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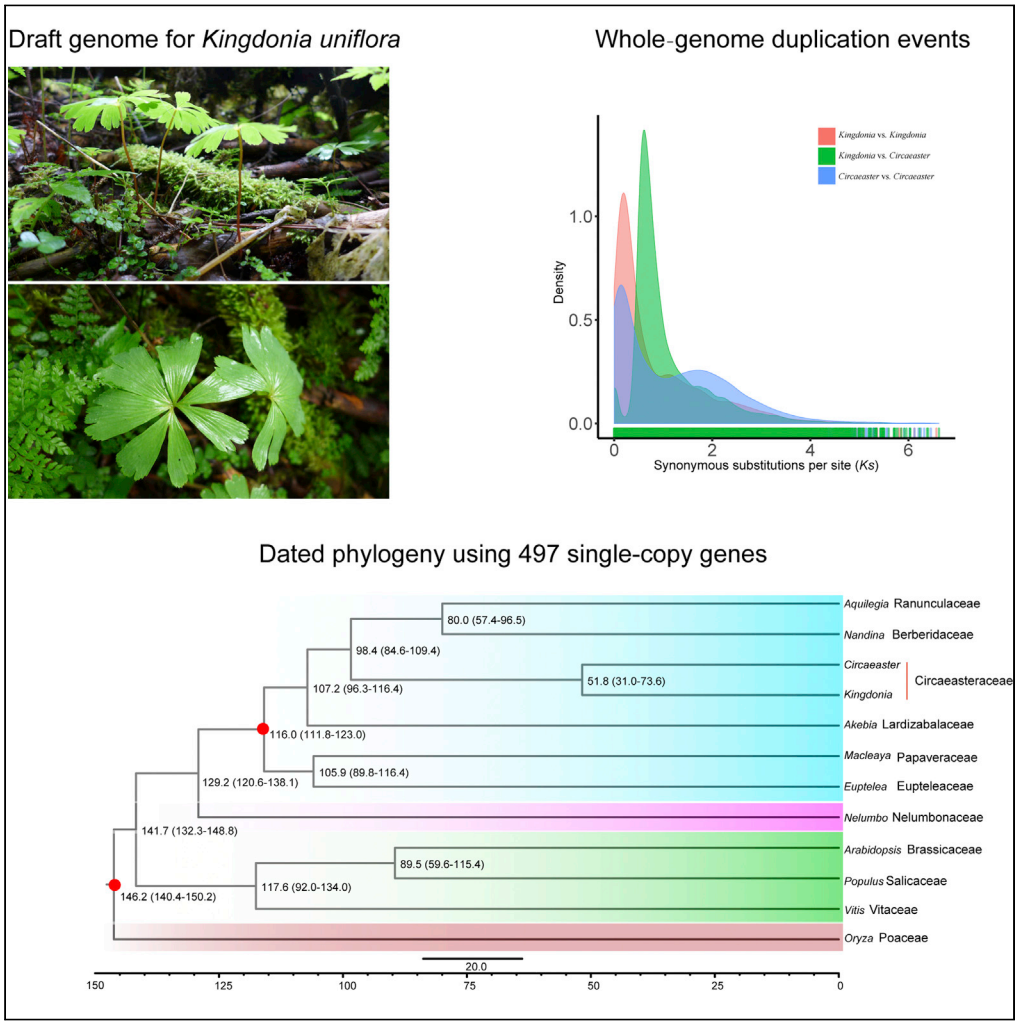

Yanxia Sun, Tao Deng, Aidi Zhang, ..., Xiujun Zhang, Hang Sun, Hengchang Wang

zhangxj@wbgcas.cn (X.Z.)
sunhang@mail.kib.ac.cn (H.S.)
hcwang@wbgcas.cn (H.W.)

HIGHLIGHTS

Provides a high-quality assembly for *K. uniflora* genome

K. uniflora shows overrepresentation in gene families associated with DNA repair

K. uniflora shows underrepresentation in gene families associated with stress response

The current endangered status of *K. uniflora* is related with adaptive degeneration

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Genome Sequencing of the Endangered *Kingdonia uniflora* (Circaeasteraceae, Ranunculales) Reveals Potential Mechanisms of Evolutionary Specialization

Yanxia Sun,[1,2,9,10] Tao Deng,[3,9] Aidi Zhang,[1,4,9] Michael J. Moore,[5] Jacob B. Landis,[6,7] Nan Lin,[1,2] Huajie Zhang,[1,2] Xu Zhang,[1,2] Jinling Huang,[8] Xiujun Zhang,[1,4,*] Hang Sun,[3,*] and Hengchang Wang[1,2,*]

SUMMARY

***Kingdonia uniflora*, an alpine herb, has an extremely narrow distribution and represents a model for studying evolutionary mechanisms of species that have adapted to undisturbed environments for evolutionarily long periods of time. We assembled a 1,004.7-Mb draft genome (encoding 43,301 genes) of *K. uniflora* and found significant overrepresentation in gene families associated with DNA repair, underrepresentation in gene families associated with stress response, and loss of most plastid *ndh* genes. During the evolutionary process, the overrepresentation of gene families involved in DNA repair could help asexual *K. uniflora* reduce the accumulation of deleterious mutations, while reducing genetic diversity, which is important in responding to environment fluctuations. The underrepresentation of gene families related to stress response and functional loss of *ndh* genes could be due to lack or loss of ability to respond to environmental changes caused by long-term adaptation to a relatively stable ecological environment.**

INTRODUCTION

Habitat destruction caused by changing climate and human activities has driven numerous plant species to endangered status (Yang et al., 2018). Current management of endangered species includes *in situ* and *ex situ* measurements (Nuijten et al., 2016). Owing to the rapidly growing number of threatened species, optimizing conservation practices based on evolutionary history and endangered mechanisms is imperative. In recent years, the development of genomic techniques has opened up new opportunities for species conservation. The main improvement is the enormous increase in loci allowing for a more detailed study of previous research questions (Nuijten et al., 2016). Specifically, genomic approaches provide the possibility to detect adaptive genetic variation across the entire genome (Kohn et al., 2006; Ouborg et al., 2010; Angeloni et al., 2012; Nuijten et al., 2016), which may help to address long-standing questions in conservation biology not fully resolved with traditional methods (Primmer, 2009; Avise, 2010; Frankham, 2010; Ouborg et al., 2010; Allendorf et al., 2010; Angeloni et al., 2012; Funk et al., 2012; Steiner et al., 2013; McMahon et al., 2014; Shafer et al., 2015; Garner et al., 2016; Benestan et al., 2016; Fuentes-Pardo and Ruzzante, 2017).

Plant lineages vary widely in their geographic distributions owing to numerous factors, e.g., ecological niche range, dispersal capacity, reproductive investments, and time since origin (Debussche and Thompson, 2003; Lavergne et al., 2004; Becker, 2010; Youssef et al., 2011), among which one crucial factor is adaptive capacity to respond to environmental changes. Lineages maintaining a small distribution are likely to possess low adaptive capacity to respond to geological and climatic changes at large scales, as well as habitat changes at small scales. For example, some asexual lineages, generating genetically and phenotypically identical individuals, are limited in their capacity to respond quickly to environmental fluctuation owing to low genetic diversity, which can lead to a status of endangerment or even extinction (Bell and Collins, 2008). In addition, species living in an equable environment for long periods might lack or lose the ability to defend against rapidly fluctuating environmental stress. Once the habitat is altered or destroyed, these species are incapable of colonizing new habitats. Shrinking habitats and low adaptive ability to new environments together lead some plants to endangered status. A recent study has shown that endangered species have more vulnerable genomes than that of congeneric non-endangered species (Hamabata et al., 2019); in fact, the endangered species exhibited significantly lower genetic diversity

[1]CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, Hubei, China

[2]Center of Conservation Biology, Core Botanical Gardens, Chinese Academy of Sciences, Wuhan, Hubei, China

[3]Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, China

[4]Center of Economic Botany, Core Botanical Gardens, Chinese Academy of Sciences, Wuhan, Hubei, China

[5]Department of Biology, Oberlin College, Oberlin, OH, USA

[6]Department of Botany and Plant Sciences, University of California Riverside, Riverside, CA, USA

[7]School of Integrative Plant Science, Section of Plant Biology and the L.H. Bailey Hortorium, Cornell University, Ithaca, NY, USA

[8]Department of Biology, East Carolina University, Greenville, NC, USA

[9]These authors contributed equally

[10]Lead Contact

*Correspondence: zhangxj@wbgcas.cn (X.Z.), sunhang@mail.kib.ac.cn (H.S.), hcwang@wbgcas.cn (H.W.)

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Genome Features	Contigs/Scaffolds
Total length, bp	1,004,656,313
Total number of contigs	2,932
Longest length, bp	11,531,354
Length of N50, bp	2,099,369
Length of N90, bp	292,588
GC content, %	38.04%
No. of genes	43,301

Table 1. Genome Assembly of *Kingdonia uniflora*

and proportion of duplicated genes and accumulated more deleterious variations than non-endangered species. Owing to limited genomic studies, genomic changes of endangered plants are largely unknown. To clarify which genetic factors contribute to vulnerability in endangered species, genome-wide genetic changes in functionality should be assessed (Hamabata et al., 2019).

Kingdonia uniflora Balf. f. and W.W. Sm. (Circaeasteraceae, Ranunculales), an alpine herb (diploid, 2n = 18), has a very narrow distribution (Figure S1). The habitat of *K. uniflora* represents an ecological environment of primeval forest with few disturbances. Specifically, *K. uniflora* is restricted to growing in high altitudes (~2,800–4,000 m); cold, damp climates with deep humus; and usually under species of *Abies*. *K. uniflora* and *Circaeaster agrestis* Maxim. together constitute the early-diverging eudicot family Circaeasteraceae (Ranunculales) (Angiosperm Phylogeny Group, 2016). Previous estimates showed *K. uniflora* diverged from *C. agrestis* around 52 mya (Ruiz-Sanchez et al., 2012). Although no fossil record is known for *K. uniflora*, fossil fruits similar to those of *C. agrestis* have been reported from the mid-Albian of Virginia, USA (Crane et al., 1994; Drinna et al., 1994; Sun et al., 2017). Remarkably, different from all other angiosperms, *K. uniflora* and *C. agrestis* possess an unusual dichotomous venation (Figure S2) similar to that found in ferns and *Ginkgo* (Sun et al., 2017). Old age for the lineage and dichotomous veins indicate an ancient relictual character for *K. uniflora*. Additionally, *K. uniflora* typically reproduces asexually, relying on rhizome systems to produce new individuals. These indications raise multiple questions, including: As an ancient relictual lineage, what history has *K. uniflora* experienced? As an endangered and habitat-dependent species, what genomic traits does *K. uniflora* possess? *K. uniflora* provides an ideal model to study the evolutionary mechanisms of ancient plant lineages that have an extremely narrow distribution and rely on a highly specialized habitat.

The *ndh* genes encode subunits of the thylakoid NADPH complex that mediate cyclic electron flow around Photosystem I and facilitates chlororespiration (Martín and Sabater, 2010). A series of studies suggests that the *ndh* genes can be dispensable under mild non-stressing environments (e.g., Casano et al., 2001; Martín et al., 2004; Rumeau et al., 2007; Martín and Sabater, 2010). A previous study (Sun et al., 2017) found rampant loss and pseudogenization of *ndh* genes in the *K. uniflora* plastome, which may indicate an association between the vulnerability of *K. uniflora* and genomic changes. In the present study, we provide a *de novo* genome sequence of *K. uniflora* using both Illumina and Pacbio sequencing technologies. We aim to investigate the evolutionary history of *K. uniflora* and reveal the potential mechanisms of its evolutionary specialization.

RESULTS

Genome Assembly and Annotation

Genome size estimation using flow cytometry suggested a haploid genome size of 1,150 Mb for *K. uniflora* (Figure S3), whereas *k*-mer statistics indicated a similar genome size of 1,170 Mb, with very low heterozygosity (Figure S4 and Table S1). In the present study, we generated 236 Gb of Illumina reads and 106 Gb Pacbio reads with an N50 length of 12.876 kb (Table S2). A total assembly of 1,004.7 Mb (representing ~86% of the estimated genome size), consisting of 2,932 scaffolds (scaffold N50 of 2.09 Mb; longest scaffold of 11.5 Mb) was achieved (Table 1). A total of 43,301 protein-coding genes were predicted (Table 1), among which 35,953 genes (83.03%) were functionally annotated (Table S3). In addition to protein-coding genes, various noncoding RNA sequences were identified and annotated (Table S4), including 1,124 transfer RNAs, 715 ribosomal RNAs, 125 microRNAs, and 1,751 small nuclear RNAs. The completeness of gene

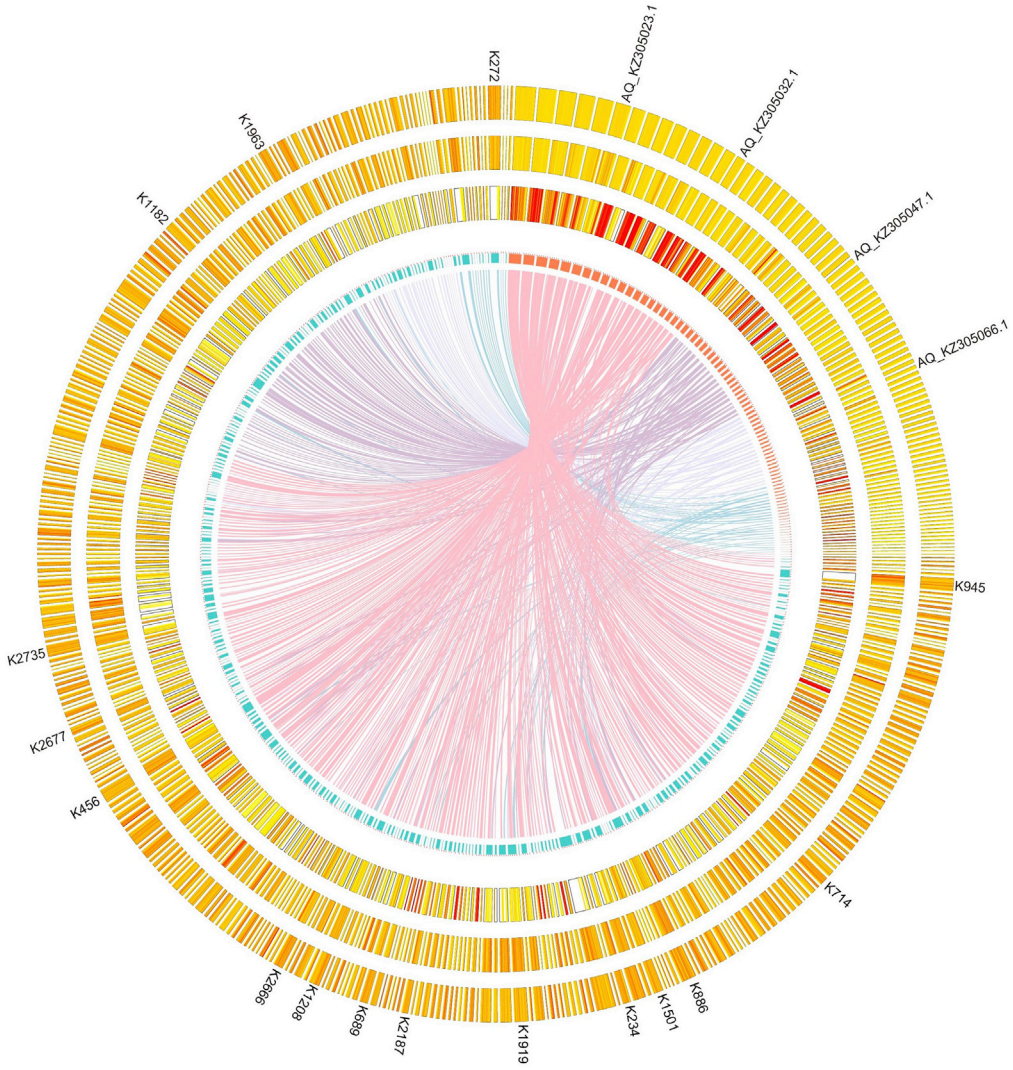

Figure 1. Comparative Analyses of Genomic Features between *Kingdonia uniflora* and *Aquilegia coerulea*
Tracks from inside to outside are collinearity between both genomes, number of chromosomes/contigs, gene density, GC content, and TE density.

regions assessed by BUSCO (Benchmarking Universal Single Copy Orthologs) showed that 90.6% of the green plant single-copy orthologs were complete (Table S5).

We compared the draft genome of *K. uniflora* with the well-annotated genomes of the model plant *Arabidopsis thaliana* (Brassicaceae) and the Ranunculales species *Aquilegia coerulea* (Ranunculaceae). The genome size of *K. uniflora* is much larger than that of both references. The *K. uniflora* genome showed strong synteny with the genome of *Aq. coerulea* (Figure 1), but weak synteny with that of *A. thaliana* (Figure S5), which is not surprising given their placement in the angiosperm Tree of Life. The gene density in *K. uniflora* is lower than that in *Aq. coerulea* and *A. thaliana*, whereas the density of TEs (transposable elements) in *K. uniflora* was higher than that in *Aq. coerulea* and *A. thaliana* (Figures 1 and S5).

We also compared our draft genome sequence with five other draft genomes of Ranunculales taxa, representing three of the seven Ranunculales families (Table S6); the quality of our assembly is comparable with that of all the five species, generating the longest N50 length and relatively fewer scaffolds. Comparatively, the genome of *K. uniflora* is larger than other Ranunculales species with sequenced genomes, such as *Aq. coerulea* (293.08 Mb), *Eschscholzia californica* (489.065 Mb), and *Macleaya cordata* (377.83 Mb) (Table S6).

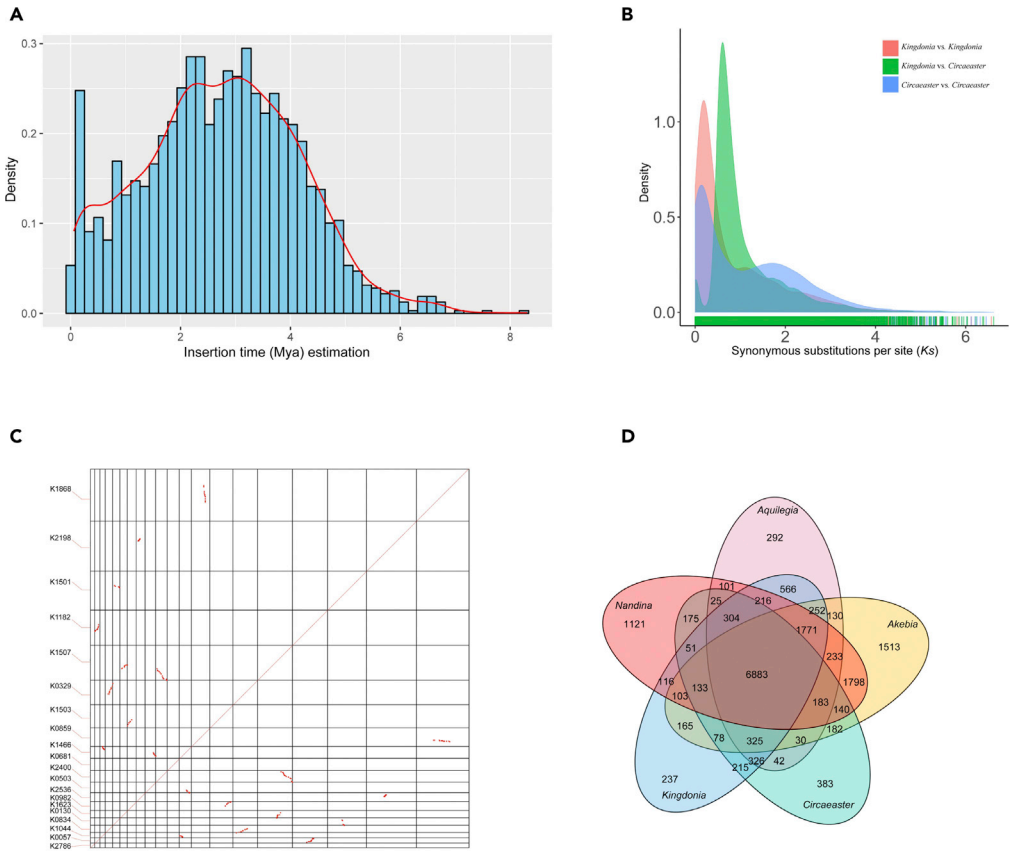

Figure 2. Evolutionary Analyses of the *K. uniflora* Genome

(A) Insertion time distribution of LTR retrotransposons. (B) Distribution of synonymous substitution rates (*Ks*) for pairs of paralogs/orthologs in/between *K. uniflora* and *C. agrestis*. (C) Dot plots of paralogs identified across contigs in the *K. uniflora* genome. (D) Venn diagram showing unique and shared gene families between genomes of *K. uniflora* and four other Ranunculales species.

Repeat Elements

Through a combination of approaches, we annotated 66.83% of the assembly as repetitive elements, among which long terminal repeats (LTRs) were the most abundant, occupying 40.62% of the genome assembly length; DNA elements and long interspersed nuclear elements occupied 5.0% and 3.0% of the genome, respectively (Table S7). The proliferation of LTR retrotransposons in *K. uniflora* was estimated to peak around 2.7 mya (Figure 2A). Analyses of age distributions built from synonymous substitutions per synonymous site (*Ks*) indicated that *K. uniflora* has undergone one recent whole-genome duplication (WGD) event, which occurred after its divergence from *C. agrestis* (Figure 2B). The inferred WGD event in the *K. uniflora* genome was further supported by dot-plot analysis of representative scaffolds, in which numerous paralogs derived from this event were identified (Figure 2C).

Phylogenetic Tree Construction and Estimation of Divergence Times

Applying OrthoFinder (Emms and Kelly, 2015) to eight whole-genome and four transcriptome sequences including monocots, basal eudicots, and core eudicots, we identified a total of 18,742 orthogroups, among which 6,883 were shared by *Kingdonia* and four other Ranunculales species (Figure 2D). Among these orthogroups, 497 were identified as putative single-copy gene families. To further investigate the phylogenetic relationships within Ranunculales, we conducted both concatenated and coalescent analyses using the sequences of 497 single-copy genes in 12 species. The topologies from two analyses were identical, confirming the sister relationship between *K. uniflora* and *C. agrestis* and resolving Circaeasteraceae as sister to the clade formed by Ranunculaceae and Berberidaceae (Figure 3); Papaveraceae + Eupteleaceae was placed as the earliest-diverging clade (Figure 3). *K. uniflora* and *C. agrestis* were estimated to have diverged ~51.8 mya in our analyses

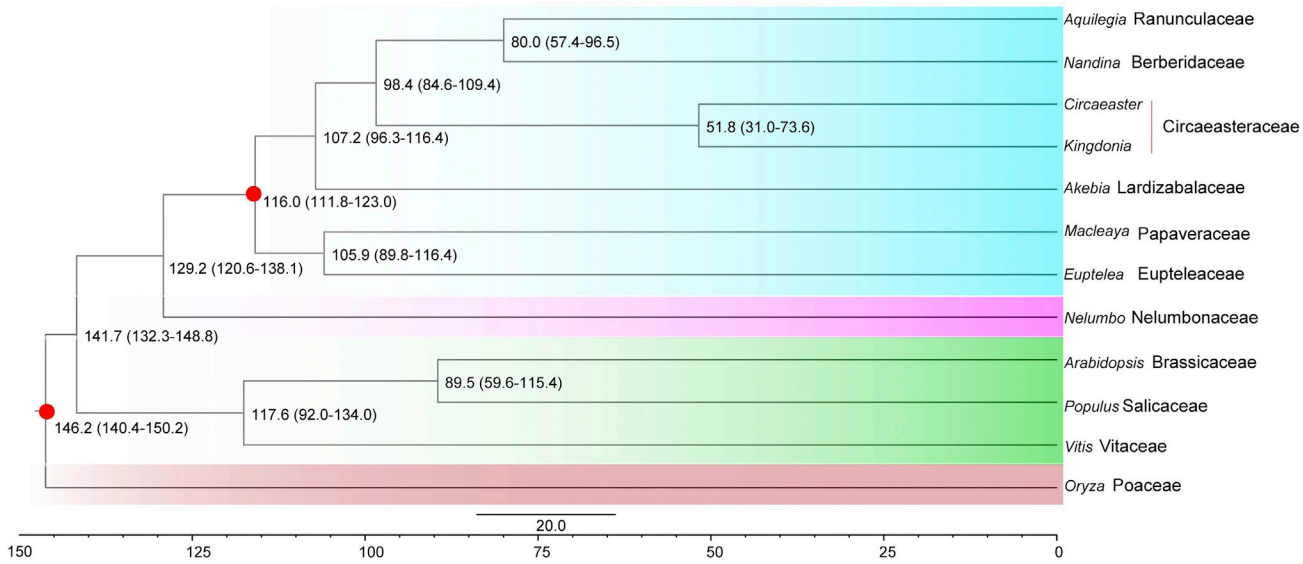

Figure 3. Dated Phylogeny for 12 Plant Species with *Oryza* as an Outgroup
A timescale is shown at the bottom, and red points in some nodes indicate fossil calibration points.

using MCMCtree with two calibration points (Figure 3). In addition, the phylogenetic analysis with an expanded group of taxa, which correspond to a larger taxonomic sampling but fewer loci indicated a similar phylogeny of Ranunculales, except the placement of Eupteleaceae (Figure S6).

Gene Family Overrepresentation and Underrepresentation

Comparisons of the genomes among 12 species identified a total of 111 gene families that are significantly ($p < 0.01$) overrepresented in *K. uniflora* and 22 gene families that are significantly underrepresented (Table S8). The results from Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) annotations showed that overrepresented gene families were considerably enriched in DNA repair pathways, such as homologous recombination, mismatch repair, DNA replication and nucleotide excision repair, whereas gene families showing significant underrepresentation in the *K. uniflora* genome were found to be involved in pathways related to stress or pest responses, such as the phenylpropanoid biosynthesis and secondary metabolites biosynthesis (Table 2).

Dispensability of Plastid *ndh* Genes

To detect whether the *ndh* genes/segments lost from *K. uniflora* plastome were transferred to the nuclear genome, we conducted a BLASTN search using 11 intact *ndh* sequences extracted from *C. agrestis* as the query, using the assembled *K. uniflora* genome sequences as target. The result showed that no intact sequences for *ndh* plastid genes were discovered with the exception of *ndhE* and *ndhJ*, indicating functional copies of these genes likely have been lost (Table S9 and Figure 4).

DISCUSSION

Species that live in stable habitats face less stress, which can cause lack or loss of ability to respond to environmental changes. In the present study, we *de novo* assembled the genome of *K. uniflora*, an ancient relictual species exclusively found in China. Given that *K. uniflora* has a larger genome than many of its close relatives, we hypothesize that the proliferation of LTR retrotransposons and the WGD event together are likely responsible for the increased genome size of *K. uniflora* (Michael, 2014). Several studies (Tenaillon et al., 2010; Michael, 2014) have suggested that the proliferation of TEs and specifically long terminal repeat retrotransposons (LTRs) in genomes is the primary driver of genome size differences in plants. This is because LTRs are expressed as RNA and reverse-transcribed into a new DNA element that can be inserted in every replication cycle (Wicker et al., 2007). A comparative study using high-quality genomes detected a correlation between intact LTR retrotransposons and genome size (El Baidouri et al., 2013). Abundant LTR retrotransposons were detected in the genome of *K. uniflora*, whereas considerably fewer LTR retrotransposons were identified in other members of the order with sequenced genomes (Figure 1).

Gene Families	KEGG Terms	Input No.	Background No.	p Value	Corrected p Value
Overrepresented gene families	Glycosphingolipid biosynthesis - globo series	9	9	5.28×10^{-7}	1.59×10^{-5}
	Homologous recombination	17	56	2.87×10^{-6}	6.74×10^{-5}
	Mismatch repair	12	39	7.31×10^{-5}	0.000968079
	Sphingolipid metabolism	9	26	0.000282866	0.003082136
	DNA replication	12	50	0.000516123	0.005446878
	Nucleotide excision repair	14	69	0.000789672	0.007779277
	Peroxisome	16	87	0.000878589	0.008505414
	Galactose metabolism	12	55	0.001064483	0.010034842
	Plant hormone signal transduction	33	271	0.002344688	0.020456082
Underrepresented gene families	Cyanoamino acid metabolism	5	60	2.06×10^{-9}	1.57×10^{-7}
	Phenylpropanoid biosynthesis	5	157	2.06×10^{-7}	6.24×10^{-6}
	Starch and sucrose metabolism	5	202	6.95×10^{-7}	1.96×10^{-5}
	Biosynthesis of secondary metabolites	6	1,076	0.00020813	0.001073645
	Metabolic pathways	6	1,910	0.004094269	0.015173186

Table 2. Functional Annotation of the Significantly Overrepresented and Underrepresented Gene Families in *Kingdonia uniflora*

To investigate the evolutionary dynamics of the LTR retrotransposons, we estimated their insertion dates. The results indicate that the proliferation of LTR retrotransposons in *K. uniflora* was likely triggered around one rapid uplift of the Hengduan Mountain region, occurring between the late Miocene and late Pliocene (Kirby et al., 2002; Clark et al., 2005; Sun et al., 2011; Wang et al., 2012, 2014; Meng et al., 2016; Xing and Ree, 2017). WGDs have been shown to pervade the evolutionary history of angiosperms (Landis et al., 2018), and *K. uniflora* is no different (Figures 2B and 2C). Therefore, the relatively larger genome size of *K. uniflora* compared with close relatives might be promoted by both LTRs proliferation and WGD events.

Based on phylogenetic inference and estimated divergence times, we speculate that the speciation of *K. uniflora* was promoted by the Himalayan orogeny. Previous studies based on DNA commonly recognize *K. uniflora* being closely related to *C. agrestis* (Kim et al., 2004; Wang et al., 2009; Sun et al., 2017). Our phylogenetic analyses confirmed the sister relationship between *K. uniflora* and *C. agrestis*. These species were resolved as sister to the clade formed by Ranunculaceae and Berberidaceae, a result incongruent with previous placements of Circaeasteraceae and Lardizabalaceae as sister pairs (Kim et al., 2004; Wang et al., 2009; Sun et al., 2017). The divergence estimation between *K. uniflora* and *C. agrestis* (~51.8 mya) is consistent with a previous estimate of ~52 mya (Ruiz-Sanchez et al., 2012) and also coincides with the timing of the first stage of Himalayan orogeny (Rowley, 1996; Huang et al., 2015). Similar to *K. uniflora*, *C. agrestis* has a narrow distribution confined to China and the Himalayas. However, the distribution range of *Circaeaster* is relatively larger; in places where *Kingdonia* occur *Circaeaster* can always be found, whereas in most regions that *Circaeaster* is distributed *Kingdonia* is absent. Previous studies have shown that orogeny could create conditions favoring speciation of resident lineages (e.g., Hoorn et al., 2013; Wen et al., 2014; Favre et al., 2015). Hence, we hypothesize that the divergence between *K. uniflora* and *C. agrestis* was likely driven by the rapid uplift of contemporary Himalayan orogeny.

An asexual reproductive system and overrepresentation of DNA repair genes together reduce genetic diversity of *K. uniflora*. Levels of genetic diversity are often associated with reproductive strategies of species (Otálora et al., 2013). Colonization, population persistence, and extinction probabilities are all influenced

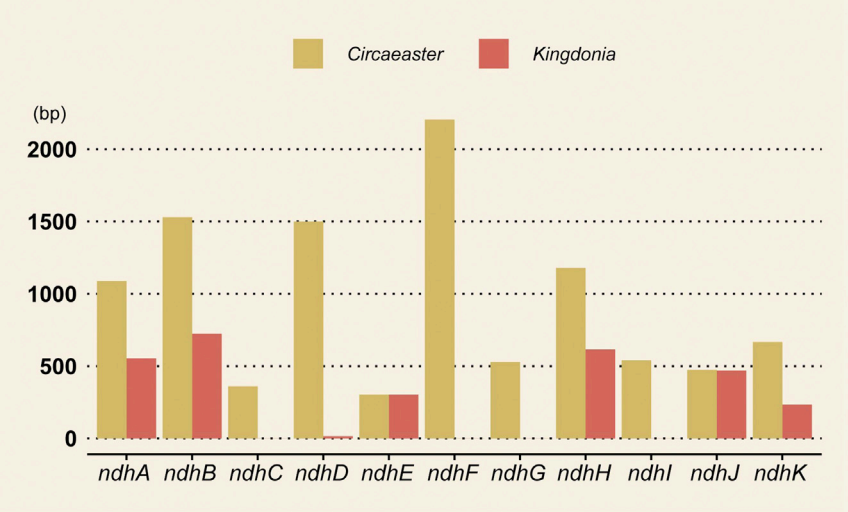

Figure 4. Length Comparison of *ndh* Genes between *K. uniflora* and *C. agrestis*

by the reproductive systems of species (Stephenson et al., 2000; Babará et al., 2009; Wornik and Grube, 2010; Beatty and Provan, 2011; Otálora et al., 2013). *K. uniflora* primarily reproduces asexually via rhizomes. This reproductive mode could produce identical individuals rapidly but lacks recombination and the possibility to create genetic variation in offspring, reducing the opportunities for adaptive evolution (Eckert, 2001; Castonguay; Angers, 2012). In addition, without segregation and recombination, obligate asexual multiplication may push a species into extinction owing to the steady accumulation of deleterious mutations (Thomas et al., 2016). During the long-term evolutionary history of *K. uniflora*, deleterious mutation accumulation cannot be ruled out; if something goes awry, such as the occurrence of a fatal mutation, whole clusters of clones can be wiped out. The integrated DNA-repair mechanism overrepresented in *K. uniflora* would allow for a reduction in the accumulation of deleterious mutations. This DNA-repair system might also reduce genetic diversity produced by mutations. Having high genetic diversity is important for plants to respond to environmental changes. We thus speculate that the extremely narrow distribution range of *K. uniflora* is associated with low genetic diversity, which restricted suitable environments to simplex, equable habitats rather than multiple, divergent habitats. Specifically, *K. uniflora* can only live in high elevations with minor human disturbances, while being characterized by perennial cold temperatures of below 0°C. The extreme temperature is likely to cause DNA, RNA, and protein damage. The overrepresentation of gene families for DNA repair might also be one kind of protection from extremely low temperature.

Underrepresentation of genes associated with stress response in *K. uniflora* leads to degeneration of adaptive ability to environmental changes. Phenylpropanoids are believed to contribute to all aspects of plant responses toward biotic and abiotic stimuli (Vogt, 2010). As concluded by La Camera et al. (2004), when plants suffer environmental stress, pests, or diseases, phenylpropanoids could evoke relevant response mechanisms to protect the plant from damage. Similarly, secondary metabolites also play a role in plant defense against environmental stresses, pests, and diseases (Bennett and Wallsgrove, 1994). Given the relatively undisturbed ecological environment of *K. uniflora* and lack of habitat stress during growth, adaptation to such conditions resulted in the functional degeneration of stress response systems. In addition, phenylpropanoids can also promote invasion of new habitats (Bais et al., 2003; Vogt, 2010). Our results indicate the underrepresentation of gene families involved in phenylpropanoid biosynthesis, which might be one reason causing low ability of *K. uniflora* to invade new habitats.

We conclude long-term living in highly equable habitats lead to the underrepresentation of stress response genes, which finally resulted in loss of ability to adapt to changing environments. The asexual reproductive strategy promoted overrepresentation of DNA repair genes, which reduced genetic diversity associated with adaptive capacity to environmental changes. Hence, both the underrepresentation of stress response genes and overrepresentation of DNA repair genes are responsible for the low adaptive ability of *K. uniflora*.

Equable habitats likely promoted the dispensability of most *ndh* genes in *K. uniflora*. Most angiosperms contain 11 plastid *ndh* genes, whereas all *ndh* genes, except for *ndhE* and *ndhJ*, were found to be either pseudogenized (*Ψndh*A, *Ψndh*B, *Ψndh*D, *Ψndh*H, and *Ψndh*K) or absent (*ndhC*, *ndhF*, *ndhI*, and *ndhG*) in the *K. uniflora* plastome (Sun et al., 2017). All 11 plastid *ndh* genes are intact in *C. agrestis* (Sun et al., 2017), indicating that the loss of *ndh* genes from *K. uniflora* occurred after the split between *K. uniflora* and *C. agrestis*, suggesting that within 52 million years most of the plastid *ndh* genes were lost from *K. uniflora* not only in the plastome but also in the nuclear genome. Among land plants, the plastid *ndh* loci have also been found absent in non-photosynthetic plants, epiphytes, Gnetales, conifers, and *Erodium* (Geraniaceae) (Kim et al., 2015; Lin et al., 2017; Nie et al., 2017). Evidence suggests that the thylakoid NADPH complex could optimize photosynthesis for plants under environmental stresses, while being found dispensable for plant growth under optimal growth conditions (Martín and Sabater, 2010). *K. uniflora* is extremely selective in habitat preference (Li et al., 2001) and is known as the indicator for natural ecological environment without disturbance. We hence speculate that the current habitats of *K. uniflora* might have promoted the dispensability of the plastid *ndh* genes. Additionally, within plants, NADPH supplies hydrogen for many anabolic processes (Antal et al., 2015). The underrepresentation of gene families related to metabolic pathways, as detected from our CAFÉ-based analyses, is likely related with the nonfunction state of plastid *ndh* genes.

Changing climate, shrinking habitats, and low adaptive ability to environmental changes together contributed to the extremely narrow distribution of *K. uniflora*. The overrepresentation of gene families involved in DNA repair could help reduce the accumulation of deleterious mutations during asexual reproduction, which is the dominant mode of reproduction in *K. uniflora*, while reducing genetic diversity, which is important in responding to environment fluctuations. The underrepresentation of gene families in charge of stress response and nonfunction of plastid *ndh* genes could be due to the adaptive degeneration caused by long-term adaptation to living in relatively stress-free environments. Considering the long evolutionary history of *K. uniflora*, and the fossil records from *C. agrestis* in the mid-Albian of Virginia, USA (Crane et al., 1994; Drinna et al., 1994), we speculate it should have been widespread around the world. Changing climate, shrinking habitats, asexual reproduction, and adaptive degeneration caused by relying on easeful environment together led it to a current status of endangerment.

Limitations of the Study

We reported the draft genome of the ancient and currently endangered *K. uniflora*. Although we revealed the evolutionary history and potential endangered mechanism of *K. uniflora* through primary analyses, population genomic analyses are required to estimate genetic diversity, reveal genetic structure and its causes, and identify loci associated with environmental adaption.

Resource Availability

Lead Contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Yanxia Sun (sunyanxia@wbgcas.cn).

Materials Availability

This study did not generate new reagents.

Data and Code Availability

The accession number for the genome assembly and raw reads reported in this paper is GenBank: PRJNA587615 and PRJNA611722, respectively.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101124.

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AUTHOR CONTRIBUTIONS

H.W., H.S., and Xi. Zhang conceived the study Y.S., H.Z., and N.L. collected the materials. Y.S. and T.D. prepared DNA and RNA for sequencing. Y.S., A.Z., and Xu Zhang performed the genome assembly and genome annotation. Y.S. and A.Z. designed genomics analyses. Y.S., T.D., M.J.M., J.B.L., and J.H. wrote the manuscript with the input of all co-authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Genome Sequencing of the Endangered *Kingdonia uniflora* (Circaeasteraceae, Ranunculales) Reveals Potential Mechanisms of Evolutionary Specialization

Yanxia Sun, Tao Deng, Aidi Zhang, Michael J. Moore, Jacob B. Landis, Nan Lin, Huajie Zhang, Xu Zhang, Jinling Huang, Xiujun Zhang, Hang Sun, and Hengchang Wang

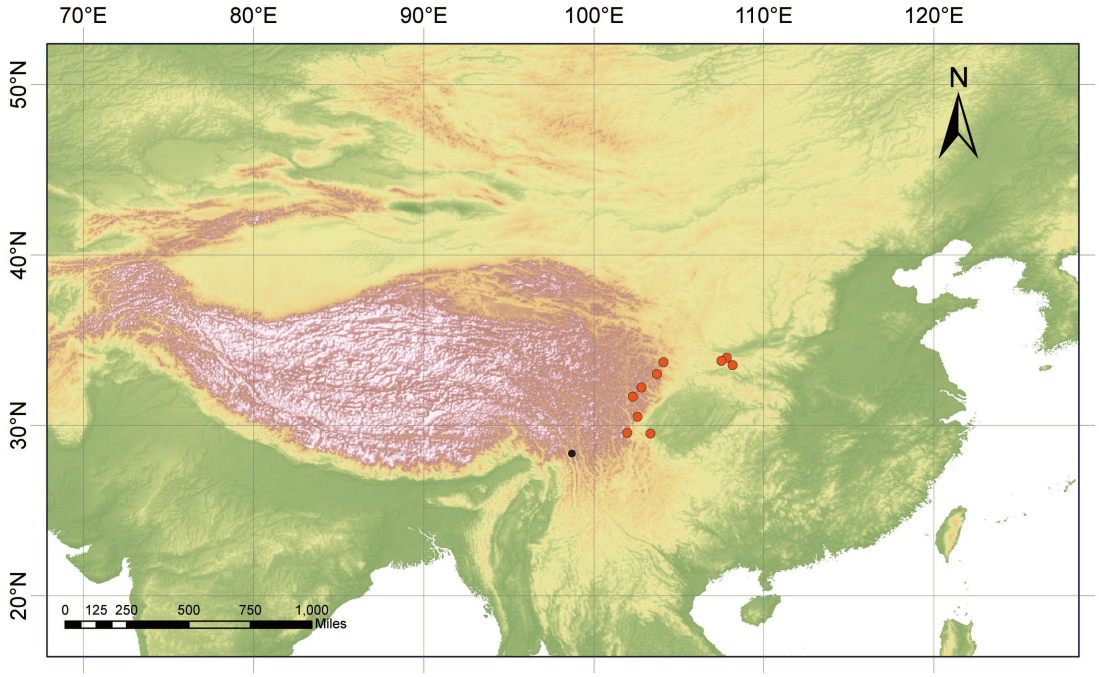

Figure S1. Distribution range of *K. uniflora.* The black dot shows the individuals with previous occurrence record but no longer have extant populations; the red dots represent the current distribution range of *K. uniflora*. Related to Table 1

Figure S2. Morphological features of *K. uniflora.* Related to Table 1.

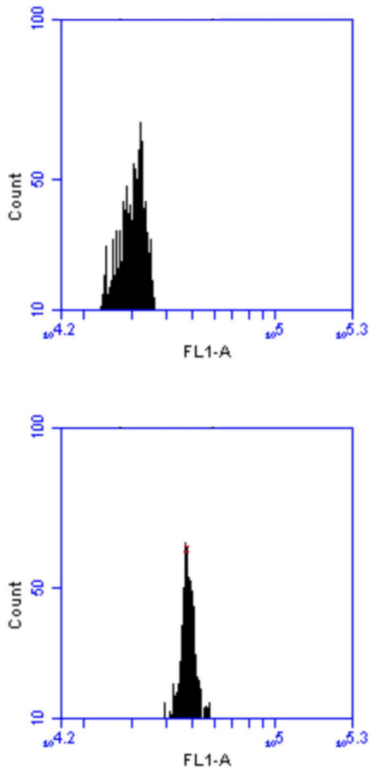

Figure S3. Estimation of *K. uniflora* genome size based on flow cytometer analysis. The above panel showing the 2C DNA of *Actinidia chinensis* (Hopping, 1994) at 32377.78, and the panel below indicating 2C DNA of *K. uniflora* at 47614.17. Related to Table 1.

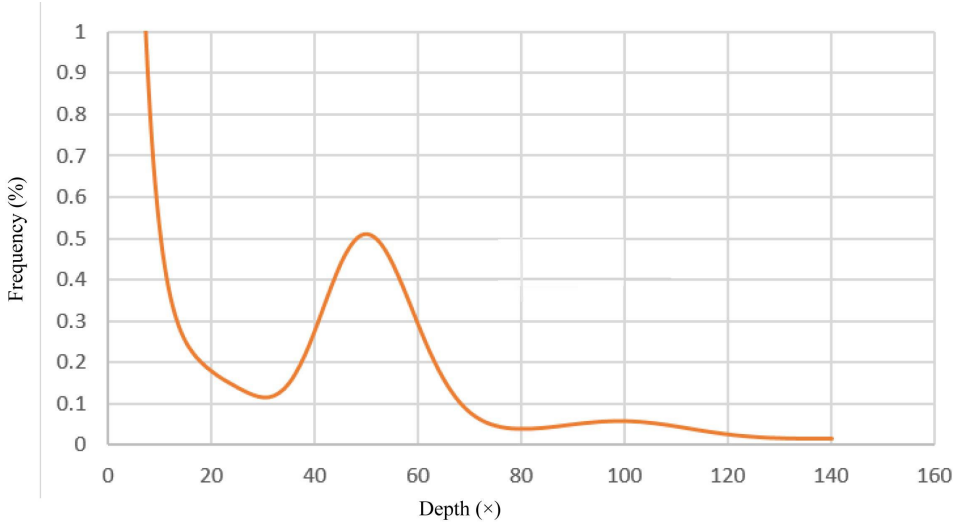

Figure S4. 17 *k*-mer frequency distribution of sequencing reads. Related to Table 1.

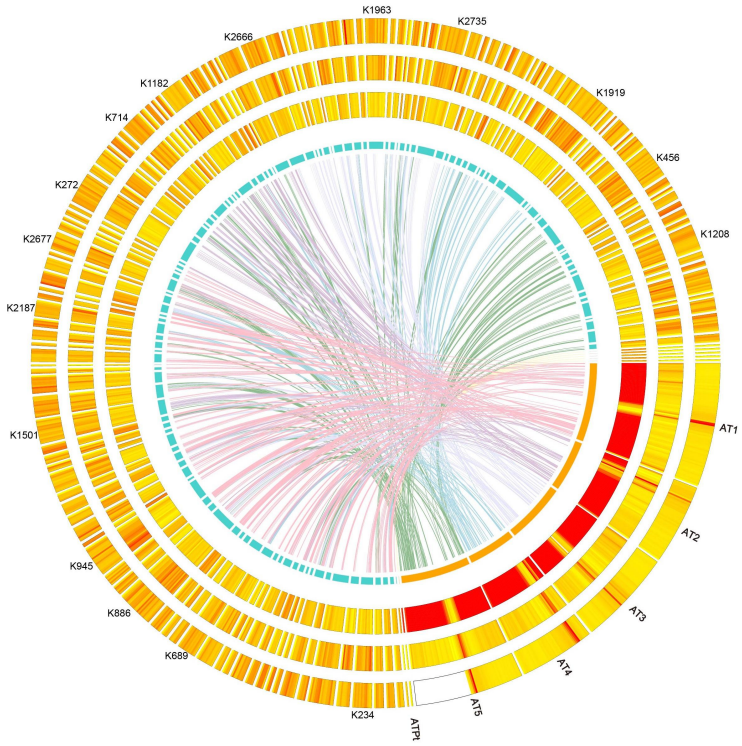

Figure S5. Comparative analyses of genomic features between *Kingdonia uniflora* and *Arabidopsis thaliana*. Tracks from inside to outside are collinearity between both genomes, number of chromosomes/scaffolds, gene density, GC content and TE density. Related to Figure 1.

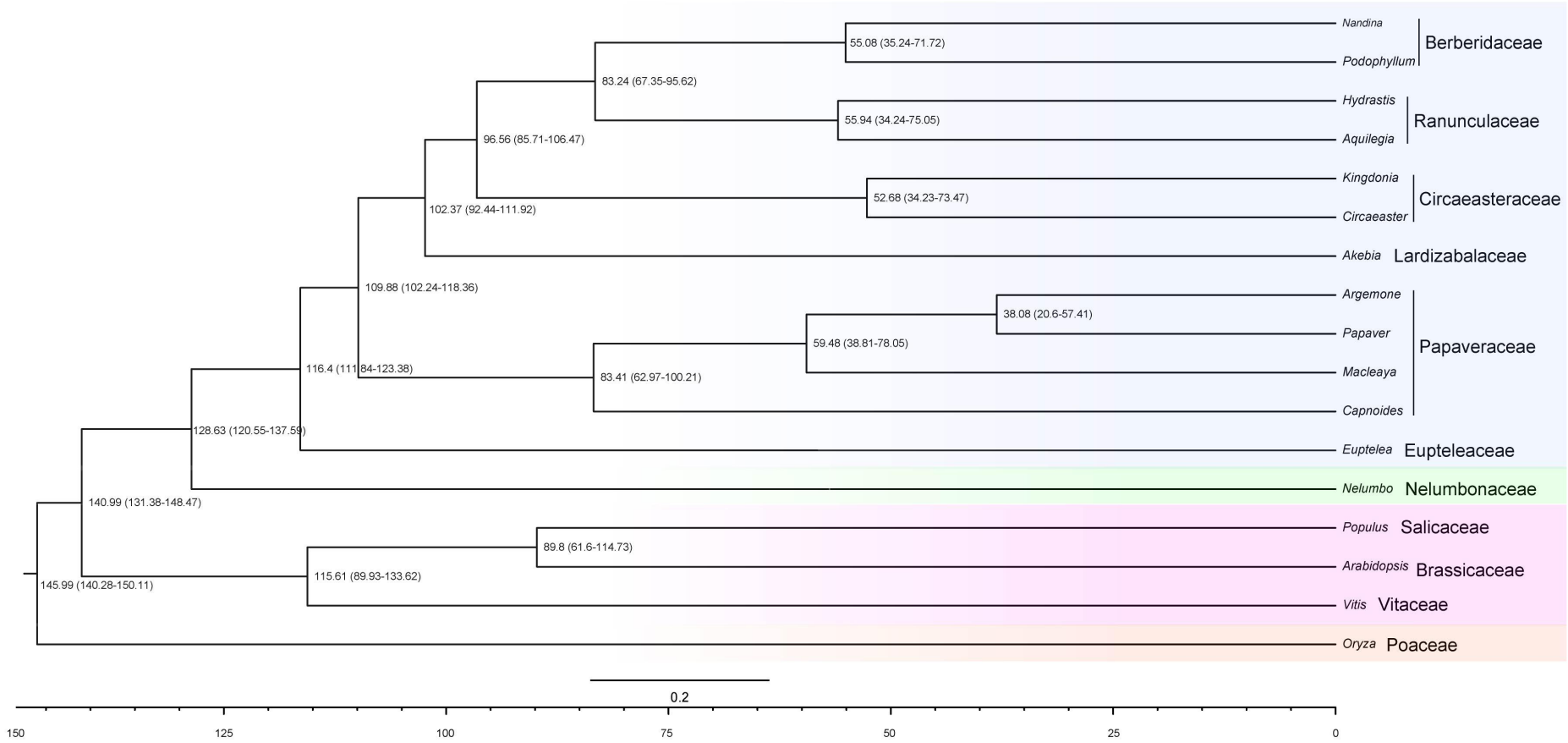

Figure S6. Dated phylogeny for 17 plant species with *Oryza* as an outgroup. A time scale is shown at the bottom. Related to Figure 3.

Table S1. Statistics of characteristics of *K. uniflora* genome (*K*-mer=17). Related to Table 1.

Chracteristics	
K-mer	17
Peak_Depth	80
N *K*-mer	58,788,492,58
Genome size	1170 Mb
Revised Genome size	1005 Mb
Heterozygous rate	nan
Repeat rate	0.6242

Table S2. Sequencing and quality filtering statistics. Related to Table 1.

	Total data (G)	Sequence coverage (X)
Illumina sequencing	236	201.7
Pacbio Sequencing	106.5	90.6
Total	342.5	292.7

Table S3. Information of function annotation in *K. uniflora* genes. Related to Table 1.

Database	Annotated Number	Annotated Percent (%)
NR	35818	82.7%
Swiss-Prot	27859	64.3%
KEGG	32104	74.1%
InterPro	25951	59.9%
Total	35953	83.03%

Table S4. Statistics of noncoding RNA in *K. uniflora* genome. Related to Table 1.

Type		Copy	Average length (bp)	Total length (bp)
tRNA		1124	76	85487
rRNA		715		
	18S	81	1802	146008
	28S	76	6732	511650
	5.8S	0	_	_
	5S	558	115	63990
snRNA	CD-box	1447	104	149886
	HACA-box	97	124	11991
	splicing	207	154	31895
miRNA		125	126	15757

Table S5. Scaffolds from the *K. uniflora* assembly were aligned to conserved genes using BUSCO method. Related to Table 1.

Species	Genome Size	BUSCO annotation assessment results
K. uniflora	1004.7 Mb	C:90.6% [D:4.1%], F:3.3%, M:6.1%, n:1375

C: Complete Single-Copy BUSCOs

D: Complete Duplicated BUSCOs

F: Fragmented BUSCOs

M: Missing BUSCOs

n: Total BUSCO groups searched

Table S6. Comparison of genome assembly within Ranunculales. Related to Table 1.

Species	Family	Genome size (Mb)	No. of scaffolds	No. of contigs	Length of N50, bp	References
Aquilegia coerulea	Ranunculaceae	301.98	970	7,189	121,821	Filiault et al., 2018
Berberis thunbergii	Berberidaceae	2240.74	11,815	11,815	397,058	NCBI available
Kingdonia uniflora	Circaeasteraceae	1004.7	2,932	2932	2,099,369	
Eschscholzia californica	Papaveraceae	489.065	53,253	85,931	20,647	Hori et al., 2018
Macleaya cordata	Papaveraceae	377.834	4,547	25,550	36,130	Liu et al., 2017
Papaver somniferum	Papaveraceae	2715.53	34,381	65,344	1,773,300	Guo et al., 2018

Table S7. Statistics of repeat sequences and transposable elements in *K. uniflora* genome. Related to Table 1.

Type	Repeat size	Percent of genome (%)
DNA	50812195	5.06
LINE	30109270	3.00
SINE	173746	0.02
LTR	408124656	40.62
Simple repeat	5211550	0.52
Unknown	179205100	17.84
Total	671481635	66.83

Table S9. Length comparasion of *ndh* genes between *K. uniflora* and *C. agrestis.* Related to Figure 4.

	C. agrestis (bp)	*K. uniflora* (bp)
ndhA	1089	553
ndhB	1530	723
ndhC	360	0
ndhD	1500	15
ndhE	303	303
ndhF	2205	0
ndhG	528	0
ndhH	1179	615
ndhI	540	0
ndhJ	474	468
ndhK	666	234

Table S10. Information of species used for phylogenetic analyses. Colored characters showing the expanded taxa in Figure S5 compared with that in Figure 3. Related to Figure 3.

Species	Family	Source
Oryza sativa L.	Poaceae	NCBI
Vitis vinifera L.	Vitaceae	NCBI
Populus trichocarpa Torr. and Gray	Salicaceae	NCBI
Arabidopsis thaliana L.	Brassicaceae	NCBI
Nelumbo nucifera Gaertner	Nelumbonaceae	NCBI
Euptelea pleiosperma J. D. Hooker and Thomson	Eupteleaceae	1 kp
Argemone mexicana L.	Papaveraceae	1 kp
Papaver bracteatum Lindl.	Papaveraceae	1 kp
Capnoides sempervirens (L.) Borkh.	Papaveraceae	1 kp
Macleaya cordata (Willd.) R. Br.	Papaveraceae	NCBI
Akebia trifoliata (Thunberg) Koidzumi	Lardizabalaceae	1 kp
Circaeaster agrestis Maxim.	Circaeasteraceae	Current study
Kingdonia uniflora Balf. f. and W.W. Sm.	Circaeasteraceae	Current study
Hydrastis canadensis L.	Ranunculaceae	1 kp

Aquilegia coerulea E. James	Ranunculaceae	NCBI
Podophyllum peltatum L.	Berberidaceae	1 kp
Nandina domestica Thunberg	Berberidaceae	1 kp

Transparent Methods

1 Genome sequencing and assembly

1.1 Plant materials and sequencing

Fresh *K. uniflora* leaves were collected from individuals growing from the same rhizome in the Taibai Mountains (altitude 2,844 m, N 34.038°, E107.715°), Shaanxi, China. Total genomic DNA (≥10 ug, ≥50 ng/ul) was isolated from fresh leaves using the conventional cetyltriethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). To help to estimate the genome size and polish genome assembly, we conducted Illumina sequencing; two paired-end sequencing libraries with insert sizes of 270 bp and 500 bp, respectively, were constructed and sequenced on the Illumina HiSeq X ten platform (Illumina Inc., CA, USA) at Beijing Genomics Institute (BGI) in Wuhan, Hubei, China. For PacBio single-molecule real-time sequencing, sequencing libraries with 20-kb DNA inserts were constructed and sequenced on the PacBio Sequel platform (Pacific Biosciences, CA, USA) at BGI. We also collected fresh leaves of *C. agrestis* in Taibai Mountains (altitude 2,837m, N34.038°, E107.68) for RNA extraction. Total RNA was extracted from young leaves (~100 mg) of both *K. uniflora* and *C. agrestis* using TRIzol Reagent RNA Purification (DSB, Guangdong, China). A cDNA library with insert sizes of 350-400 bp was prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, MA, USA) and paired-end sequenced on the HiSeq X ten platform (Illumina Inc., CA, USA) at

BGI. The transcriptome data of *K. uniflora* was used for the prediction of protein-coding genes; and the transcriptome data of *C. agrestis* was used to detect single copy genes.

1.2 *De novo* assembly

The PacBio long reads were first error corrected and then *de novo* assembled using Canu v1.8 (Koren et al., 2017) with default parameters (rawErrorRate=0.300, correctedErrorRate=0.045, minReadLength=1000, minOverlapLength=500, canuIterationMax=2) except for setting the genome size to 1.2 G to obtain contigs. Then iterative polishing was conducted on the Canu derived contigs using Pilon v1.2.3 (Walker et al., 2014) in which adapter-trimmed paired-end Illumina reads from DNA sequencing were aligned with the raw assembly with default parameters to fix bases and correct local misassembles. RNA-seq reads were assembled into transcripts using Trinity v2.6.6 (Grabherr et al., 2011) with the paired-end option and remaining default parameters.

2 Genome annotation

2.1 Annotation of repetitive sequences

We identified *de novo* repetitive sequences in the *K. uniflora* genome using RepeatModeler (http://www.repeatmasker.org/RepeatModeler/) based on a self-blast search. We further used RepeatMasker (http: //www.repeatmasker.org/) to search for known repetitive sequences using a

cross-match program with a Repbase-derived

RepeatMasker library and the *de novo* repetitive sequences constructed by RepeatModeler. Intact LTR (long terminal repeat) retrotransposons were identified by searching the genome of *K. uniflora* with LTRharvest (Ellinghaus et al., 2008) (-motif tgca -motifmis 1) and LTR_Finder (Xu and Wang, 2007) (-D 20000 -d 1000 -L 5000 -I 100). We combined results from both analyses and filtered false positives using LTR_retriever (Qu and Jiang, 2018), which also calculated the insertion date (*t*) for each LTR retrotransposons (*t*= *K/2r*, K: genetic distance) using a substitution rate (*r*) of 1.4*10−9 substitutions per site per year calculated by MCMCtree in PAML (Yang, 2007) .

2.2 Structural and functional annotation of genes

Putative protein-coding gene structures in the *K. uniflora* genome were homology predicted using the Maker package v2.31.10 (Holt and Yandell, 2011) with protein references from the published Ranunculales genomes and the *de novo* assembled transcripts of *K. uniflora* transcriptome data generated in this study, and *de novo* predicted using Augustus v3.3.2 (Stanke et al., 2006). The rRNAs were predicted using RNAmmer v1.2 (Lagesen et al., 2007), tRNAs were predicted using tRNAscan-SE v1.4 (Lowe and Eddy, 1997), and other noncoding RNA sequences were identified using Rfam v12.0 by inner calling using Infernal v1.1.2 (Nawrocki and Eddy, 2013).

Functional annotation of the protein-coding genes was carried out by performing BLASTP analyses (e-value cut-off 1e-05) against the NCBI nonredundant protein sequence database and SwissProt. Searches for gene motifs and domains were performed using InterProScan v5.16.55

(Jones et al., 2014). Completeness of the genome was assessed by performing gene annotation using the BUSCO (v3.0.2) method (Simão et al, 2015) by searching the Embryophyta library.

3 Investigation of whole-genome duplication

We identified paralogs (within *K. uniflora* and *C. agrestis*, respectively) and orthologs (between *K. uniflora* and *C. agrestis*) using BLASTP (E value = 1E-07). For each gene pair, the number of synonymous substitutions per synonymous site (*Ks*) based on the NG method was calculated using TBtools (Chen et al., 2018); *Ks* values of all gene pairs were plotted to identify putative whole-genome duplication events. In addition, MCScanx (Wang et al., 2012) was used to identify syntenic blocks within the *K. uniflora* genome. Dot-plot analysis of syntenic blocks with at least five gene pairs was conducted using the dot plotter program within the MCScanX package to further detect whole-genome duplication events.

4 Gene family and phylogenomic analysis

Orthogroups were constructed with 11 other sequenced plant species (Table S10). To get reliable tree topology, all basal eudicot species (*Nandina domestica, Aquilegia coerulea, Circaeaster agrestis, Akebia trifoliata, Macleaya cordata, Euptelea pleiosperma,* and *Nelumbo nucifera*) with available genome sequences were included for the phylogenetic analyses. In addition, the frequently-used outgroups (*Arabidopsis thaliana, Populus trichocarpa, Vitis vinifera* and *Oryza sativa*) in previous studies (e.g., Chen et al., 2018; Song et al., 2018; Yang et al., 2019)

were also included in our analyses. CD-HIT (Huang et al., 2010) was employed to remove redundancy caused by alternative splicing variations (-c 0.8 -aS 0.8). To exclude putative fragmented genes, genes encoding protein sequences shorter than 50 aa (amino acids) were filtered out. All filtered protein sequences of the 12 species were compared with each other using BLASTP (E value = 1E-5) and clustered into orthologous groups by OrthoFinder (Emms and Kelly, 2015). Protein sequences of single-copy gene families identified by OrthoFinder were used for phylogenetic tree construction. MAFFT version 7.0 (Katoh and Standley, 2013) was used to generate multiple sequence alignment for protein sequences in each single-copy family. Poorly aligned regions were further trimmed using the Gblocks (Castresana, 2000; Talavera and Castresana, 2007). The alignments of each gene family were concatenated to a super alignment matrix, which was then used for phylogenetic tree reconstruction through the PROTCATJTT model in RAxML version 8.1.2 (Stamatakis, 2014). To assess species tree clade support, a coalescent-based analysis was also conducted using RAxML bootstrap gene trees as input for ASTRAL v. 4.7.6 (Mirarab et al., 2015). To test the accuracy of above phylogenetic analyses, a second data set consisting of 17 taxa was also used following the same steps which consisted of increased taxonomic sampling with the tradeoff of fewer loci.

To investigate the evolutionary history of *K. uniflora*, divergence time between 12 species was estimated using MCMCtree in PAML (Yang, 2007) with the options “independent rates” and “HKY85” model. A Markov chain Monte Carlo analysis was run for 100,000,000 generations, using a burn-in of 1,000 iterations. Two constraints were used for time calibrations: (1) 140–150 Mya for the monocot-dicot split (Gaut et al., 1996; Yang et al., 2018); 112-124 Mya for the Ranunculales crown group (Magallón et al., 2015; Sun et al., 2018).

5 Gene family overrepresentation and underrepresentation

Overrepresentation and underrepresentation of the OrthoFinder-derived orthologous gene families were determined using CAFÉ v. 4.1 (De Bie et al., 2006). The program uses a birth and death process to model gene gain and loss across a user-specified phylogenetic tree. The distribution of family sizes generated under this model can provide a basis for assessing the significance of the observed family size differences among taxa. For each significantly overrepresented and underrepresented gene family in *K. uniflora*, functional information was inferred via KOBAS (http://kobas.cbi.pku.edu.cn/anno_iden.php) using KEGG Pathway database.

6 Plastid *ndh* gene searching

To examine whether the plastid *ndh* genes have been completely lost from *K. uniflora* or transferred to the nuclear genome, intact sequences of all (11) plastid *ndh* genes (*ndhA*, *ndhB*, *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ* and *ndhK*) were extracted from the plastome of *C. agrestis* (Sun et al., 2017). Then BLASTN analyses (E value = 1E-5) between the 11 gene sequences and assembled *K. uniflora* genome sequences was conducted.

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