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RADseq-based population genomic analysis and environmental adaptation of rare and endangered recretohalophyte *Reaumuria trigyna*

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Abstract

Genetic diversity reflects the survival potential, history, and population dynamics of an organism. It underlies the adaptive potential of populations and their response to environmental change. *Reaumuria trigyna* is an endemic species in the Eastern Alxa and West Ordos desert regions in China. The species has been considered a good candidate to explore the unique survival strategies of plants that inhabit this area. In this study, we performed population genomic analyses based on restriction-site associated DNA sequencing to understand the genetic diversity, population genetic structure, and differentiation of the species. Analyses of 92,719 high-quality single-nucleotide polymorphisms (SNPs) indicated that overall genetic diversity of *R. trigyna* was low ($H_O = 0.249$ and $H_E = 0.208$). No significant genetic differentiation was observed among the investigated populations. However, a subtle population genetic structure was detected. We suggest that this might be explained by adaptive diversification reinforced by the geographical isolation of populations. Overall, 3513 outlier SNPs were located in 243 gene-coding sequences in the *R. trigyna* transcriptome. Potential sites under diversifying selection occurred in genes (e.g., *AP2/EREBP*, *E3 ubiquitin-protein ligase*, *FLS*, and *4CL*) related to phytohormone regulation and synthesis of secondary metabolites which have roles in adaptation of species. Our genetic analyses

AMOVA, analysis of molecular variance; FPKM, fragments per kilobase of exon per million mapped fragments; GO, gene ontology; IBD, isolation by distance; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; PCR, polymerase chain reaction; PIC, polymorphism information content; SNP, single-nucleotide polymorphism; SM, secondary metabolite.

provide scientific criteria for evaluating the evolutionary capacity of *R. trigyna* and the discovery of unique adaptions. Our findings extend knowledge of refugia, environmental adaption, and evolution of germplasm resources that survive in the Ordos area.

1 | INTRODUCTION

Climate changes due to alternating glacial and interglacial periods since the Quaternary period have caused exceptionally drastic environmental changes that have had a dramatic impact on the distribution of terrestrial plant populations (Barnosky, 2005). The occurrence of extreme events and impact of human activities have accelerated global environmental issues, further altering species distribution patterns (Cambronero et al., 2018). Loss of habitats threaten the ability of organisms to efficiently respond to environmental changes, leading to significant population decline as species with small ranges become vulnerable to extinction (Purvis et al., 2000; Sanz-Arnal et al., 2022).

The survival and reproduction of an organism depends on the genetic variation from generation to generation (Hughes et al., 2008). Adaptative changes made in response to environmental changes are probably the only way for many organisms to survive (Meyers & Bull, 2002; Nogues-Bravo et al., 2018). In response to environmental changes, particularly climate change, organisms may use heritable adaptation strategies to increase their intrinsic fitness, genetic diversity, and adaptive potential (Becker et al., 2013). Thus, measurements of genetic diversity are often used as an indicator to evaluate the current survival status of a species and populations (Ellegren & Galtier, 2016; Mhemmed et al., 2008) to investigate population differentiation and relationships and to provide valuable information from a conservation perspective (Booy et al., 2000). Characterizing adaptive genetic variation and investigating the molecular mechanisms underlying adaptation are critical to understand the resilience of an organism to environmental change (Hoffmann et al., 2015).

High-throughput sequencing technologies have developed rapidly, and these have revolutionized life science research. The development of restriction-site associated DNA sequencing (RADseq/GBS) technology is considered one of the most important scientific breakthroughs. It has improved the ability to obtain information on population genetic diversity, genetic structure, and other genetic variation and has greatly accelerated the study of adaptive evolutionary mechanisms (Feng et al., 2020; F. Guo et al., 2015). The technology takes advantage of high throughput next-generation sequencing, is simple in operation, and has relatively low cost. It enables large numbers of polymorphic single nucleotide polymorphism (SNP) markers to be identified in the genomes of organisms and their populations (Andrews et al., 2016; Emerson et al., 2010; Lowry et al., 2017). Compared with anonymous markers (e.g., amplified fragment length polymorphisms and restriction fragment length polymorphisms), a small number of microsatellites, or some sequence-based markers (e.g., mitochondrial DNA and nuclear ribosomal DNA), SNPs obtained by RADseq have much greater genome coverage, come from a rich diversity of loci, have strong genetic stability, and can be processed with a high level of automation. Moreover, they are more suitable for large sample sizes (Grover & Sharma, 2016). In addition, many SNPs are well known to play a role in functional and ecological divergence (e.g., polymorphisms in coding or promoter regions) and are important for the evolutionary processes of an organism (Reitzel et al., 2013).

Plants distributed in the Eastern Alxa–Western Ordos area are excellent objects to study the genetic basis of adaptation. This area is located at the northern edge of the Tethys Ocean. It is characterized by a strong continental, weak monsoon, and arid/semi-arid plateau climate with soil salinity as high as 0.7%, average annual precipitation of 140.9–302.2 mm, and average annual temperature of 6.0–9.2°C (B. Li, 1990). The region is a well-known refuge for relict plants with origins in the Tethys Ocean, including ancient rare species such as *Reaumuria trigyna*, *Tetraena mongolica*, *Helianthemum songaricum*, *Potaninia mongolica*, *Ammopiptanthus mongolicus*, *Tugarinovia mongolica*, and *Prunus mongolica* (Meng et al., 2015). This region contains a large number of genetic resources for studying external stress and unique adaptation mechanisms in plants.

Reaumuria trigyna (family Tamaricaceae) is a strongly xerophilous, dicotyledonous small shrub (Y. Q. Ma, 1989; Zhao, 2006). As a long-time survivor in a severe environment, *R. trigyna* has evolved with many adaptive features including a well-developed root system, succulent leaves, sunken stomata, fissurate growth, multicell salt excretion glands, integrative regulation of endogenous hormones, and various physiological and molecular mechanisms for stress resistance (Dang et al., 2013; N. N. Li et al., 2021; B. J. Ma et al., 2021; S. L. Shi et al., 2011; Xue & Wang, 2008; Xue et al., 2012). Moreover, the microenvironment of habitats occupied by *R. trigyna* populations varies in soil salinity, moisture, and vegetation community structure. These factors are likely to contribute to the local adaptation of *R. trigyna* populations. If so, *R. trigyna* will also be a model to explore the habitat adaptations of plants in this area. Improving knowledge of the unique adaption strategies used by *R. trigyna* will provide a valuable model for investigating adaptive diversification in

plant species growing in the Ordos desert and other adverse environments.

In the last half century, the deterioration of natural conditions and disturbances by human activities have exerted considerable stress on *R. trigyna*, leading to its habitat loss. Its population size has been drastically reduced, and the population patchiness is a serious issue (T. Zhang et al., 2006). In this study, we conducted a population genomic analysis of *R. trigyna* using RADseq technology. Using a large number of SNP loci, we attempted to clarify the types of genetic diversity and genetic structure of *R. trigyna*. We assessed whether genetic differentiation has occurred among populations of the species and investigated variations associated with the adaptation of the population with the habitat. Our results provide insights into adaptive diversification of *R. trigyna* and lay a foundation for conservation of germplasm resources.

Core Ideas

- The overall genetic diversity of Reaumuria trigyna was low ($H_O = 0.249$ and $H_E = 0.208$).
- There was no apparent genetic differentiation among *R. trigyna* populations.
- A subtle population genetic substructure was detected among the investigated *R. trigyna* populations.
- Single-nucleotide polymorphisms important in adaptation to environmental stress and heterogeneous habitats of *R. trigyna* were identified.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Extensive field surveys were conducted in the entire distribution area of *R. trigyna* in July 2019 (Zhao, 1996), and eight geographically isolated populations were selected for the study (Figure 1 and Table 1). In each population, 10 plants with similar heights and canopies were selected at intervals of 30 m to avoid sampling progeny from the same maternal parent. Young healthy leaves were collected, immediately frozen in liquid nitrogen, further transported to the laboratory, and stored at −80°C.

2.2 | DNA preparation and RADseq

Genomic DNA was extracted from frozen leaves of *R. trigyna* using the Plant Genomic DNA kit (DP305; TianGen, Beijing, China) as per the manufacturer's instructions and was digested using a restriction enzyme *EcoR*I. The digested fragment was ligated to a modified Illumina P1 adapter containing individual-specific 4- to 8-bp-long nucleotide barcodes for sample tracking. All barcodes differed by at least two nucleotides to minimize sample misassignment due to sequencing errors. Adapter-ligated fragments were subsequently pooled and sheared. Sheared DNA was separated using electrophoresis on a 2% agarose gel, and fragments in the size range of –0–550 bp were isolated using a MinElute Gel Extraction kit (Qiagen, Valencia, CA, USA). After dsDNA ends were blunt ended and 3′-adenine overhangs added, a modified Illumina P2 adapter was ligated to these fragments. Finally, libraries were enriched using PCR amplification and further quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). RADs for each individual were paired-end sequenced (10 individuals per sequencing lane) on an Illumina HiSeq 2000 platform at Beijing Genomics Institute (BGI, Shenzhen, China).

2.3 | Quality filtering

Sequencing reads were sorted according to their unique barcode tag, and reads with ambiguous barcodes and those with poor quality were omitted from the analysis. Using the FastX toolkit (Version 0.0.14), reads containing one or more bases with a Phred quality score below 10 (equivalent to 90% probability of being correct) or more than 5% of the positions below 30 were discarded (Ratan et al., 2014). The final read length was trimmed to 84 bp using Trimmomatic (Version 0.36) (Bolger et al., 2014). For subsequent analyses, only the first (left) paired-read was used because DNA fragments created by RAD tag library preparation have a restriction site at one end and are randomly sheared at the other end. This results in sampling of each restriction-site sequence many times by the first read and random sampling of the genomic DNA sequence in the nearby region at a lower coverage in the second paired-end read (Etter & Johnson, 2012). Therefore, the second paired-end reads are less suitable to analyze SNPs.

2.4 | Rad tags clustering and SNP calling

Files containing all RAD tags for all individuals were analyzed using Stacks (Version 2.53) (Catchen et al., 2013) for the *de novo* assembly. Initially, putative orthologous tags (stacks) per individual were assembled using ustacks with a minimum depth of coverage required to create a stack (m) of 2 and a maximum distance (M) allowed between stacks of 2. The value of -N was always defined as M + 2. Catalogues of loci were assembled based on all the individuals using cstacks, and the number of mismatches allowed between

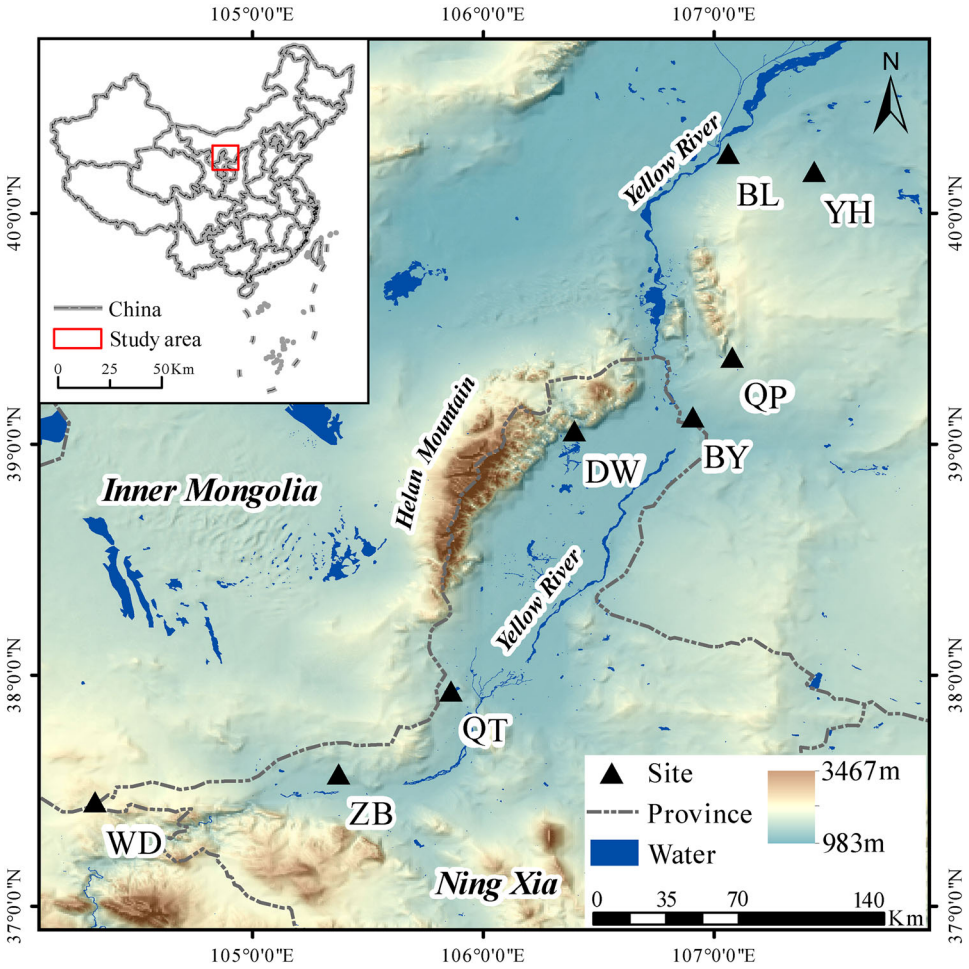

FIGURE 1 The geographical location of eight *Reaumuria trigyna* populations in this study. The geographical position of the study area in China is given in the top left corner. BL, Balagong; BY, Bayintaohai; DW, Dawukou; QP, Qipanjing; QT, Qingtongxia; WD, Wendu'ertu; YH, Yihewusu; ZB, Zhaobishan

TABLE 1 Habitat characteristics of *Reaumuria trigyna* populations

Population ID	Longitude (E)	Latitude (N)	Habitat	Altitude (m)
BL	107°03′38"	40°15′41''	Foothills	1123
BY	106°54′30''	39°07′17''	Foothills	1134
DW	106°23′42''	39°03′39''	Foothills	1252
YH	107°26′02''	40°11′02''	Piedmont plain	1225
QP	107°04′42''	39°22′47''	Foothills	1397
QT	105°51′37''	37°55′60''	Piedmont plain	1260
WD	104°19′05''	37°27′18''	Foothills	1693
ZB	105°22′28''	37°34′35''	Foothills	1280

Abbreviations: BL, Balagong; BY, Bayintaohai; DW, Dawukou; QP, Qipanjing; QT, Qingtongxia; WD, Wendu'ertu; YH, Yihewusu; ZB, Zhaobishan.

sample tags while generating the catalogue (n) was 3. Matches of individual RAD loci to the catalogue were searched using sstacks. Further, the tsv2bam program was used to transpose the data so that it is oriented by locus instead of sample.

2.5 | SNP filtering

Based on the population program in Stacks, parameter values were set for all individual RAD loci with $p = 8$ and $r = 0.6$. This means that to process a locus, it must be present in all

eight *R. trigyna* populations and in 60% of individuals in each population. The main parameter value *R* was set to 0.7, and the minor allele frequency (–min-maf) was set to 0.075. To avoid linkage bias for the SNP calling, only the first SNP per locus was included in the final analysis (–write-single-snp). Finally, VCF and genepop files with the filtered SNPs were created.

2.6 | Detection of SNPs under selection

SNPs under selection were identified using BayeScan (Version 2.1) (Gaggiotti, 2008) and Arlequin (Version 3.5) (Excoffier & Lischer, 2010). PGDSpider (Version 2.1.1.2) (Lischer & Excoffier, 2012) converted genepop data into BayeScan format data for identifying outliers. BayeScan was run under default settings, except for a modification to increase the number of prior odds runs to 100. The false discovery rate was set at 0.1. The hierarchical island model implemented in Arlequin accounts for the hierarchical structure of the populations in which migration was higher within group than between groups. We determined the outlier loci with a *p*-value of 0.005 and the adjusted starting parameters (number of simulations: 200,000; number of groups: 10; demes per group: fixed at 100).

2.7 | Genetic diversity and genetic differentiation in population

After removing SNPs under selection, 92,719 SNPs were used for population genetic analysis. General genetic variation metrics such as mean observed (*Ho*) and expected (*He*) heterozygosity were estimated using the R package PopGenKit (Version 1.0) (Paquette, 2012). Polymorphism information contents (PIC) were measured using PowerMarker (Version 3.0) (Lui, 2005). The populations program of Stacks was used to calculate the nucleotide diversity (π), average individual *F*-statistics (F_{IS}), and coefficient of genetic differentiation (F_{ST}) for each population pair (Catchen et al., 2013). Analysis of molecular variance (AMOVA) was performed on all populations using the Arlequin software to identify the distribution of genetic variation between and within populations (Excoffier & Lischer, 2010). The correlation between the matrix of the genetic distance of pairwise populations and corresponding geographical distances (km) between each pair of sampled locations was analyzed for the presence of isolation by distance via a Mantel test with 10,000 random permutations using the R package vegan (Version 2.5) (Oksanen et al., 2013).

2.8 | Population genetic structure and gene flow

For elucidation of the genetic structure of the eight *R. trigyna* populations, the variation information of the SNPs was analyzed using principal components analysis (PCA) to examine the separation of populations with PLINK (Version 1.9) (Purcell et al., 2007) and further visualized using the R package ggplot2 (Gómez-Rubio, 2017). The phylogenetic tree of the 80 individuals was constructed using IQ-TREE (Version 1.6.12) (Nguyen et al., 2015). ModelFinder was used to determine the best model for constructing the phylogenetic tree, and the best model was determined using the Bayesian Information Criterion. Finally, maximum likelihood was used for tree building. We used the software Admixture (Version 1.3.0) to estimate the genetic structure (Alexander & Lange, 2011). First, the vcf files containing all the used SNPs were transformed using Vcftools (Version 0.1.13) (Danecek et al., 2011) and PLINK to generate med and bed files. The predefined genetic clusters (*K*) were set from 2 to 8. We analyzed the gene flow among eight populations, and the *N*m was evaluated using the formula $Nm = (1 - F_{ST})/4\ F_{ST}$. The directions of the historical gene flow among the *R. trigyna* populations were estimated using Migrate-n (Version 3.6.11) (Beerli, 2008), and the following five possible models were assumed:

1. All samples were considered a whole population.
2. The population was divided into several local populations, with random mating within each local population.
3. Migration occurred between adjacent populations only.
4. North to south, the direction of the prevailing wind in this area.
5. South to north, the direction of the Yellow River in this area.

For each model, the number of recorded steps in the chain was set to 8000. The number of discarded plants per chain was set to 10,000, setting up three independent operations and a default heating mechanism. Finally, to determine the most likely migration model, log Bayes factors were calculated from the Bezier approximation score and compared for all gene flow models.

2.9 | Identification, quantification, and functional annotation of outlier-loci-located genes

Our previously published transcriptomic data (BioProject: PRJNA73445, BioSample accessions: SAMN00737691) were used to construct a local reference dataset of

R. trigyna (Dang et al., 2013). The transcriptome data were assembled *de novo* using Trinity (Version 2.4.0), with an optimized k-mer length (25-mer) (Haas et al., 2013). The subsequent clustering and elimination of redundancies were completed using TGICL (Version 2.1) to obtain unigenes (Pertea et al., 2003). Based on the coordinates of the SNP loci, the sequences of the corresponding RAD-tags were extracted using TBtools (Version 1.098) (Chen et al., 2020). We used the mem function of BWA (Version 0.7.17) to match the RAD-tags with the assembled transcripts and to locate the associated genes with outlier loci (H. Li, 2013). Bowtie 2 (Version 2.3.4.1) was used to map the reads to the assembled transcriptome to quantify the transcript levels (Langmead & Salzberg, 2012). Further, the number of mapped reads was estimated using RSEM (Version 1.2.12) (B. Li & Dewey, 2011) with the default setting. The value of normalized fragments per kilobase of exon per million mapped fragments (FPKM) for each unigene was used to represent gene expression levels. To obtain functional information about the outlier-located unigenes, we aligned the transcripts against the NCBI nonredundant (nr) database using BLASTX (Version 2.2.27+) with an *E*-value cut-off of 1.0×10^{-5}. Subsequently, gene ontology (GO) functional categories were identified using Blast2GO (Version 2.5.0, https://www.blast2go.com/), and pathways were assigned by sequence searches against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using the BLASTX algorithm with an *E*-value threshold of 1.0×10^{-5}.

2.10 | SNP genotyping

Overall, 40 outlier-loci-located genes containing polymorphic SNPs were randomly selected. The primers used to amplify the flanking sequences of these SNPs were designed using the NCBI primer-blast program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Tsingke BioTech Co., Ltd. (Beijing, China). The 60 DNA samples (7–8 individuals per population) were run on 1% TAE agarose gels and quantified using NanoDrop 2000c (Gene Company Limit, Beijing, China). PCR was performed as follows: 5 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and the final extension step at 72°C for 10 min. The successfully amplified PCR products were sequentially subjected to 2% TAE agarose gel electrophoresis, gel extraction, and DNA fragment purification (Tsingke BioTech Co., Ltd.) and further put into an ABI 3730xl DNA analyzer for sequencing. SNPs with typing were screened using Bioedit (Version 7.0.9) (Hall, 1999).

3 | RESULTS

3.1 | Sequencing output and data filtering

A total of 180.55 Gb of raw data were obtained from the 80 sequencing samples. Overall, 161.21 Gb passed initial quality filters, with an average Q20 percentage nearly of 99%, and an average GC content of 35.38%. The retained data presented 1859.73 million reads with a length of 84 bp, and the mean number of reads per individual was 23.25 million (ranging from 1.16 to 57.92 million). The complete details of sequencing output are provided in Table S1.

3.2 | RAD tag clustering and SNPs calling

Using ustacks, 577,557 to 28,962,075 left paired-reads for each individual were clustered into 88,989 to 2,565,323 stacks. The utilization rate of reads per individual ranged from 48.10% to 78.60%, with a mean of 73.58% (Table S2). The minimum depth of the clustered tags individually ranged from 3.15× to 9.1×, with a mean of 5.5× (SD = 4.15). After merging all the stacks of each individual, 7,891,440 RAD-tags were constructed, of which 29,041,789 SNPs were identified. Most of the SNPs were biallelic (80.47%), except for a low proportion of 15.33% triallelic SNPs and 4.19% tetrallelic SNPs. Finally, 96,232 SNPs met the SNP filtering criteria executed by population programs of Stacks and were used in the following analyses.

3.3 | Outlier detection

Using two outlier detection methods (BayeScan and Arlequin), 3513 outlier SNPs were identified. For the Bayescan-based scan, 51 outliers were identified (Figure S1). The F_{ST} outlier test in Arlequin produced 3513 outliers at the 0.5% level of significance (Figure 2). Arlequin confirmed all 51 BayeScan loci as being under selection.

3.4 | Population genetic diversity

Except the outlier SNPs, 92,719 SNPs were used for population genetic analysis. In the analyzed *R. trigyna* populations, H_O varied from 0.139 (WD) to 0.271 (YH), whereas H_E ranged from 0.133 (WD) to 0.224 (YH) with mean values of 0.249 and 0.208, respectively (Table 2). The PIC ranged from 0.112 (WD) to 0.190 (YH) with an average of 0.176. The nucleotide diversity (π) varied between 0.150 (WD) and 0.239 (YH) with an average of 0.223. The F_{IS} values of all

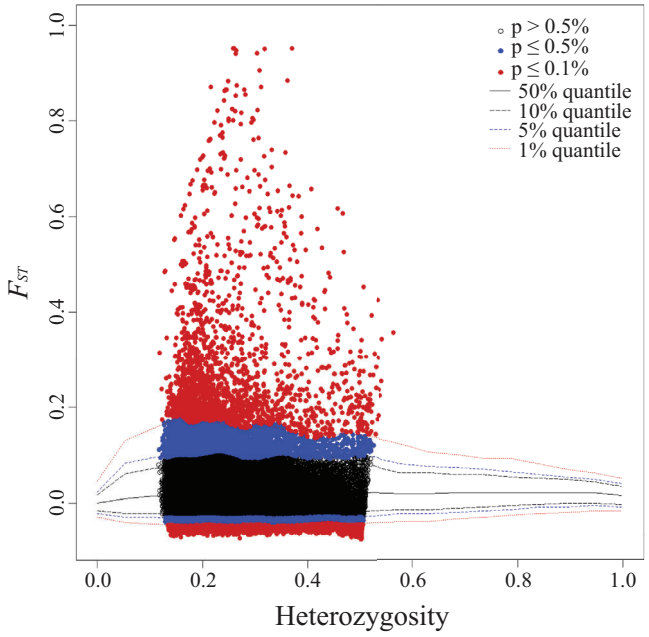

FIGURE 2 Detection of loci under selection based on F_{ST}. Loci significant at the 0.5% and 0.1% levels are the candidate loci under selection, which have been indicated as blue and red circles, respectively. Negative F_{ST} values indicate that there are no significant differences in the frequencies of markers between populations. This can occur when sample sizes are small and the number of SNPs analyzed is large (Gerlach et al., 2010)

TABLE 2 Genetic diversity statistics for the eight *R. trigyna* populations

Population ID	POP	PIC	π	F_{IS}	H_O	H_E
BL	1	0.182	0.231	−0.075	0.262	0.215
BY	2	0.187	0.236	−0.081	0.269	0.221
DW	3	0.182	0.231	−0.070	0.260	0.215
YH	4	0.190	0.239	−0.079	0.271	0.224
QP	5	0.186	0.233	−0.076	0.264	0.219
QT	6	0.184	0.231	−0.082	0.263	0.216
WD	7	0.112	0.150	0.014	0.139	0.133
ZB	8	0.185	0.233	−0.085	0.266	0.218
Mean		0.176	0.223	−0.067	0.249	0.208

Note: PIC, π, F_{IS}, *Ho*, and *He* represent polymorphism information content, average nucleotide diversity of population, *F*-statistics, observed heterozygosity, and expected heterozygosity, respectively.
Abbreviations: BL, Balagong; BY, Bayintaohai; DW, Dawukou; QP, Qipanjing; QT, Qingtongxia; WD, Wendu'ertu; YH, Yihewusu; ZB, Zhaobishan.

populations varied from −0.084 (ZB) to 0.014 (WD) with an average of −0.067. Among the eight *R. trigyna* populations, YH had the highest and WD had the lowest H_O, H_E, PIC, and π.

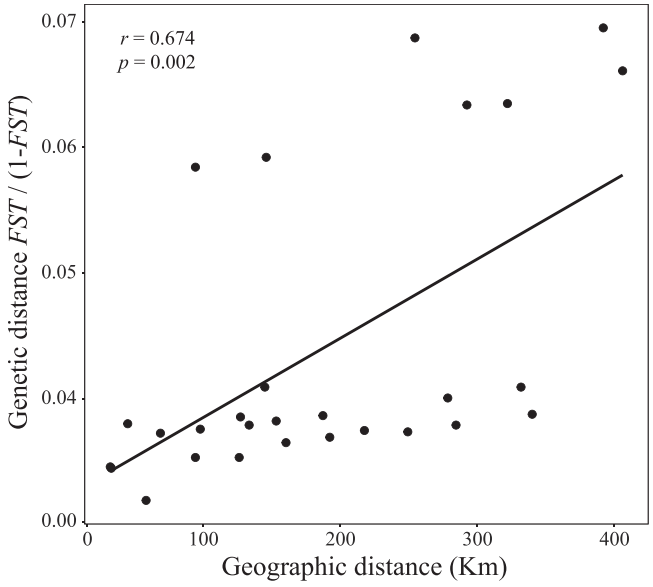

FIGURE 3 Mantel test between genetic distance and geographical distances (km) among eight *R. trigyna* populations, based on isolation-by-distance analyses ($r = 0.674$, $p = 0.002$)

3.5 | Genetic differentiation

The relationship between various populations of *R. trigyna* was analyzed using pairwise F_{ST} (Table 3). The F_{ST} value among the eight populations varied from 0.031 (between ZB and QT) to 0.070 (between BL and WD) with an average of 0.043. Among which there was a moderate genetic differentiation between the WD population and other populations, with the mean F_{ST} of 0.065 between WD and other populations. When the total variance was partitioned in a hierarchical AMOVA, 98.24% of the total variance was observed within populations, and only 1.76% variance was observed among populations (Table S3). Mantel's test revealed that genetic divergence was significantly correlated with geographical distance across the studied populations ($r = 0.674$, $p = 0.002$), conforming to expectations for isolation-by-distance (Figure 3 and Table S4).

3.6 | Population genetic structure

Admixture analysis (Figure 4A) showed that cross-validation errors increased from 0.450 to 0.793 with K from 2 to 8 (Figure S2). Based on the optimal K value ($K = 2$), the eight populations were divided into two groups which separated WD from the other populations. At $K = 4$, the four groups comprised WD, QT-ZB, BL-YH, and BY-QP-DW (Figure 4A). PCA revealed that the two principal components accounted for 27.26% (PC 1) and 10.68% (PC 2) of the total variability, respectively. In the Admixture analysis with $K = 4$, individuals from different populations

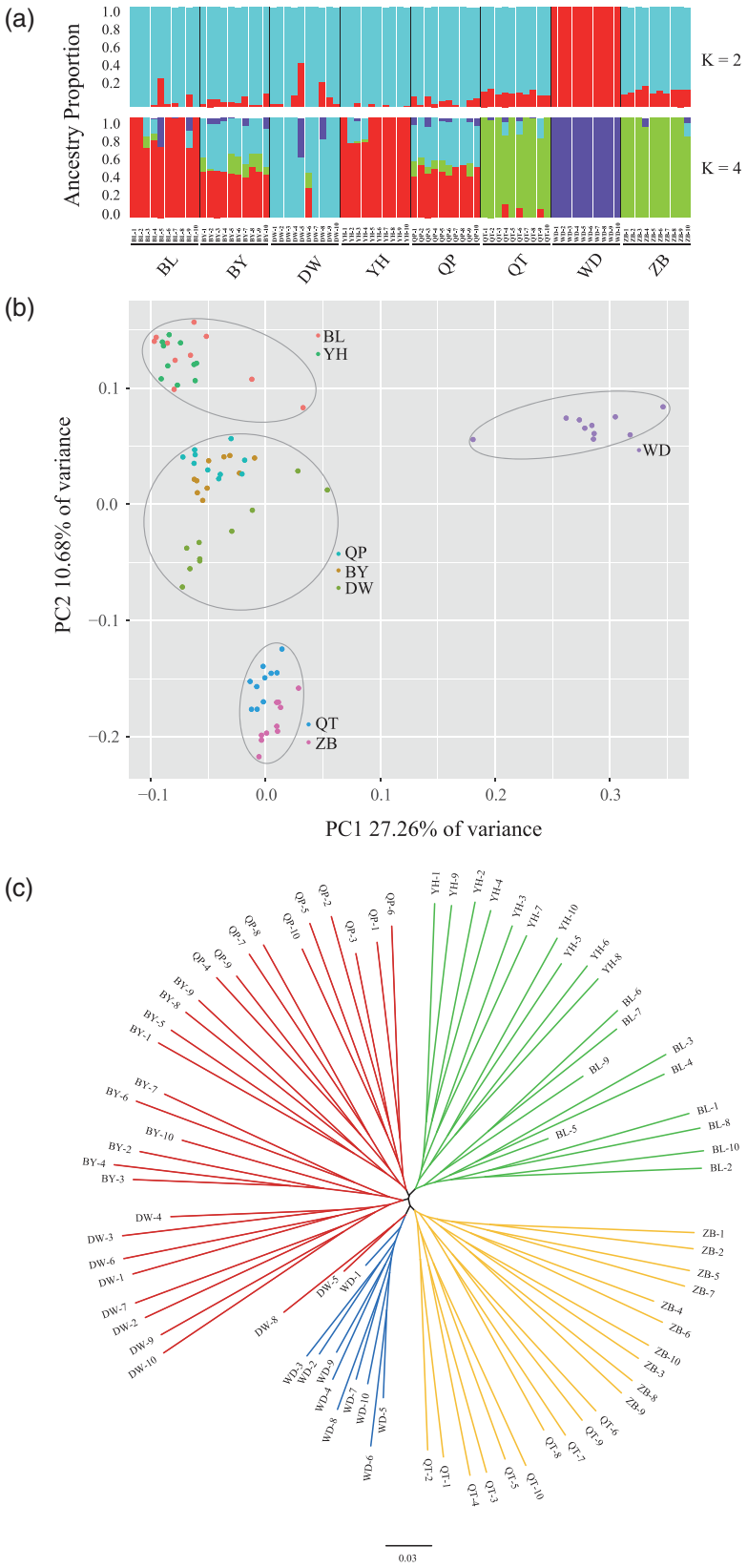

FIGURE 4 Population genetic structure of the eight *R. trigyna* populations. (a) Results of the Admixture analysis when *K* at 2 and 4. (b) Clustering of *R. trigyna* populations based on principal component analysis (PCA). Each point represents an individual, and different populations are colored differently. (c) Phylogenetic tree of 80 *R. trigyna* individuals. BL, Balagong; BY, Bayintaohai; DW, Dawukou; QP, Qipanjing; QT, Qingtongxia; WD, Wendu'ertu; YH, Yihewusu; ZB, Zhaobishan.

TABLE 3 Genetic differentiation coefficient (F_{ST}) and gene flow between each pair of populations

Population ID	BL	BY	DW	YH	QP	QT	WD	ZB
BL		0.037	0.039	0.033	0.036	0.039	**0.070**	0.040
BY	6.479		0.037	0.034	0.033	0.035	**0.064**	0.036
DW	6.086	6.561		0.037	0.036	0.037	**0.069**	0.037
YH	7.249	7.057	6.511		0.034	0.037	**0.067**	0.038
QP	6.638	7.249	6.707	7.080		0.036	**0.064**	0.036
QT	6.205	6.824	6.554	6.551	6.761		0.060	0.031
WD	3.338	3.666	3.391	3.506	3.660	3.912		0.059
ZB	6.067	6.642	6.431	6.387	6.667	7.861	3.989	

Note: The upper triangle represents the interpopulation F_{ST} of the eight *R. trigyna* populations, and the lower triangle indicates the pairwise gene flow of the populations. Significant pairwise F_{ST} values at the 0.5 levels are indicated by bold values.
Abbreviations: BL, Balagong; BY, Bayintaohai; DW, Dawukou; QP, Qipanjing; QT, Qingtongxia; WD, Wendu'ertu; YH, Yihewusu; ZB, Zhaobishan.

TABLE 4 Log Bayes factor and log-probability of *R. trigyna* single nucleotide polymorphism data detected by Migrate-n software with different models (based on Bezier score)

Model	Log-probability	Log Bayes factor	Rank
Full model	−490936.66	−2094.47	2
N-island model	−490956.67	−2134.49	3
Adjacent model	−491928.24	−4077.63	4
North to south	−489889.43	0	1
South to north	−496021.17	−12263.48	5

grouped into four clusters (Figure 4B). Similarly, phylogenetic analysis also placed individuals into four main phylogenetic clusters (Figure 4C). In this phylogenetic tree, some individuals from different populations clustered with individuals from another population. Notably, individuals of ZB clustered with individuals of QT, some individuals of BY clustered with individuals of QP, some other individuals of BY clustered with DW, and individuals of YH clustered with individuals of BL. In all cases within the four main phylogenetic clusters, individuals from different populations remained most closely related to individuals of the same population.

3.7 | Gene flow

The *N*m values between the eight *R. trigyna* populations ranged from 3.338 (between WD and BL) to 7.861 (between QT and ZB), with a mean value of 5.930 (Table 3). The *N*m value between the WD and other populations differed significantly. The directions of the historical gene flow estimation demonstrated that the north-to-south migration model (model 5) was the most suitable, suggesting that the BL population might be an ancestral population spreading southward (Table 4).

3.8 | Identification and annotation of genes associated with outlier loci

Of the 3513 outlier loci located in RAD tags, 251 were fully or partially mapped to 243 unigenes in the assembled transcriptome of *R. trigyna*. Functional annotation indicated that 136 (55.97%) returned at least one match with known proteins at the *E*-value > 10^{-5}; 48 (19.75%) were homologous to hypothetical, uncharacterized, or unknown proteins, and the remaining 59 did not match to known genes in the database (Table S5). Based on sequence homology, 159 unigenes were categorized into 40 GO terms. Under the biological process category, "metabolic process" was the most abundant term, followed by "cellular process" and "single-organism process." Within the cellular component category, the most abundant terms were "cell," "cell part," and "organelle." In the molecular function category, "catalytic activity" was the most abundant term, followed by "binding" (Figure 5A and Table S6). Overall, 112 unigenes were assigned to 103 KEGG pathways (Figure 5B and Table S7). Among the annotated pathways, "metabolic pathways" (27 unigenes) contained the highest number of unigenes, followed by "biosynthesis of secondary metabolites (SMs)" (12 unigenes). Quantitative analysis of gene expression revealed that 10 unigenes were highly expressed, with FPKM values >100.0. Overall, 80 unigenes had the FPKM values ranging from 10.0 to 100.0, and 153 unigenes were expressed with the FPKM <10.0 (Table S5). The top 10 highly expressed transcripts encoded proteins including a light-harvesting chlorophyll a/b-binding protein (unigene23195), a transcription factor BTF3 (CL5282.Contig2), two histones (CL59.Contig7 and unigene13953), a fructose-1,6-bisphosphatase protein (unigene27886), a proactivator polypeptide-like protein (unigene23195), a Frigida-like protein (unigene29804), a E3 ubiquitin-protein ligase protein (unigene717), and a flavonol synthase protein (unigene1722).

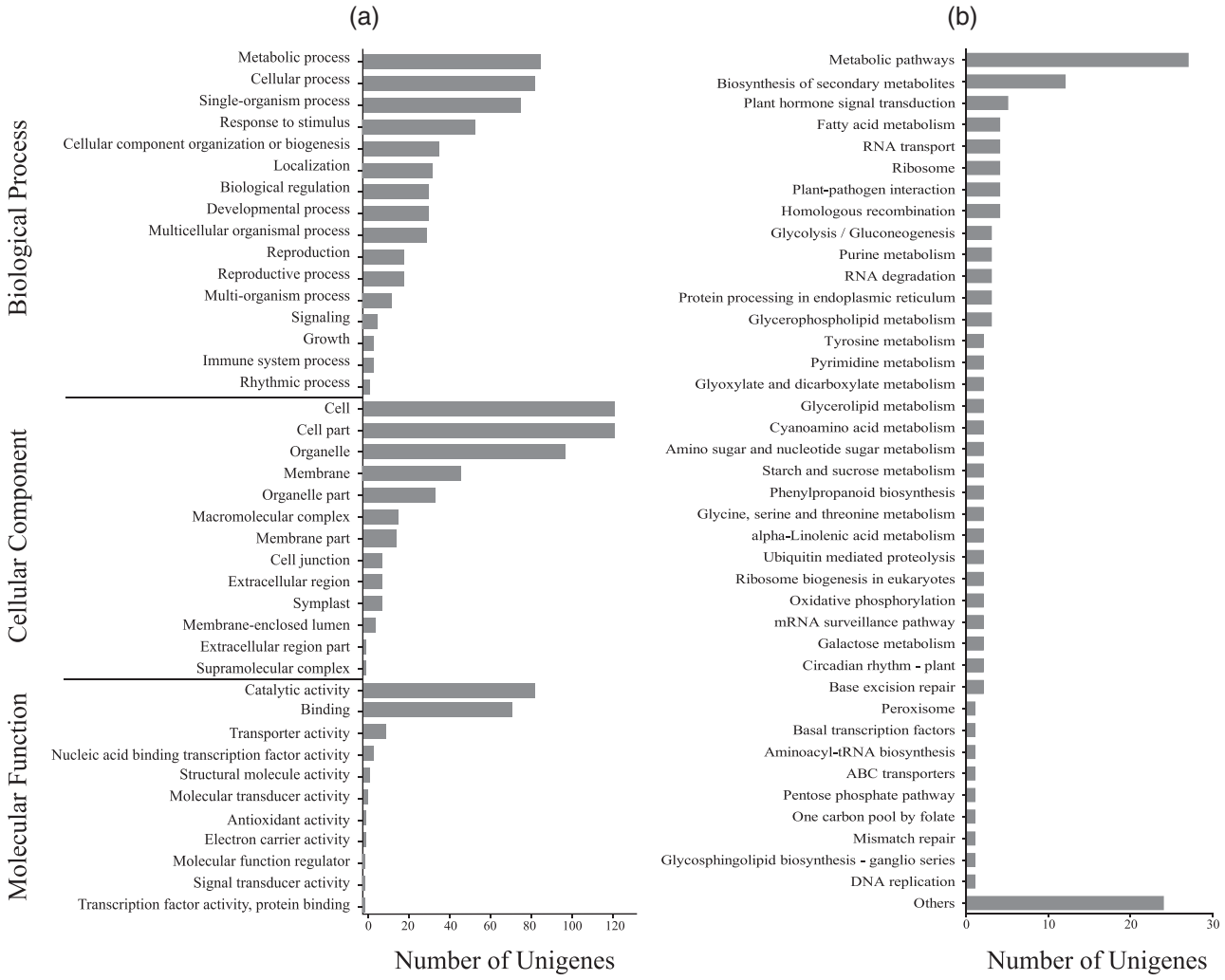

FIGURE 5 Functional annotation of the outlier-loci-associated unigenes. (a) Gene ontology (GO) functional annotation of the outlier-loci-associated unigenes. (b) Kyoto Encyclopedia of Genes and Genomes (KEGG) functional classification of the outlier-loci-associated unigenes. The *y* axis indicates the GO terms/KEGG pathway to which the gene was assigned. The *x* axis indicates the number of genes in a category/pathway

3.9 | SNP genotyping

Among the 40 selected outlier SNPs, 32 were successfully genotyped in 60 *R. trigyna* individuals using the designed primers (Figure S3 and Table S8). Among them, 13 and 6 outlier SNPs were homozygous or heterozygous in all investigated individuals, respectively, and the other 13 were polymorphic SNPs (Table S9). All the validated SNPs had the same genotypes compared with the RADseq-based SNPs.

4 | DISCUSSION

4.1 | *R. trigyna* had a low level of genetic diversity

Genetic diversity and geographical range are strongly correlated (Q. F. Guo, 2012). For endemic species with narrow distributions and small population sizes, the genetic diversity is generally lower than that reported for widely distributed species (Daco et al., 2019; Xu et al., 2021). *Reaumuria trigyna* is an endangered dicotyledonous shrub that is endemic to the Eastern Alxa–Western Ordos area in China. In this study, we obtained 96,232 high-quality SNPs using RADseq technology and performed a population genomic analysis of *R. trigyna*. A low level of genetic diversity ($H_E = 0.208$, $H_O = 0.249$) was reported in the species. Probably explained by the different marker systems employed, the values reported in our study differed and were significantly lower than those detected using RAPD ($H = 0.3303$) and ISSR ($H = 0.3083$) markers (Y. J. Zhang & Wang, 2008a, 2008b). The large number of SNP markers used in this RADseq study provided more comprehensive information on genetic variation in the *R. trigyna* genome. Thus, the values obtained in this study may provide the best indication of limitations to the adaptive potential of

this species (Benestan et al., 2015; Rodriguez-Ezpeleta et al., 2016). However, it is important to note that previous studies have reported that RAD data can underestimate genetic diversity when polymorphism in restriction sites is high (Arnold et al., 2013). Diversity estimates using different molecular markers have exhibited differences in measures of variation, with SSR markers showing more variation than SNP markers (PCR-SSR > RAD-SSR > RAD-SNP). Nevertheless, short of full genome sequencing of population samples, RADseq is perhaps the optimal compromise to assess genome diversity (Cariou et al., 2016).

Plant genetic diversity is influenced by several factors, including reproductive patterns, life history, and evolutionary history of the organism (Ellegren & Galtier, 2016). Moreover, quaternary climatic variations and associated environmental changes have led to the disruption of plant habitats and have been associated with diversification of species (Comes & Kadereit, 1998). As the geographical range of plants has repeatedly narrowed and then expanded in response to these changes, disjunct populations of species have undergone divergence through natural selection (local adaptation) and genetic drift (Qiu et al., 2011). Through this process, many populations and their species have become extinct or greatly reduced, with only a few species or individuals surviving in refuges or relatively stable habitats before expanding their ranges (Tzedakis et al., 2002). The Ordos region is a well-known refuge in China and has retained many germplasm resources of plants that originated in Tethys Ocean, such as *R. trigyna*, *T. mongolica*, and *H. songaricum* (Meng et al., 2015). The genetic diversity of the plants retained in such a refugium might only represent a fraction of the total genetic diversity under the circumstance of dramatic climate fluctuations (Ge et al., 2003); however, through hybridization and adaptive introgression they might also be hotspots for the generation of biological novelty (Becker et al., 2013). The ability of relictual populations to expand their population size, disperse and successfully establish in novel habitats is crucial for longer term survival and resilience. *Reaumuria soongarica* is a tertiary relic shrub widely distributed in all the desert regions across Arid Central Asia (Y. Shi et al., 2013). This species exhibits an overall high level of genetic diversity, which is associated with the successful adaptation of this species, particularly for populations in the monsoonal climate zone (Yin et al., 2015). However, endemic monoecious species such as *R. trigyna* do not appear to have capacity for range expansion. Its seed is an oblong capsule, approximately 1 cm in length, and relatively heavy in mass. Due to the large size of seeds and gravel habitats, long-distance wind-borne seed dispersal is likely to be uncommon. Presumably, this limits genetic connectivity and admixture between disjunct populations. Sympatric *T. mongolica* tends to be distributed in sandy habitats. Its seeds are relatively lighter and smaller than those of *R. trigyna*. These features may facilitate seed dispersal and pollen flow between disjunct populations and/or individuals, resulting in opportunities for admixture, recovery, and renewal of genetic diversity. In a recent population genomic analysis using RAD-seq combined with 38,097 SNP markers, Cheng et al. (2020) reported that *T. mongolica* had a moderate level of genetic diversity ($H_O = 0.446$ and $H_E = 0.348$), very low genetic differentiation and high levels of gene flow between populations. A total of 12 nuclear microsatellites identified that the overall H_O and H_E of *T. mongolica* were as high as 0.840 and 0.868, respectively (Zhi et al., 2018). Compared with *T. mongolica*, it was suggested that *R. trigyna* has a higher risk of extinction and may require more protection to maintain genetic diversity using germplasm resources (Cheng et al., 2020).

4.2 | Weak genetic differentiation and strong gene flow among *Reaumuria trigyna* populations

If *R. trigyna* has contracted into Ordos refugia because of habitat loss with glacial and interglacial oscillations of global climate change, the current populations of the species might represent only a small part of a historically widely distributed species (X. Yang et al., 2014; Y. J. Zhang & Wang, 2008a). Consistent with this hypothesis, the mean F_{ST} between the eight studied *R. trigyna* populations was 0.043. According to Wright (1978), genetic differentiation among populations will be low and moderate when $F_{ST} < 0.05$ and between 0.05 and 0.15, respectively. In this study, the low F_{ST} indicated weak genetic differentiation existing among *R. trigyna* populations. Such low genetic differentiation has been reported for other narrowly distributed endangered species, such as *Aquilaria malaccensis*, *Thuja sutchuenensis*, and *T. mongolica* (Cheng et al., 2020; Qin et al., 2021; Singh et al., 2015). AMOVA demonstrated that 98.24% of the overall genetic variation in *R. trigyna* populations occurred within populations (Table S3), confirming weak genetic differentiation among *R. trigyna* populations.

Genetic differentiation of populations is influenced by gene flow (Gamba & Muchhala, 2020). When effective gene flow is high, for example, when $Nm > 4$, genetic drift will have less affect (Whitlock & McCauley, 1999). The average Nm between populations of *R. trigyna* was 6.055, indicating the frequent gene flow between these populations. However, in *T. mongolica*, the gene flow between populations was extremely high, ranging from 9.750 to 39.169 (average 12.25) (Cheng et al., 2020). The results reinforced the feeble seed and pollen flow estimates between *R. trigyna* populations compared with those of *T. mongolica*. Between WD and other populations, an average F_{ST} of 0.06 was estimated. The values indicate a moderate degree of genetic differentiation between WD and other populations (Benestan et al., 2015). The estimates for gene flow between WD and other populations do not mean that

gene flow existed between these populations (Wright, 1949). The values are likely explained by isolation of the WD population from other populations by geographical barriers, such as the Yellow River, Helan Mountain, and Zhaobi Mountain, and with relatively large geographical distances. These geographical barriers may have hindered genetic connection between WD and other populations, resulting in gradual differentiation of the WD population.

Gene flow is crucial for the stability of population genetic structure (Ellstrand, 2014). The differentiation pattern detected in this study likely represented a historical signal of genetic variation. The gene flow inferred by Migrate-n revealed that the north-to-south migration model was the most likely migration model for *R. trigyna* (Table 4), which is consistent with the direction of gene flow for *T. Mongolica* (Cheng et al., 2020). We speculate that in the context of interaction among the Asian–European, Indian, and Pacific plates and geological and climatic environmental changes caused by the quaternary glacial movement, the plants expanded their range by a short distance southward with the direction of prevailing winds (Yin et al., 2015). The complex topography of the Ordos region (high mountains and hills, dry valleys, and flow deserts) may mean that gene flow has been restricted to a narrow area with dispersal only into adjacent areas.

4.3 | Subtle genetic structure of *R. trigyna* populations

Genetic structure is regarded as a crucial factor important for understanding the short-term evolutionary processes of a population (Kevin et al., 2004). Although no apparent genetic differentiation was reported among previously studied *R. trigyna* populations, the present study provides higher resolution data for inferring genetic structure among the eight *R. trigyna* populations. This structure may result from various factors, for example, human activities, local adaptation, and other environmental factors (Quiñones-Pérez et al., 2014).

Comprehensive PCA, phylogenetic, and admixture analyses demonstrated that the eight *R. trigyna* populations could be divided into four subpopulations (Figure 4). It is possible that gene flow between the populations was historically relatively high. However, increasing habitat fragmentation caused by geographical barriers and human activities in recent decades greatly reduced gene flow between the populations and influenced genetic composition (Delnevo et al., 2021). Since the 1950s, Wuhai city and its surrounding areas have been vigorously developed; thus, the plants growing in those areas were seriously impacted by human activities, such as industrial production, coal mining, gas exploration, agriculture, grazing, and road construction. This has led to the reduction and fragmentation of plant populations resulting in loss of genetic diversity and formation of the genetic structure. Less adaptability to the environment has been reported in these plants (Elshibli & Korpelainen, 2021; Jacquemyn et al., 2012; Young et al., 1996). From the perspective of geographical distribution, the growth of Wuhai city has weakened the genetic connection between subpopulations, making them gradually genetically disconnected. Mantel test revealed that the geographical distance between the studied *R. trigyna* populations was significantly correlated with their genetic distances ($r = 0.674$, $p = 0.002$), indicating that the formation of the genetic structures was highly related to the isolation of the geographical factors (Bolnick & Otto, 2013).

In recent years, numerous lines of evidence have indicated that some other landscape components can influence gene flow and population connectivity independent of geographical distance. These components include bioclimatic variables (elevation, humidity, and precipitation; Bontrager & Angert, 2019; Gharehaghaji et al., 2017), discrete variables (habitat or substrate type; Hopley & Byrne, 2018; Roffler et al., 2016), abiotic factors (heat, drought, and salt; Quiñones-Pérez et al., 2014)), or biotic factors (vegetation density and diversity; Lobato-de Magalhaes et al., 2020). For *R. trigyna*, the geographical characteristics among the subpopulations vary in latitude, longitude, altitude, and climate conditions, leading to the heterogeneity of habitats; thus, they might contribute to the identified patterns of population differentiation (Bontrager & Angert, 2019; Duan et al., 2018; Gharehaghaji et al., 2017; Lobato-de Magalhaes et al., 2020; Roffler et al., 2016; Y. ZYang et al., 2019). For example, differences in soil physicochemical properties among habitats affect the distribution patterns of plant communities and pollinators, which in turn result in differences in pollen flow between populations and alteration in the genetic composition of populations (Gaudeul et al., 2000). Soil salinity measurements of various *R. trigyna* populations revealed that this plant can grow and complete its life cycle in nonsaline, mildly saline, severely saline, and saline soils, where the soil composition is dominated by Na_2SO_4 and $CaSO_4$ (Xue & Wang, 2008). Therefore, soil salinity conditions may be one of the abiotic inducing factors related to the genetic differentiation and local adaptation of the species. However, further studies are needed to investigate whether the landscape components explain the genetic divergence among *R. trigyna* populations. In that case, allele frequencies of SNPs and other types of allelic data would be expected to exhibit a significant correlation with ecological heterogeneity that is linked to different selection pressures (I. J. Wang & Bradburd, 2014).

4.4 | Signatures of environmental adaptation or habitat-related adaptation in *R. trigyna*

SNPs present in genes could have a considerable influence on protein function. RADseq techniques can locate variation

in the coding region of genes and have been widely used in reverse genetic studies of local or population-level adaptations (B. J. Liu et al., 2020). Under considerable drought and windy and varied soil salinity conditions, *R. trigyna* may have undergone adaptive evolution. In the present study, 3513 outlier SNPs were identified, and these could potentially contribute to adaptive evolution of *R. trigyna* through changes in the expression of specific genes.

Previous studies have reported that *R. trigyna* has evolved with various physioecological adaptation strategies in heterogeneity habitats. S. L. Shi et al. (2011) reported that the levels of endogenous hormones (indole-3-acetic acid, abscisic acid [ABA], gibberellin, and zeatin riboside) varied in different habitats of *R. trigyna* populations, and that individuals growing in salinized soil had the highest level of endogenous hormones, followed by those growing in nonsalinized soil and heavily salinized soil. Zhou et al. (2009) identified that the ability of *R. trigyna* to resist to water deficiency was different under heterogeneous saline conditions. These studies indicated that *R. trigyna* has evolved with distinct adaptive mechanisms in response to differences in soil salinity, and the regulation of endogenous hormones and drought-resistance physiological processes under various saline environments may play crucial roles in habitat-related adaptation of the species. In this study, several outlier SNPs containing transcripts were related to plant hormone regulation and signal transduction and may function as "the turning knobs" in regulating hormones that are relevant to biological processes.

The APETALA2/ethylene-responsive element binding protein (AP2/EREBP) transcription factors play essential roles in stress responses (e.g., heat, drought, high salinity, and cold) and in various other plant processes (e.g., pathogen infection) by directly responding to stresses or regulating the expression of downstream target genes, and they are implicated in various signal transduction pathways related to hormones including ethylene, ABA, cytokinin, and jasmonate (C. Liu & Zhang, 2017). In *Arabidopsis thaliana*, AP2/ERFs can inhibit the GA biosynthesis enzyme GA20oxs and reduce GA content and its signal transduction, thus inducing growth retardation. RAP2.6L and RAP2.6 were induced by ABA to upregulate genes containing drought-responsive elements and ABA-responsive elements, further enhancing the tolerance of *A. thaliana* to adapt to drought stress (Xie et al., 2019). Overexpression of the *DREB5* in *Syntrichia caninervis* can enhance salt tolerance in transgenic *A. thaliana* by enhancing JA biosynthesis (J. Y. Liu et al., 2022). In rice, *OsERF71* positively regulates ABA signaling to alter root architecture and confer drought tolerance of the plant (Lee et al., 2017; J. J. Li et al., 2018). In this study, we identified that unigene30001 exhibited high homology (*E*-value = $5E^{-73}$) with the members of AP2/EREBP family genes, and its expression profile (with a FPKM value of 99.51) in the *R. trigyna* transcriptome suggested the versatile roles of the gene in stress tolerance or environmental adaptation in the species. However, further studies are needed to assess whether there are mechanisms modulating the expression of the hormone pathways in the *R. trigyna*.

E3 ubiquitin ligases are the key regulators of hormone signaling in plants, including hormone perception and regulation of hormone biosynthesis. They participate in regulating post-translational modifications of various regulatory polypeptides and enzymes that play roles in multiple plant developmental stages (e.g., seed dormancy and germination, root growth, flowering time control, self-incompatibility, and chloroplast development) and several abiotic stress responses (e.g., drought and high salinity) (Shu & Yang, 2017). *Oryza sativa* E3-ubiquitin ligase (high expression of osmotically responsive gene1) directly regulates the stability of two ERF transcription factors (EREPB1 and EREBP2), which thereby regulates the JA-mediated root curling response (Lourenco et al., 2015). Dwarf and short grain 1 encodes U-box E3, which is collectively regulated by BR, ET, auxin, and SA and positively regulates cell division and elongation in rice (N. Wang et al., 2017). The U-box E3 ubiquitin ligase gene *PalPUB79* is significantly induced by drought, salinity, and ABA treatments. Overexpression of the gene can enhance the drought tolerance in transgenic *Populus* by positively regulating ABA signaling pathway (Tong et al., 2021). In this study, we identified the coding gene of E3 ubiquitin-protein ligase MARCH6 (unigene717), which may correlate with the phytohormone induced or regulated adaptive strategies in the plant. Besides, Beyer et al. have reported that E3 ubiquitin-protein ligase MARCH6 can be a viable target for wheat improvement. When upregulating this gene in a cultivar with high branching, the plant could increase root nitrate uptake at tillering, thus increasing the overall nitrogen content (Beyer et al., 2019). Azni et al. (2017) identified that the gene was a potential dwarf gene regulating the growth and development of oil palm. The high degree of similarity and inferred homology of unigene717 with E3 ubiquitin-protein ligase MARCH6 (*E*-value = 0) suggested that selection for SNPs in this gene may also play important roles in the growth and survival of *R. trigyna* under the general barren desert environments.

Plants growing under natural conditions are prone to damage due to biotic (e.g., insects and pathogens) and abiotic (e.g., drought and high salinity, high temperature, low temperature, and ultraviolet-B) stresses. Environmental stresses trigger the synthesis of certain SMs in plants by inducing certain genes. Subsequently, these SMs enable the plants to improve their growth and survival under unfavorable conditions (Ashraf et al., 2018). Our previous transcriptome comparison study performed between non- and salt-stressed *R. trigyna* reported that differentially expressed genes were significantly enriched in pathways related to biosynthesis of SMs in which the pathways of phenylpropanoid biosynthesis and flavonoid biosynthesis were very active in salt-stressed

plants (Dang et al., 2013). To identify the functional importance of these metabolic pathways in stress adaptation in *R. trigyna*, a series of experiments were performed. H. R. Zhang et al. (2014) identified that the expression levels of two *F3H* genes, *RtF3H1* and *RtF3H2*, were increased under not only salt stress but also drought, cold, and abscisic acid treatment to maintain the growth of *R. trigyna* under such abiotic stresses. Recombinant *Escherichia coli* overexpressing each of these genes exhibited better growth than a control line under various stress treatments. Under NaCl and ultraviolet-B (UV-B) radiation treatments, the expression of genes (e.g., *RtC4H*, *RtCHS*, *RtF3H3*, *RtFLS1*, *RtFLS2*, and *RtF3'5'H*) related to flavonol biosynthesis and flavonol content were generally increased in *R. trigyna* (H. Zhang et al., 2017). Leucoanthocyanidin dioxygenase genes (*RtLDOX*/*RtLDOX2*) were rapidly upregulated in *R. trigyna* under salt, drought, and UV-B stresses, and overexpression of these two genes in *Arabidopsis* could enhance the primary root length, biomass accumulation, and chlorophyll content in transgenic plants under the same stress conditions. In addition, the transgenic plants accumulated more flavonoids, anthocyanins, and aromatic phenols, and these SMs in turn increased scavenging of reactive oxygen species and activated other stress responses to improve tolerance to abiotic stresses (N. N. Li et al., 2021; H. R. Zhang et al., 2016). Interestingly, two outlier SNPs identified in this study were located on coding genes of flavonol synthase (*FLS*; unigene1722) and 4-coumarate: coenzyme A ligase (*4CL*; unigene32263). *4CL* contributes to channelizing flux of various phenylpropanoid biosynthetic pathways; the product of the gene catalyzes the reaction converting hydroxy or methoxy cinnamic acid derivatives to corresponding thioesters, which are further used for the biosynthesis of phenylpropanoids (Lavhale et al., 2018). The expression of *4CL* is optimized at developmental stages and in response to environmental triggers such as biotic and abiotic stresses. As identified in *Populus*, *4CL2*, *4CL11*, and *4CL12* were upregulated significantly in response to salt stress, and the accumulated lignin in the xylem might play a positive role for the plant to adapt to the extremely arid environments (C. H. Zhang et al., 2015). In *O. sativa*, upon wounding *Os4CL3*, *Os4CL4*, *Os4CL5*, and other genes of phenylpropanoid biosynthesis pathway were significantly upregulated (Lavhale et al., 2018). After reviewing these findings, we believe that it is not a coincidence that vital roles have been identified for biosynthesis of SMs involved in stresses tolerance and environmental adaptation of *R. trigyna*. Our results suggest that the metabolic pathways in *R. trigyna* are under strong habitat selection and are important for adaptative diversification of this species.

AUTHOR CONTRIBUTIONS

Zhenhua Dang: Formal analysis; Funding acquisition; Investigation; Project administration; Supervision; Visualization; Writing - original draft; Writing - review & editing. **Jiabin Li**:Formal analysis; Investigation; Validation; Visualization; Writing - original draft. **Yanan Liu**: Investigation; Validation; Writing - original draft. **Miaomiao Song**: Investigation; Validation; Writing - original draft. **Peter J. Lockhart**: Writing - original draft; Writing - review & editing. **Yunyun Tian**: Formal analysis; Investigation; Validation; Writing - original draft. **Miaomiao Niu**: Investigation; Validation; **Qinglang Wang**: Investigation; Validation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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