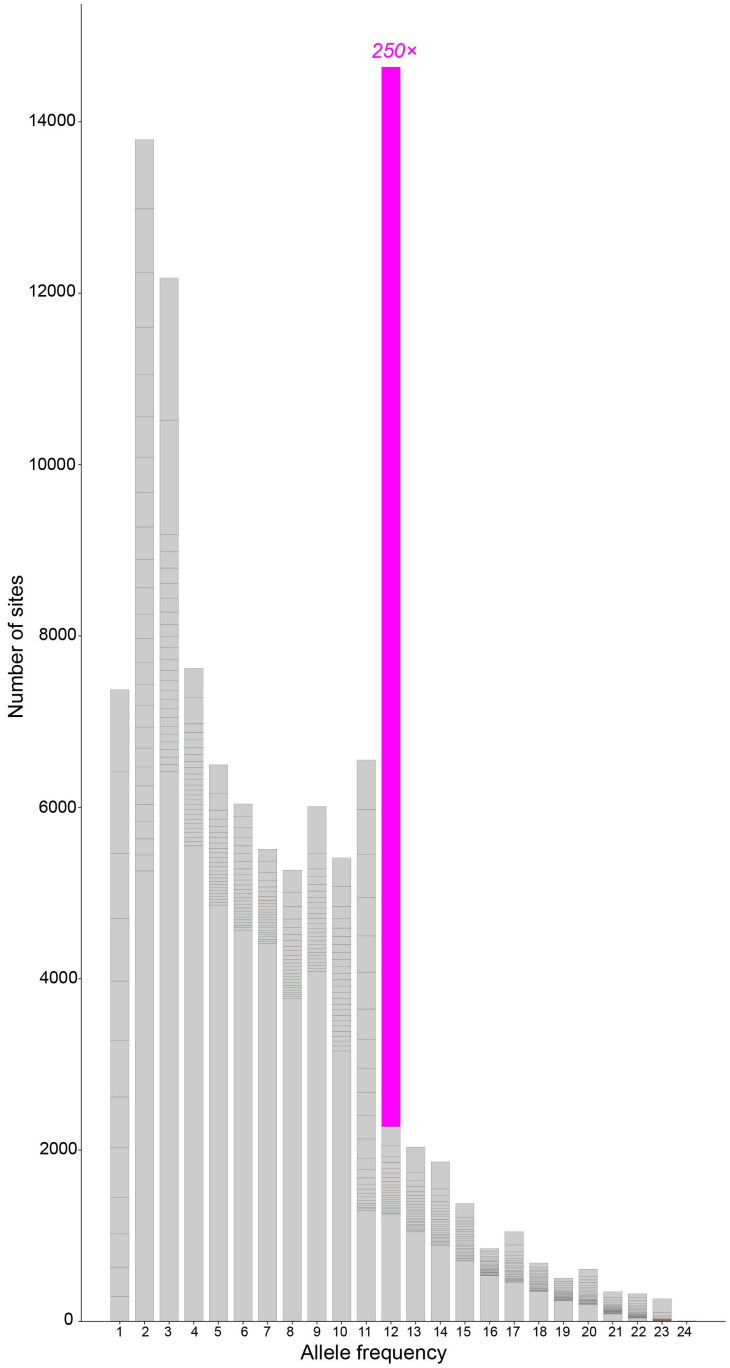
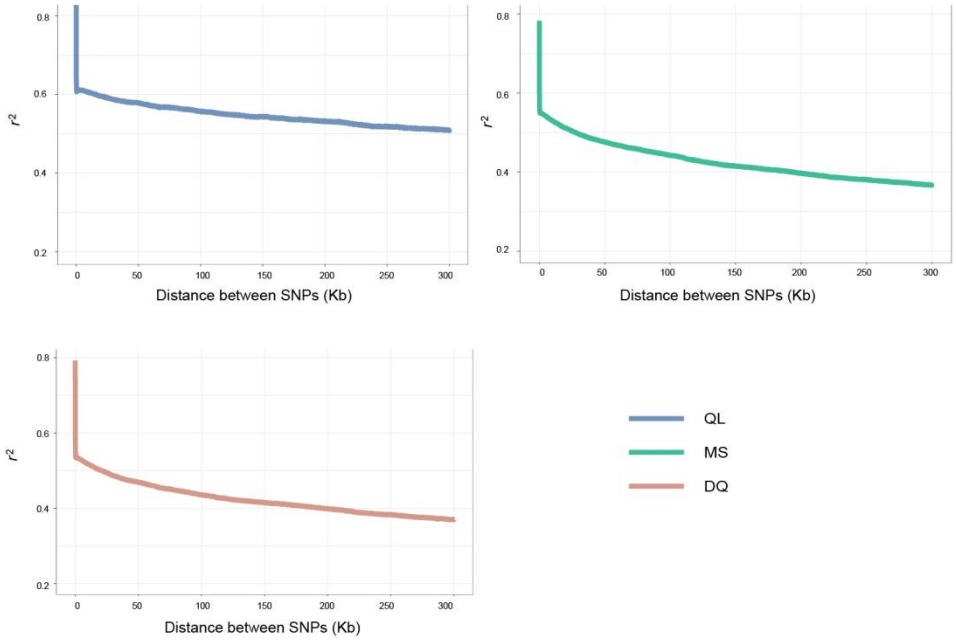


Fig. 3 The site frequency spectrum (SFS) depicting the number of sites with different allele frequency across 12 *K. uniflora* individuals (one allele can display a maximum frequency of 24 among 12 diploid individuals). Heterozygous genotypes shared among all 12 populations are highlighted using the purple color and its excess over HWE indicated (*c*. 250 times as frequent as expected under HWE).

227 Result from Linkeage Disequilibrium (LD) analyses showed sites within 0-25 kb
228 region had an average r^2 of 0.60, 0.50 and 0.50, which slowly decay to *c*. 0.50, 0.40
229 and 0.40 after a physical distance of 300 kb in QL, MS and DQ, respectively (Fig. 4),
230 implying very slow LD decay over very large distance. In addition, the LD analysis
231 between pairs of SNPs up to 1000 kb obtained similar results (Fig. S4).



232

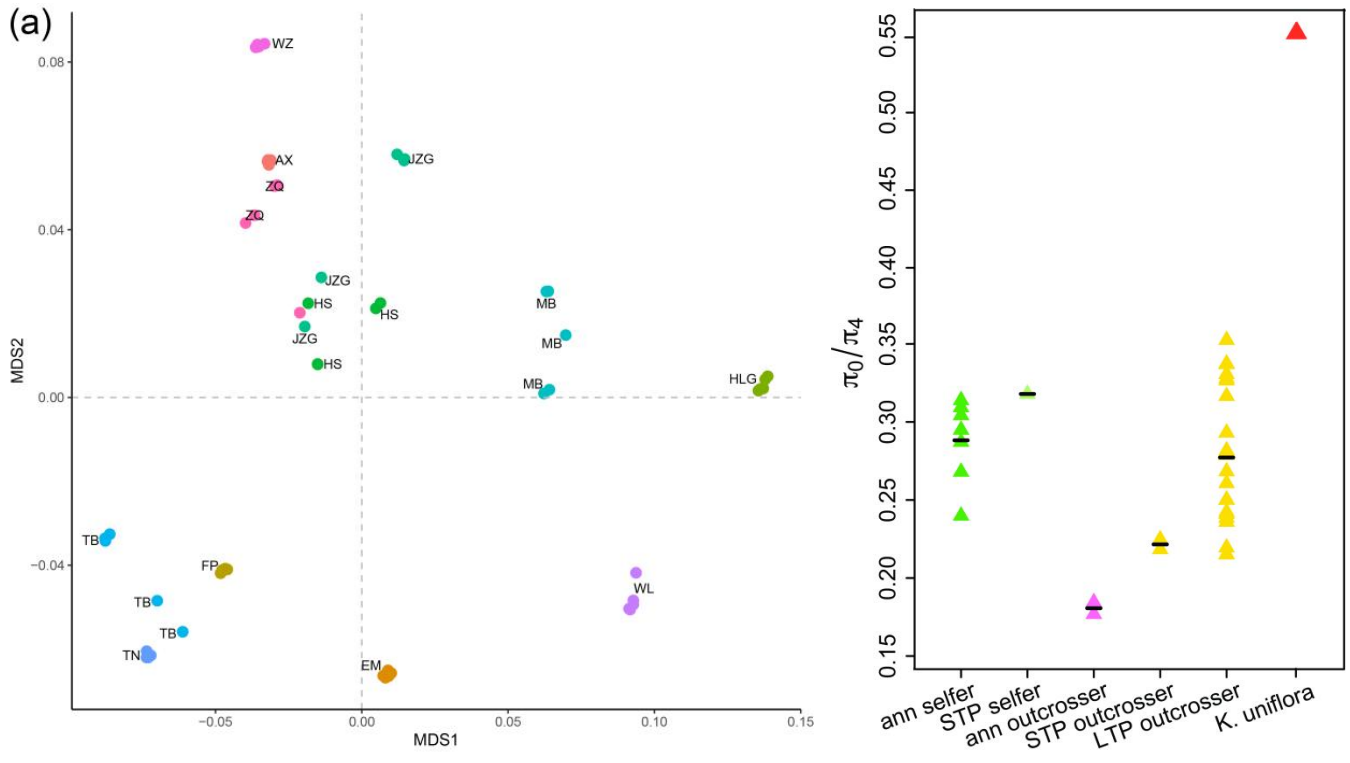
233 Fig. 4 Decay of linkage disequilibrium (LD) with physical distance in three genetic groups of
234 *Kingdonia uniflora*. Averages of pairwise linkage disequilibrium measures r^2 are plotted for each
235 bin of distances between pairs of SNPs. The displayed data are for the bins with pairs of SNPs
236 separated by ≤300 kb

237

238 We used the ratio of nonsynonymous to synonymous polymorphisms (π_N/π_S) to
239 assess the efficiency of purifying selection in *K. uniflora*. We defined a total of 24
240 genotypic groups according to multidimensional scaling (MDS) clustering (Fig. 5a).
241 Among the 24 genotypic groups, genetic diversity at zero-fold degenerate

242 (nonsynonymous) and four-fold degenerate (synonymous) sites (π_0 and π_4) is 0.000546

243 and 0.000992, respectively, resulting in π_0/π_4 (π_N/π_S)=0.55 (Fig. 5b).



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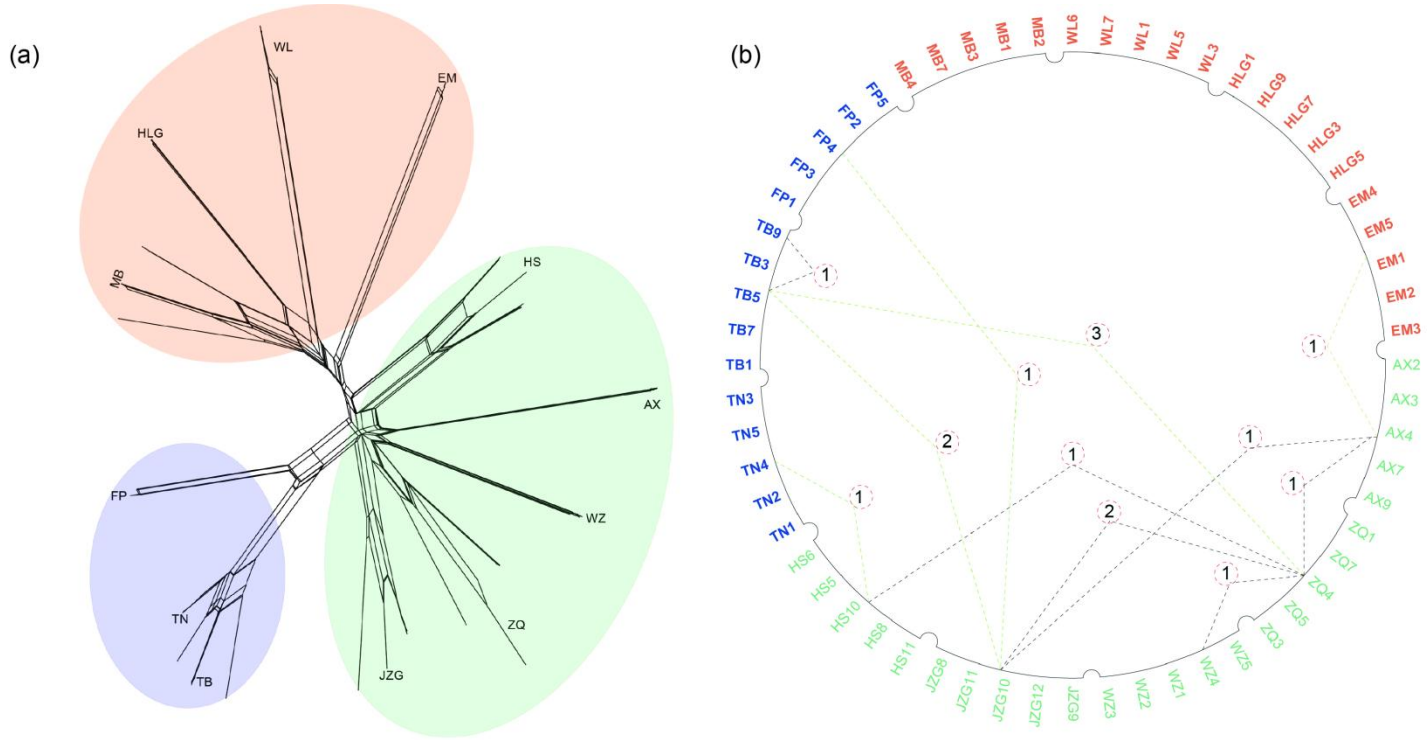
245 Fig. 5 Calculation of π_N/π_S in *K. uniflora*. (a) Multidimensional scaling analysis of identity-by-state pairwise distances between individuals. Individuals in the same

246 population are coded with the same color. Individuals that come from the same population and cluster together are grouped into one genotypic group, and a total of

247 24 genotypic groups are identified. (b) π_0/π_4 difference between *K. uniflora* and other plants reported in Chen et al. (2017). The figure is modified based on Fig. 2B in

248 Chen et al. (2017). STP, short-term perennial; LTP, long-term perennial.



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250 Fig. 6 Recombination events detected in *Kingdonia uniflora* (a) Recombination events identified by SplitsTree phylogenetic networks using a total of 114,476 SNPs .

251 The closed loops in the network shows recombination events. (b) Recombination events identified by RDP4. Black dotted lines indicate recombination between

252 different individuals in the same genetic group; green dotted lines represent recombination between different individuals from different genetic groups; the number of

253 corresponding DNA recombination events are indicated in circles.

Results from SplitsTree v4.13.1 (Huson and Bryant 2006) showed reticulations on the phylogeny of *K. uniflora*, which are signals of recombination (Fig. 6a). When all SNPs were concatenated into a single sequence, the RDP4 (Recombination Detection Program 4) (Martin et al. 2015) analysis identified 15 recombination events that were unevenly distributed among populations (Supplemental Table S4; Fig. 6b).

Inferring population demographic history and distributions

The model with the highest score found by simulations of demographic history using FSC2 (*fastsimcoal2*) (Excoffier et al. 2013) showed a divergence between the DQ and QL + MS groups 513,000 years ago and a divergence between the MS and QL groups 37,800 years ago (Table 3; Fig. 7a). The population sizes of DQ, MS and QL were estimated to be 98,800, 32,300 and 23,200, respectively (Table 3; Fig. 7a). The ancestral effective population size of the species ($N_{A1;}$ Fig.7a) was estimated to be 29,000 (Table 3), slightly larger than that of QL, but smaller than those of DQ and MS (Table 3; Fig. 7a). The model also indicates asymmetric gene flow among the three genetic groups, with the rates of migration from QL to DQ and MS being higher than in the reverse direction. Between DQ and MS, the rate of migration from MS to DQ is higher than in the opposite direction (Table 3).

Results from MAXENT ENM (ecological niche modeling) (Phillips and Dudik 2008) analyses showed a greater suitable habitat for *K. uniflora* in the LGM than at present, especially in the Hengduan Mountains region, and that there is more suitable habitat at present than is currently occupied (Fig. 7b).

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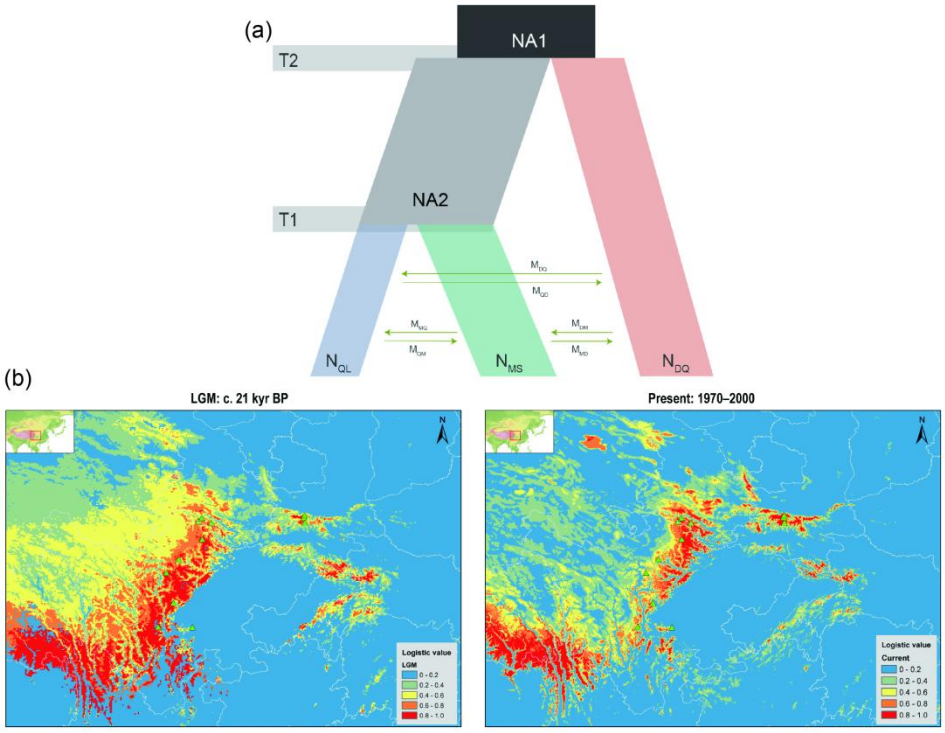

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278 Fig. 7 Inferred evolutionary history of *K. uniflora*. (a) Schematic of demographic scenarios

279 modeled using FSC2, with the ancestral population shown in gray. Column width represents the

280 relative effective population size. Arrows indicate gene flow between populations. (b) Predicted

281 suitable distributions of *K. uniflora* at different historical periods based on species distribution

282 modeling (SDM). Area color indicates probability (0-1) of suitable habitat for *K. uniflora*. LGM,

283 last glacial maximum. The map image was derived from ArcGIS v10.3.

284

285 **Table 3.** Inferred demographic parameters for the best-fitting FSC2 model shown in Figure 7a,

286 including 95% confidence intervals.

Parameter	Point estimation	95% confidence intervals	
		Lower bound	Upper bound
T_1	3.78E+04	2.65E+04	4.91E+04

T_2	5.13E+05	4.60E+05	5.65E+05
N_{A1}	2.90E+04	2.46E+04	3.33E+04
N_{A2}	1.08E+05	1.00E+05	1.15E+05
N_{DQ}	9.88E+04	6.58E+04	1.32E+05
N_{MS}	3.23E+04	2.74E+04	3.71E+04
N_{QL}	2.32E+04	3.55E+03	4.29E+04
M_{DM}	2.30E-04	9.76E-05	3.63E-04
M_{DQ}	1.53E-04	8.08E-05	2.26E-04
M_{MD}	6.13E-04	3.26E-04	9.01E-04
M_{MQ}	4.13E-04	1.58E-04	6.67E-04
M_{QD}	1.08E-03	4.67E-04	1.69E-03
M_{QM}	1.12E-03	6.84E-04	1.56E-03

Notes: Parameters included here comprise population size measures (N_{A1}, N_{A2}, N_{DQ}, N_{MS}, and N_{QL}, indicating ancestral populations, QL+MS, DQ, MS and QL, respectively), population divergence times (T1 and T2, years), migration per generation after diversification between QL and MS, QL and DQ, and DQ and MS.

Assessing the correlation between genetic differentiation and environmental variables

Among the seven variables used for the GF (gradient forest) analysis, precipitation seasonality (bio15) and temperature seasonality (bio04) were identified as the two most important predictors of genetic variation. Mean temperatures of the warmest

297 quarter (bio10), annual precipitation (bio12) and precipitation of the wettest month
298 (bio13) were also of high importance (Fig. 8). The redundancy analysis (RDA)
299 revealed a significant amount of genetic variation among populations associated with
300 the seven important environmental variables (27.06%, $P = 0.001$). Each of the two
301 axes explained a significant amount of variation (Axis 1: 25.80%, $P = 0.001$**; Axis 2:
302 17.03%, $P = 0.001$**; Fig. 8). Separate analyses of the seven environmental variables
303 individually also found significant association with genetic variation (Table 4).
304 Consistent with the GF analysis, temperature seasonality (bio04; 6.498%, P = 0.001**)
305 and precipitation seasonality (bio15; 6.27%, P = 0.001**) were the two most
306 important predictors (Table 4).
307
308 **Table 4.** Redundancy analysis (RDA) results based on seven important environmental variables
309 identified by GF analysis. **, $P < 0.01$.

Environmental variables	Constrained proportion (%)	*F*-statistic	*P*
Bio04	6.498	4.0309	0.001**
Bio15	6.27	3.88	0.001**
Bio02	5.138	3.1418	0.001**
Bio13	4.477	2.7183	0.001**
Bio11	4.02	2.4294	0.001**
Bio10	3.973	2.3996	0.001**
Bio12	3.936	2.3762	0.001**

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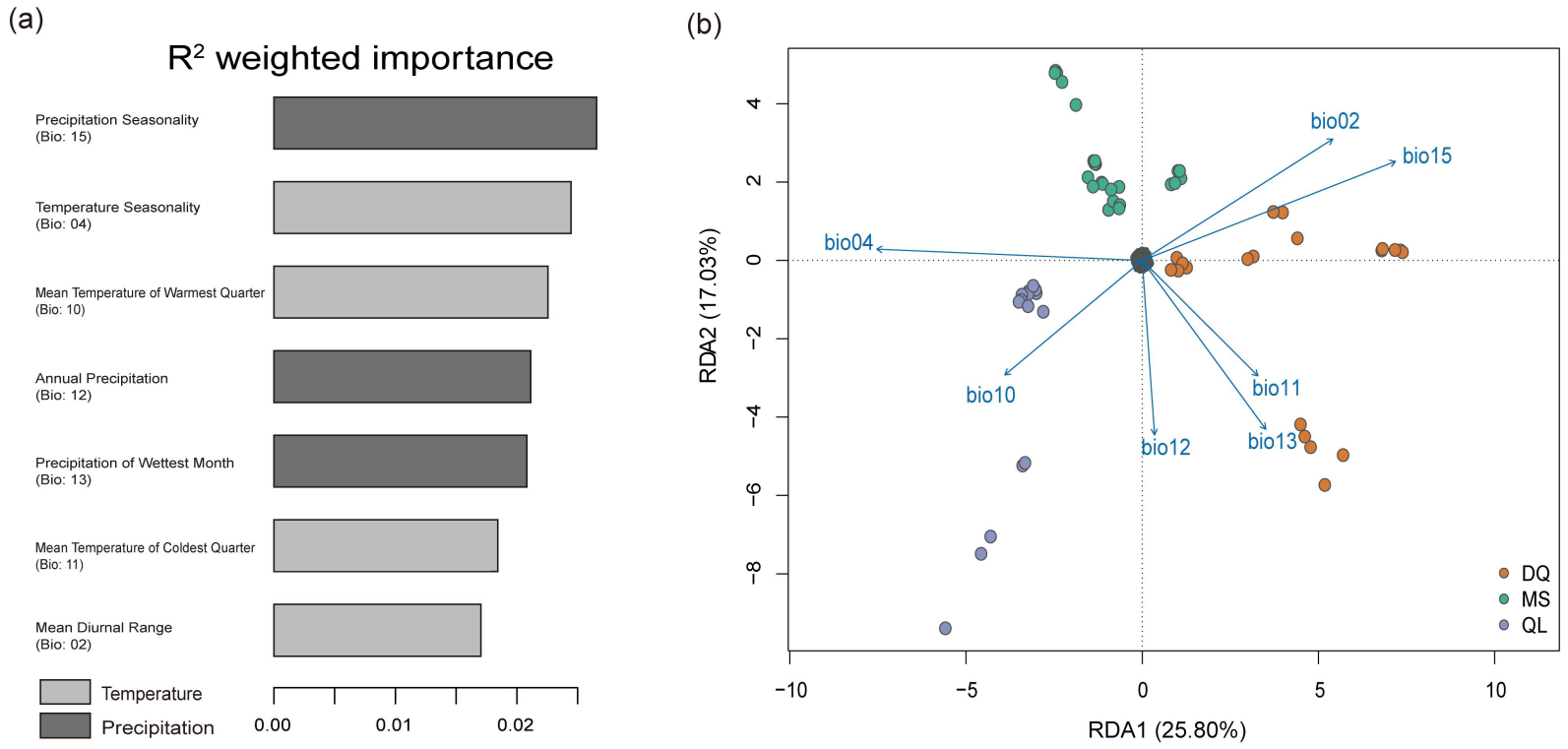
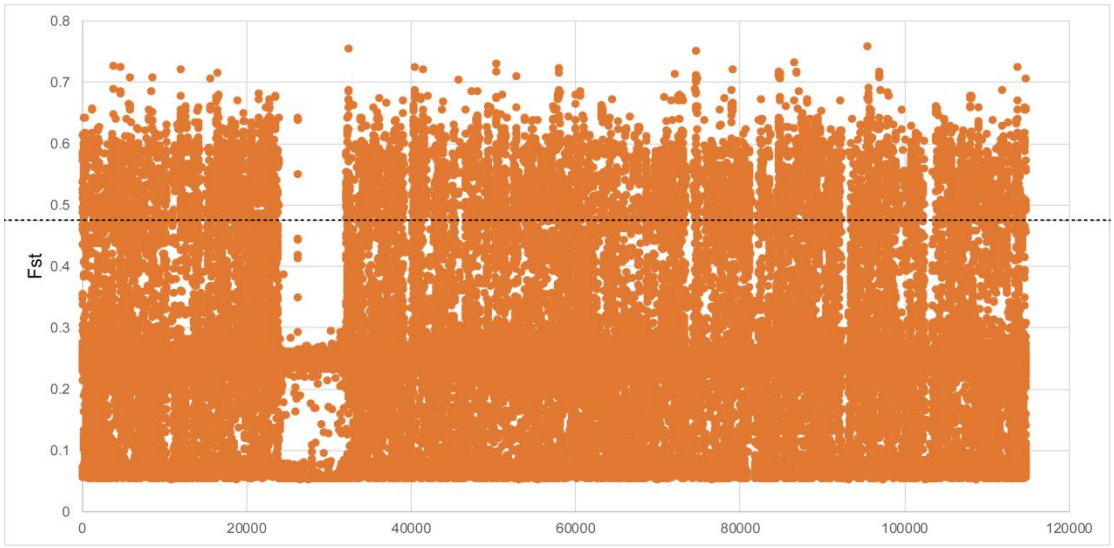


Fig. 8 Correlation between genetic distance and environmental difference. (a) R^2-weighted importance of environmental variables that explain genomic variation from GF analysis. (b) Redundancy analysis showing the relationship between the independent climate parameters and population structure of *K. uniflora*. Individuals are colored points and colors represent three groups (QL, MS, DQ). Small black points are SNPs.

316 **Detecting and annotating outlier loci**

317 The average F_{ST} between populations was estimated as 0.24, but ranged from 0.05
318 to 0.76 (Fig. 9). BAYESCAN identified 7,917 outlier (*F*st values ranging from 0.47 to
319 0.76) sites out of the total 114,746 SNPs. Through comparisons with the reference
320 genome, we found these outliers were located in 159 genes, of which 87 had BLAST
321 hits in the Unified Protein Database, related to 15 biological processes (second-level
322 gene ontology (GO) terms) (Fig. 10a). Gene set enrichment (GSE) analysis for all 87
323 genes detected five significantly over-represented terms ($p < .05$) associated with
324 reproduction, including: "reproduction" (13 genes), "reproductive process" (13 genes),
325 "reproductive developmental process" (11 genes), "fruit development" (6 genes), and
326 "seed development" (6 genes) (Fig. 10b; Table 5).

327



328

329 Fig. 9 F_{ST} values of 1, 114,746 SNPs among 60 *K. uniflora* individuals calculated by BAYESCAN.
330 The x-axis represents the sequence number of SNPs. The black dash line indicates the cut-off line
331 between identified outliers and non-outliers.

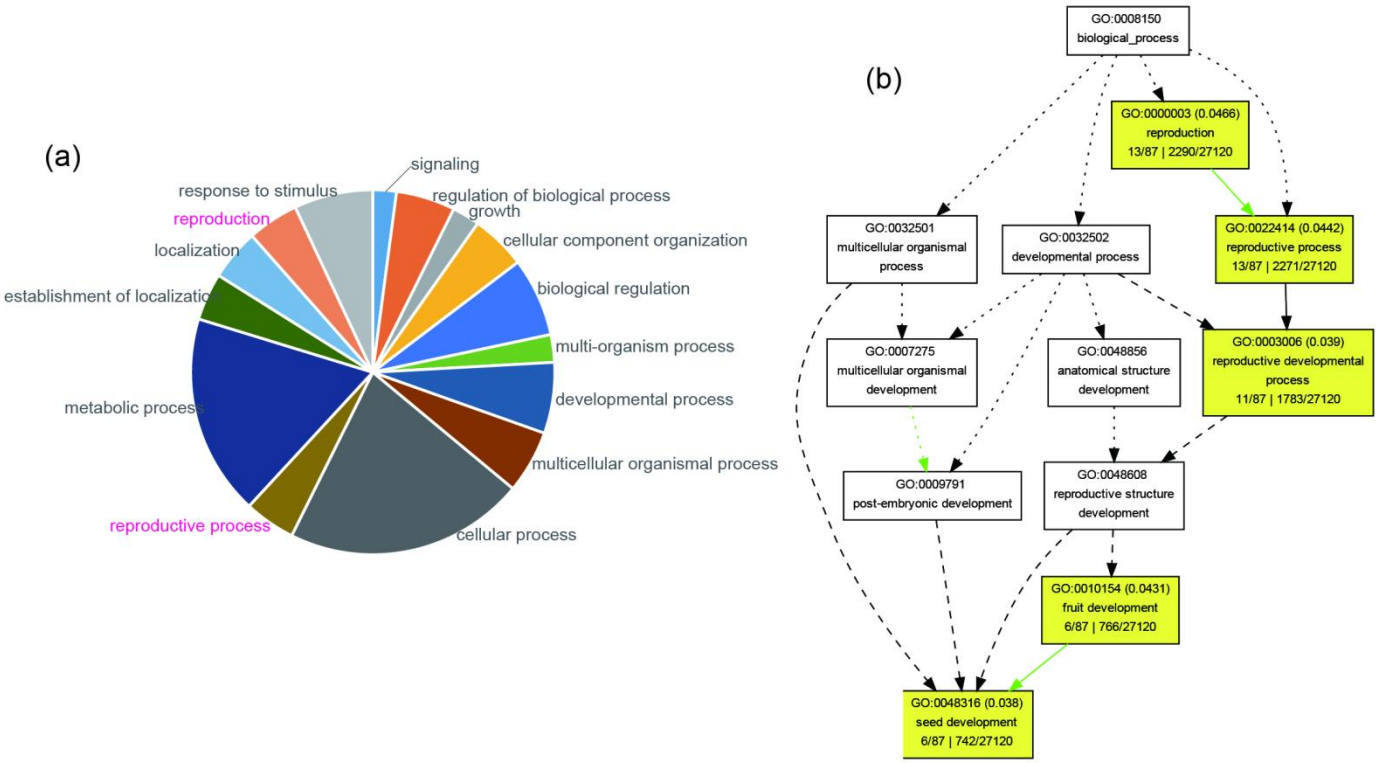


Fig. 10 Gene set enrichment (GSE) analysis of genes containing outlier SNPs. (a) Pie chart of second-level gene ontology (GO) terms (biological process) of genes showing outlier *F*st values. (b) Graphical results showing enriched GO terms (second to sixth level; highlighted with yellow color) of genes containing outlier SNPs.

336

337 **Table 5.** The significantly over-represented GO terms of genes containing outlier SNPs in *K. uniflora*.

GO term	Class	Description	Significant	Annotated	*p*-value
GO:0048316	P	seed development	6	742	0.038
GO:0003006	P	reproductive developmental process	11	1783	0.039
GO:0010154	P	fruit development	6	766	0.043
GO:0022414	P	reproductive process	13	2271	0.044
GO:0000003	P	reproduction	13	2290	0.047

338

Discussion

Kingdonia uniflora is characterized by high allelic heterozygosity, slow LD decay, reduced efficacy of purifying selection, and unseen sexual reproduction

In diploid asexuals, high levels of allelic divergence are expected to result from two factors: (1) long-term evolution under obligate asexuality, i.e., Meselson effect (Birky 1996; Mark Welch and Meselson 2000) and (2) transition to asexual reproduction via hybridization between sexual species (Jaron et al. 2021). The Meselson effect is usually considered to be a strong indicator of long-term evolution under obligate asexuality (Hartfield 2016; Brandt et al. 2021), yet this phenomenon has been shown to appear in young lineages of less than 100,000 years age (Pellino et al. 2013). Here we detected high allelic heterozygosity indicating obligate or high levels of asexuality in the flowering plant species *K. uniflora*: (1) an excess of observed individual heterozygosity over Hardy-Weinberg expectation as indicated by negative F_{IS} values (Fig. 2) (which is different from what is observed in the sister sexual species *C. agrestis* (mean F_{IS} = 0.02; Table 1 in Zhang et al. 2020) and (2) greater genetic divergence within individuals than that between populations revealed by the AMOVA analysis (Table 2). Theoretically, as mentioned above, both the Meselson effect and hybrid origin can explain high allelic heterozygosity in diploid asexuals. Hence high levels of allelic divergence detected in *K. uniflora* could be explained as accumulation of heterozygous variants caused by independent evolution of alleles after the transition to obligate or high levels of asexuality. Alternatively, *K. uniflora* could have switched to obligate or high level of asexuality via interspecific

361 hybridization, as reported in asexual species of *Meloidogyne*, *Lineus* ribbon worms,
362 and the *Ranunculus auricomus* complex (Lunt 2008; Pellino et al. 2013; Lunt et al.
363 2014; Ament-Velasquez et al. 2016). In the current study, although we cannot
364 formally exclude a hybrid origin of asexuality, this is unlikely to be the case in *K.*
365 *uniflora*. Different from the above hybrid origins of asexual species with multiple
366 congeneric sister species, *Kingdonia* is a monotypic genus and has no fossil record
367 that indicates the presence of parental species for hybridization. The closest related
368 species, *C. agrestis*, is from a different genus and diverged from *K. uniflora*
369 tens of millions of years ago (Sun et al. 2020; Zhang et al. 2020). The site frequency
370 spectrum reveals the level of the heterozygous genotypes shared among
371 lineages/populations greatly exceed those under Hardy-Weinberg equilibrium (Fig. 3),
372 indicating high allelic heterozygosity occurred prior to lineage differentiation. Hence,
373 in any event, both explanations (i.e., long-term asexuality and hybrid origin) provide
374 support that *K. uniflora* has engaged in obligate or high levels of asexuality before
375 lineage differentiation around 0.5 mya.

376 Asexual reproduction prevents free exchange of alleles among individuals and
377 results in allele linkage disequilibrium (LD) and in extreme cases (obligate asexuality)
378 may result in complete physical linkage of markers over the entire genome
379 (Flint-Garcia 2003; Simko et al. 2006; Schurko et al. 2009). Various genetic processes
380 in outcrossing can reduce LD and free random mating results in linkage equilibrium
381 of alleles. Therefore, the level of LD in a species reflects the extent of inbreeding
382 (non-random mating) or asexuality. Our data show that LD in *K. uniflora* has an

383 average r^2 up to 0.6, and the decay of LD with physical distance is extremely slow
384 (Fig. 4). This pattern of LD decay is comparable to that seen in the highly
385 self-fertilizing/asexual plant species, e.g., *Arabidopsis thaliana* (Nordborg et al. 2005;
386 Kim et al. 2007), *Medicago truncatula* (Branca et al. 2011), and *Spirodela polyrhiza*
387 (Ho et al. 2019), and completely different from outcrossing populations that often
388 show rapid LD decay over several hundred bp (Foxe et al. 2009; Mackay et al. 2012;
389 Ho et al. 2019). The LD decay pattern provides genetic evidence supporting *K.*
390 *uniflora* as a species is undergoing reproduction by high asexuality.
391 Interference among loci caused by high linkage disequilibrium will decrease the
392 efficiency of selection by preventing selection from acting individually on each locus
393 (Gordo and Charlesworth 2001; Otto 2021). Such kinds of selective interference, e.g.,
394 a selective sweep, can result in a higher ratio of non-synonymous (selected) to
395 synonymous (neutral) polymorphisms in asexual lineages (Ament-Velásquez et al.
396 2016; Hartfield 2016). The value of π_N/π_S ratio (0.55) in *K. uniflora* is relatively
397 higher than that (ranging from 0.20 to 0.35) observed in outcrossing plants (Fig. 5b),
398 implying a higher rate of non-synonymous substitution caused by reduced efficacy of
399 purifying selection in *K. uniflora*. Under high levels of asexuality, mutations are
400 expected to be an important source of variation, with mutations typically occurring in
401 a heterozygous state in asexual species due to independent evolution of alleles (as
402 found in *K. uniflora* by the F_{IS} and SFS analyses). However, recessive mutations are
403 not exposed to selection (Ament-Velásquez et al. 2016), which could be another
404 reason why a higher rate of non-synonymous substitution is detected in *K. uniflora*.

405 Although high allele divergence, LD level and π_N/π_S ratio are characterized by *K.*
406 *uniflora*, low levels of sexual reproduction cannot be excluded. We detected
407 recombination events in RDP4 analysis and genealogical network (Supplemental
408 Table S4; Fig. 6), suggesting occurrence of sexual reproduction. The evidence
409 supports *K. uniflora* as likely not an obligate asexual species. Although the detected
410 recombination events could have been from mitotic recombination, due to the
411 following evidence, we argue that occasional successful sexual reproduction exists in
412 the species. First, the species still produces seeds, although no seedling has been
413 observed in the field (Ren et al. 2003; Xu 2015). Second, high allele divergence has
414 been shown to be compatible with low-rate sexual reproduction (Ceplitis 2003).
415 Lastly, SVDQuartets showed a quarter of the quartets were incongruent with the
416 species tree, indicating a portion of the SNPs did not diverge congruently with the rest
417 of the SNP sites due to incomplete lineage sorting or recombination. Therefore, we
418 hypothesize that the species likely engages, to an extent, in sexual reproduction to
419 maintain genetic variation and slow down the speed of Muller's ratchet, supporting the
420 theory that recombination is necessary for long-term survival. In sum, our results
421 indicate that *K. uniflora* is not a true obligate asexual species although the asexuality
422 likely has evolved before 0.5 mya.
423
424 **Evolutionary history of *Kingdonia uniflora***
425 In asexual lineages, reduced efficacy of purifying selection caused by linked selection
426 will lead to decreased fitness and a reduction in the effective population size N_e,

(Nordborg and Donnelly 1997; Nordborg 2000; Ho et al. 2019). Populations with small N_e values usually show reduced capacity to respond to changing environmental pressures (Higgins and Lynch 2001; Siol et al. 2007). Currently, our knowledge of the evolutionary history of relict lineage with highly asexual reproduction, specifically the origins of genetic differentiation and demographic dynamics, has been extremely limited. In our study, results from the LEA and PAUP analyses indicate that populations within each of the three mountain systems, i.e., the Qinling Mountains, Minshan Mountains, and Daxue-Qionglai Mountains, belong to the same genetic cluster (Table 2; Fig. 1d). This observation suggests an important role of mountain isolation in shaping the population genetic structure of the species due to constraints in dispersal by distance (Fig. 1e) and/or abiotic environments, as evidenced by results from the GF and RDA analyses (Table 4; Fig. 8). The initial divergence of *K. uniflora* lineages was estimated to have occurred *c*. 0.51 million years ago (mya) (Table 3; Fig. 7a). The divergence time coincides with the occurrence of the Naynayxungla Glaciation (0.78-0.50 mya), one of the two largest glaciations on the Qinghai-Tibetan Plateau (QTP) (Zheng et al. 2002). We speculate that the climate caused by the Naynayxungla Glaciation triggered intraspecific differentiation in *K. uniflora* by shifting the distribution range, while the synergistic effects following geographic distance and environmental difference have further driven *K. uniflora* to eventually evolved into different genetic lineages.

Gene flow is an important way to weaken differentiation within species inhabiting montane regions, but is usually limited due to the aforementioned reasons. Gene flow

449 in highly asexual species of flowering plants is especially limited due to the generally
450 poor dispersal ability of propagules for asexual reproduction. Nevertheless, the FSC2
451 analysis support ancient gene flow between MS and both QL and DQ (Table 3; Fig.
452 7a). Our data from ecological niche modeling suggests gene flow likely occurred
453 during the LGM when the species expanded its size and distribution to more suitable
454 habitats as detailed below. Similar to many other cold adapted/tolerant species (e.g.,
455 Anderson et al. 2006; Tian et al. 2010; Opgenoorth et al. 2010; Gao et al. 2015), *K.*
456 *uniflora* had a significant population expansion during the LGM (10,000-20,000 years
457 ago) (Fig. 7b), well after the Naynayxungla Glaciation in the QTP or the divergence
458 of the three lineages (Table 3; Fig. 7a). This provided opportunities for population
459 admixture of the three previously (~0.5 mya) diverged lineages of *K. uniflora*.
460 Therefore, the gene flow likely have occurred recently during the LGM.
461

462 **Uneven distribution of sexual reproduction among *Kingdonia uniflora***
463 **populations is associated with differentiation in genes regulating seed**
464 **development**

465 Local adaptation involving diversifying selection is viewed as the best and longest
466 manifestation of an evolutionary response to selection in nature (Kawecki and Ebert
467 2004; Lovell et al. 2014). Our data suggests such process occurred in *K. uniflora* in
468 genes controlling seed development. The fact that field surveys and lab experiments
469 failed to observe successful seed germination indicates failure or low rate of seed
470 development and germination is likely to be the major factor that has led to

non-evident (unseen/infrequent) sexual reproduction in *K. uniflora*. Our results showed that genes playing a strong role in divergence among *K. uniflora* populations are enriched for functions involved in seed development (Fig. 10; Table 5), suggesting differential selection of different alleles in different populations. An obvious divergence of DNA recombination frequency among lineages, e.g., extremely rare recombination events in DQ vs. relatively more frequent recombination events within QL and MS (Fig. 6b,7a) and between QL and MS, suggests different levels of sexual reproduction among and between population groups in different local environments. These together indicate the genetic divergence in seed development associated genes is likely to be the key factor of varied seed germination rate and sexual reproduction extent among *K. uniflora* populations. Although local adaptation might be constrained due to reduced efficacy of selection caused by genome-wide interference between loci (Jaron et al. 2021), our results show genetic differentiation in genes associated with seed development that, however, appear not to be random. The differences among populations in environmental variables explains a significant amount of genetic variation within *K. uniflora* (Fig. 8; Table 4), suggesting putative selection pressure on genes regulating seed development. This indicates the adaptive constraints posed by the high LD level in *K. uniflora* may be buffered by infrequent sexual reproduction. The differences in environmental variables, e.g., seasonal temperature and precipitation (Fig. 8), may directly affect seed germination rates and the corresponding genes among populations, further driving diversifying selection in shaping the diversity of genes regulating seed development. Overall, our results reveal

493 that the infrequency or uneven distribution of sexual reproduction in *K. uniflora* is
494 associated with genetic differentiation among populations.
495
496

497 Materials and Methods

498 Sample collection and resequencing

499 We carefully selected a total of 12 populations (Supplemental Table S5) to represent
500 all known localities of *K. uniflora*. We collected fresh leaves of five individuals that
501 were at least ten meters away from each other in each population to reduce the
502 possibility that each sample was not a physiologically independent ramet. We dried
503 the leaves in silica gel in the field, then stored them at -20°C before DNA extraction.
504 Genomic DNA extraction, library construction, and amplification followed the
505 protocols of Novogene (Beijing, China) (Supplementary Methods). All samples were
506 sequenced using the Illumina HiSeq 4000 platform with a pair-end read length of 150
507 bp by Novogene (https://en.novogene.com/). Illumina raw reads were filtered by
508 removing adapters and low-quality reads using Trim Galore v0.6.5
509 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore) with default
510 options (Phred quality threshold 20; adapter auto-detection) (Krueger 2021)
511

512 SNP calling and quality control

513 We mapped the filtered reads of each individual to the *K. uniflora* genome (Sun et al.
514 2020) using BWA-MEM v0.7.12-r1039 (Li 2013) with default parameters. We then

converted sequence alignment/map (SAM) format files to BAM and sorted the BAM files using SAMtools v1.6 (Li et al. 2009) and conducted the following analyses in Genome Analysis Toolkit (GATK, v4.0) (Depristo et al. 2011). We first marked duplicate reads using MarkDuplicates. We then processed the data with AddOrReplaceReadGroups to replace all read groups in the INPUT file with a single new read group name and assign all reads to this read group in the OUTPUT BAM. To identify SNPs, we first conducted single-sample haplotype calling with HaplotypeCaller, and then identified Multi-sample SNPs using GenotypeGVCFs after merging the haplotype caller results from each sample using CombineGVCFs which aligned the haplotypes among samples. To obtain high quality SNPs, we filtered multi-sample SNPs using VariantFiltration with strict filter settings "QD < 2.0 || MQ < 40.0 || FS > 60.0 || SOR > 3.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0". We further filtered the SNPs data to exclude monomorphic or triallelic variants, indels, and SNPs missing in any samples via VCFtools v0.1.16 (Danecek et al. 2011) for further analyses.

Genetic diversity and population structure

To assess the population structure of *K. uniflora*, we performed the following analyses. We used Landscape and Ecological Association (LEA) (v3.3.2) R package (Frichot and François 2015) to determine the number of ancestral populations. LEA was developed for large genotypic matrices and does not rely on the genetic assumptions of the absence of genetic drift, Hardy-Weinberg or linkage equilibrium in ancestral

537 populations (Pritchard et al. 2000). We also used a coalescent-based method to
538 estimate a "species tree" based on the SNP data for comparison with results from
539 LEA. Specifically, we used the SVDQuartets method (Chifman and Kubatko
540 2014) implemented in PAUP* v4.0a166 (Swofford, 2003;
541 http://paup.phylosolutions.com/) with 100 bootstrap replicates and the quartet
542 assembly method QFM to produce a species tree (Reaz et al. 2014).
543 We estimated nucleotide diversity (π) and genetic differentiation (F_{ST}) between
544 groups identified in the aforementioned analyses using VCFtools v0.1.16 (Danecek et
545 al. 2011), and used Analysis of Molecular Variance (AMOVA) in Arlequin v3.5.2.2
546 (Excoffier and Lischer 2010) to estimate relative contributions of genetic variation
547 from within and between groups. To determine if genetic differentiation is associated
548 with geographic distance, we performed an IBD (isolation by geographic distance)
549 analysis. We first estimated population-level genetic differentiation F_{ST} using the Weir
550 and Cockerham method (Weir and Cockerham 1984) implemented in HIERFSTAT
551 (Goudet 2005) in R v3.6.1 (R Core Team 2014). We then calculated genetic distance
552 with the formula $F_{ST}/(1-F_{ST})$ and computed the pairwise geographic distance among
553 12 populations using GENALEX v6.5 (Peakall and Smouse 2012). We then tested the
554 significance for the relationship between geographical distances and genetic distance
555 among populations by conducting Mantel tests with ADE4 v1.7
556 (https://CRAN.R-project.org/package=ade4) using *mantel.rtest* with 9999
557 permutations.
558

559 **Detecting genomic signatures and signs of asexual and sexual reproduction**

560 To understand the reproductive strategies, we first employed VCFtools v0.1.16
561 (Danecek et al. 2011) (option-het) to calculate F_{IS}=1-H_{obs}/H_{exp} for each individual,
562 where H_{obs} and H_{exp} are the observed and expected heterozygosity, respectively. A
563 negative F_{IS} value indicates an excess of observed individual heterozygosity. We then
564 examined the distribution pattern of the excessive heterozygosity within and among
565 the genetic groups identified in the aforementioned analyses by generating a site
566 frequency spectrum (SFS) using Pop-Con with standard parameters
567 (https://github.com/YoannAnselmetti/Pop-Con). Twelve randomly selected
568 individuals that cover all populations were used for the SFS analysis.

569 We further examined other genetic consequences of asexual reproduction (i.e.,
570 linkage disequilibrium (LD) and reduced efficiency of purifying selection) and
571 genetic evidence of unseen sexual reproduction (i.e., recombination). We calculated
572 pairwise linkage disequilibrium (r^2 value) and modeled the decline of LD with
573 physical distance using PopLDdecay v3.40 (Zhang et al. 2019) with default settings.
574 Within each contig we calculated LD between pairs of sites up to 300 kb and 1000 kb,
575 respectively. In obligate asexuals, genome-wide LD between loci is expected, and the
576 decline of LD between loci does not depend on their physical distance (Vakhrusheva
577 et al. 2020).

578 To test if *K. uniflora* is characterized by reduced efficacy of purifying selection, we
579 calculated the π_N/π_S ratio of the species. For asexual species, individuals from the
580 same population may be clones via descending from a common ancestor via only

clonal reproduction, possibly over many generations (Ho et al. 2019). Therefore, prior to the calculation, we grouped individuals by conducting multidimensional scaling (MDS) analysis in plink v1.9 with the options -cluster, -mds-plot 2 eigvals and -allow-extrachr (Purcell et al. 2007) to group individuals that were genotypically highly similar into the same genotypic group within a population. We then randomly selected one individual from each genotypic group and generated a concatenated coding sequence for each selected individual based on SNP loci from coding regions using a custom script (Supplemental Methods). The sequence matrix comprising all above selected individuals was used for following nucleotide diversity calculation. Designated nucleotide diversity at 0-fold and 4-fold degenerate positions, π_N (π_0) and π_S (π_4), were calculated in MEGA (v10.1.6) (Kumar et al. 2018).

To assess if any unseen sexual reproduction may have occurred in *K. uniflora*, we reconstructed the relationship of individuals using SNP data with the NeighborNet method in SplitsTree v4.13.1 (Huson and Bryant 2006) with default settings. The method does not force a tree-like phylogeny in the analysis and can reveal phylogenetic networks resulting from recombination events. We further used Recombination Detection Program (RDP4) (Martin et al. 2015) to detect recombination events: "RDP4 implements a range of methods for both detection and characterization of recombination events that are evident within a sequence alignment without any prior user indication of a non-recombinant set of reference sequences" (Martin et al. 2015). We ran the analyses using six algorithms (RDP, BootScan, MaxChi, Chimera, GeneConv and SiScan) of recombination signal detection with

603 Bonferroni correction and the highest acceptable *p* value 0.05. Recombination events
604 that were identified by at least three of the six methods were considered.
605
606 **Inferring population demographic history and species distributions**
607 To obtain a comprehensive view of the species' evolutionary history, we first inferred
608 the demographic history of *K. uniflora* using *fastsimcoal2* (FSC2; Excoffier et al.
609 2013) with the following details. We tested eight possible N_e models (Supplemental
610 Fig. S5) to find the best model explaining our data for the genetic groups revealed by
611 PAUP and LEA analyses. Then we estimated the composite likelihood of the observed
612 data given a specified model using SFS (which was constructed here using
613 easySFS.py instead of Pop-Con to generate a direct input file for FSC2)
614 (https://github.com/isaacovercast/easySFS). Each model was run 20 times with
615 1,000,000 simulations for the calculation of the composite likelihood, and 40
616 expectation-conditional maximization (ECM) cycles. We compared the models based
617 on the maximum likelihood value across 50 independent runs using the Akaike
618 Information Criterion and Akaike's weight of evidence and chose the model with the
619 maximum Akaike's weight value as the optimal model. Finally, we calculated
620 confidence intervals of parameter estimates from 100 para-metric bootstrap replicates
621 by simulating SFS from the maximum composite likelihood estimates and
622 re-estimating parameters each time.
623 We also performed ecological niche modeling (ENM) to reconstruct the distribution
624 range of the species in the present and past using MAXENT (Phillips and Dudik 2008)

625 with population present occurrence data and climatic variables of the occurrence
626 locations. Species' presence occurrence data were compiled from the Chinese Virtual
627 Herbarium (http://www.cvh.ac.cn) and our own field collections (Supplemental Table
628 S5). We downloaded the climate layers of 19 bioclimatic variables (Supplemental
629 Table S6) at a 2.5 arc minute resolution at present (average for the years 1970-2000)
630 and during the last glacial maximum (LGM: *c.* 21 thousand years before present (kyr
631 BP)) from WorldClim database website (http://www.worldclim.org/) (Fick and
632 Hijmans 2017). To avoid multicollinearity, a Pearson correlation analysis was
633 conducted to eliminate one of the variables in each pair with a correlation higher than
634 0.75, which resulted in seven climatic layers retained for analyses (Supplemental
635 Table S6).
636
637 **Assessing the correlation between differentiation and environmental variables**
638 We performed a gradient forest (GF) analysis implemented in GRADIENTFOREST
639 v0.1 (http://gradientforest.r-forge.r-project.org/) to identify potential key
640 environmental drivers of genomic variation in *K. uniflora*. The gradient forest method
641 captures complex relationships between potentially correlated predictors (e.g.,
642 climatic variables) and multiple response variables (e.g., genetic variation), and
643 provides the overall importance for each predictor weighted by R^2 (Ellis et al. 2012;
644 Ma et al. 2020). The seven climatic layers retained in the ENM analysis were used as
645 environmental predictors in the GF analysis for analyses of all SNPs. The analysis
646 was run 1000 times to obtain the variability of R^2, and the run with the highest overall

647 performance (R^2) for calculating weighted importance of predictors on response
648 variables. To verify the results of GF analysis, we performed a redundancy analysis
649 (RDA) to evaluate the associations between genetic variation and the seven
650 environmental variables. We constrained the dependent variables (individuals) by the
651 explanatory variables (environmental variables). The RDA analysis was performed
652 using the rda function in VEGAN v2.5 (Oksanen et al. 2018;
653 http://CRAN.R-project.org/package=vegan). The *anova.cca* function was used to
654 calculate overall significance and significance of each climate variable using 9999
655 permutations.
656
657 **Detecting and annotating outlier loci**
658 To detect potential loci under diversifying selection, we performed an overall F_{ST}
659 outliers test in BAYESCAN v2.1 (Foll and Gaggiotti 2008) with default parameters.
660 BAYESCAN identifies outliers using differences in allele frequencies between
661 populations. A locus-specific component (α) was used to differ loci under-diversifying
662 selection (α>0) from that under balancing or purifying selection. Significance is based
663 on FDR-corrected *q*-values (<0.01). Loci under strong diversifying selection are those
664 whose *F*st values are larger than expected from coalescent simulation of neutral
665 evolution (Strand et al. 2012).
666 To determine what functions the genes containing SNP outliers may have, we
667 annotated each of these genes using gene ontology (GO) terms with TBtools (Chen et
668 al., 2018). We then used the Singular Enrichment Analysis (SEA) tool in agriGO v2.0

669 (Tian et al. 2017) to analyze gene enrichment and tested for statistical significance of
670 gene enrichment using the chi-squared test.
671
672

673 ## Data accessibility

674 *K. uniflora* resequencing reads have been deposited in the NCBI Short Read Archive
675 (SRA) under accession SRA------.
676
677

678 ## Competing interest statement

679 The authors declare no competing interests.
680
681

682 ## Acknowledgments

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688
689

690 ## Author contributions

HW, JX, and HS developed the idea and designed the experiment; YS, XZ, and HZ collected the leaf materials; YS, XZ, and AZ performed the statistical analyses; YS, JBL, and JX interpreted the results and wrote the manuscript. All authors read, edited and approved the final manuscript. YS, XZ, and AZ contributed equally to this work.

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