



# Genetic Diversity Study on Geographical Populations of the Multipurpose Species *Elsholtzia stauntonii* Using Transferable Microsatellite Markers

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*Elsholtzia stauntonii* Benth. (Lamiaceae) is an economically important ornamental, medicinal and aromatic plant species. To meet the increasing market demand for *E. stauntonii*, it is necessary to assess genetic diversity within the species to accelerate the process of genetic improvement. Analysis of the transferability of simple sequence repeat (SSR) markers from related species or genera is a fast and economical method to evaluate diversity, and can ensure the availability of molecular markers in crops with limited genomic resources. In this study, the cross-genera transferability of 497 SSR markers selected from other members of the Lamiaceae (*Salvia* L., *Perilla* L., *Mentha* L., *Hyptis* Jacq., *Leonurus* L., *Pogostemon* Desf., *Rosmarinus* L., and *Scutella* L.) to *E. stauntonii* was 9.05% (45 primers). Among the 45 transferable markers, 10 markers revealed relatively high polymorphism in *E. stauntonii*. The genetic variation among 825 individuals from 18 natural populations of *E. stauntonii* in Hebei Province of China was analyzed using the 10 polymorphic SSR markers. On the basis of the SSR data, the average number of alleles ( $N_A$ ), expected heterozygosity ( $H_E$ ), and Shannon's information index ( $I$ ) of the 10 primers pairs were 7.000, 0.478, and 0.688, respectively. Lower gene flow ( $N_m = 1.252$ ) and high genetic differentiation ( $F_{st} = 0.181$ ) were detected in the populations. Analysis of molecular variance (AMOVA) revealed that most of the variation (81.47%) was within the populations. Integrating the results of STRUCTURE, UPGMA (Unweighted Pair Group Method with Arithmetic Mean) clustering, and principal coordinate analysis, the 825 samples were grouped into two clusters associated with geographical provenance (southwestern and northeastern regions), which was consistent with the results of a Mantel test ( $r = 0.56$ ,  $p < 0.001$ ).

Overall, SSR markers developed in related genera were effective to study the genetic structure and genetic diversity in geographical populations of *E. stauntonii*. The results provide a theoretical basis for conservation of genetic resources, genetic improvement, and construction of a core collection for *E. stauntonii*.

**Keywords:** cross-transferability, *Elsholtzia stauntonii* Benth., simple sequence repeat (SSR) markers, genetic diversity, population structure

## INTRODUCTION

Plants provide the fundamental support system for life on Earth and plant diversity is a key property of ecosystems (Moreira et al., 2016). Lamiaceae, with more than 7,000 species around the world, is a large family of flowering plants and an important part of plant diversity (Harley et al., 2004). Some species of the family have been commercially exploited worldwide (e.g., *Lavandula angustifolia*) (Pistelli et al., 2017), while many others are known locally for their ornamental, biological, and medicinal properties (e.g., *Hedeoma patens*) (Leyva-Lopez et al., 2016). *Elsholtzia stauntonii* Benth., a perennial deciduous shrub of the Lamiaceae family, is widely distributed in China, especially in Hebei Province (Editorial committee of the Flora of China, and Chinese Academy of Sciences, 1977). *Elsholtzia stauntonii* is aromatic and its essential oils show insecticidal activity against certain pests of stored products (Lv et al., 2012). Consequently, *E. stauntonii* is used in novel biological insecticides (Liang et al., 2021). With its dense flowers, late flowering and bright colors, *E. stauntonii* is a popular bedding plant cultivated in public gardens and a wild nectar plant (Tian et al., 2007). Its branches and leaves are also used as a traditional Chinese medicinal material for treating common colds, toothache, and digestive system diseases (Zheng et al., 1999; Pekhova et al., 2020). Furthermore, the species is of ecological importance owing to its strong tolerance of drought and barren environments (Si and Peng, 2017). Previous studies on *E. stauntonii* have mainly focused on its morphological traits (Zhou et al., 2016), cultivation and propagation (Zhou et al., 2014), and extraction and identification of volatile oils (Guo et al., 2012; Xing et al., 2019). Little information is available on the genome and genetic diversity of this species. Studies of genetic diversity have been performed on many Lamiaceae species to clarify the genetic relationships among species (Bahadırli and Ayanoglu, 2020) and to identify molecular markers associated with agronomic traits for marker-assisted breeding (Lim et al., 2021). Therefore, with recognition of the value of this important but neglected species, it is crucial to evaluate the genetic variation in *E. stauntonii*.

Understanding the genetic diversity and structure of germplasm resources is a prerequisite for formulating effective breeding and conservation strategies (Gepts, 2006; Engelhardt et al., 2014; Vanavermaete et al., 2021). In recent years, with advances in molecular biological technologies, molecular marker-based analysis of genetic variation has avoided the limitations of morphological and biochemical indicators that are readily affected by environmental factors, and are more accurate for evaluation of population genetic parameters (Agarwal et al., 2008; Nadeem et al., 2018). Among the various types of molecular markers, microsatellites (or simple sequence repeats;

SSRs) have become the preferred choice because of their high degree of reproducibility, ability to identify high levels of genetic polymorphism, codominant inheritance, and abundance in plant genomes (Powell et al., 1996; Grover and Sharma, 2016; Jose et al., 2018). Currently, SSR markers have been widely used in assessments of population genetic diversity (Gadissa et al., 2018), cultivar identification (Pinto et al., 2018; Sulu et al., 2020), and association mapping (Yang et al., 2020) as effective tools to describe genetic variation in plants. Using available genomic or expressed sequence tag databases (Vieira et al., 2016), a large number of SSR markers have been developed in Lamiaceae species, such as *Scutellaria baicalensis* (Zhang et al., 2014), *Perilla frutescens* (Ha et al., 2021), and *Salvia splendens* (Jiao et al., 2020). However, owing to the lack of genetic information, to date no SSR markers are available for evaluation of the genetic diversity within *E. stauntonii*, which limits its breeding and commercial development.

An alternative approach to overcome the time-consuming and costly development of SSR primers for target species is to exploit the transferability of SSR primers between related species or genera (Peakall et al., 1998; Pan et al., 2018). The transfer of SSR markers has been successful in many cases, as documented among Lamiaceae species (Karaca et al., 2013), from *Gossypium hirsutum* and *Corchorus olitorius* to Malvaceae species (Satya et al., 2016), among *Allium* species and among members of the Alliaceae (Barboza et al., 2018), and from *Ricinus communis* to Euphorbiaceae species (Dharajiya et al., 2020).

In this study, cross-genera amplification of SSR markers in Lamiaceae species was used to enrich the genetic database of *E. stauntonii*. On this basis, the genetic diversity and population structure of 825 *E. stauntonii* individuals sampled from 18 regions in Hebei Province, China, which is an important center in the distribution range of the species, were successfully evaluated using 10 highly polymorphic cross-transferable SSR markers. The results provide insight into the genetic resources of *E. stauntonii*, and represent a foundation for the effective management and genetic improvement of the species.

## MATERIALS AND METHODS

### Plant Materials

A field survey of extant *E. stauntonii* populations in Hebei Province, China was conducted from July to September 2020. Samples of 825 mature and disease-free individuals were randomly collected from 18 populations in the natural distribution area; the sample size per population ranged from 43 to 50 individuals (**Table 1**). To ensure sufficient geographical representation of the samples, the distance between sampled

**TABLE 1** | Location and sampling site characteristics of 18 *Elytholzia stauntonii* geographical populations (825 individuals).

Population name	Sample size	Location	Latitude (N)/Longitude (E)	Altitude (m)
HS	50	Shexian, Handan	N:36°36'/E:113°52'	880
XW	44	Wangnaocun, Xingtai	N:36°54'/E:114°4'	540
XM	47	Malingguan, Xingtai	N:37°20'/E:113°57'	900
SZ	46	Zanhuangxian, Shijiazhuang	N:37°39'/E:114°23'	969
SP	48	Pingshanxian, Shijiazhuang	N:38°15'/E:114°11'	1,200
BF	46	Fupingxian, Baoding	N:38°53'/E:114°1'	1,200
BZ	46	Zoumayizhen, Baoding	N:39°8'/E:114°36'	915
ZQ	46	Qiaomaichuancun, Zhangjiakou	N:40°2'/E:115°16'	1,200
ZF	47	Feihuyu, Zhangjiakou	N:39°43'/E:114°38'	1,200
BL	43	Laishuixian, Baoding	N:39°23'/E:115°46'	984
BY	47	Yixian, Baoding	N:39°14'/E:114°58'	975
ZC	46	Chichengxian, Zhangjiakou	N:40°58'/E:115°57'	1,000
CF	43	Fengningxian, Chengde	N:41°46'/E:116°20'	1,100
CY	47	Yunwushan, Chengde	N:41°9'/E:116°45'	800
CL	43	Longhuaxian, Chengde	N:41°22'/E:117°48'	800
CX	46	Xinglongxian, Chengde	N:40°25'/E:117°30'	700
CC	46	Chengdexian, Chengde	N:40°46'/E:118°10'	800
QD	44	Dushan, Qinhuangdao	N:40°29'/E:118°48'	700

individuals was more than 50 m. The GPS coordinates were recorded for each population. The altitude of all sampled populations ranged from 540 to 1,200 m, with an average altitude of 937 m. Fresh leaves were desiccated in silica gel and stored at  $-20^{\circ}\text{C}$  until use.

## DNA Extraction and Genotyping With Simple Sequence Repeats Markers

Total genomic DNA of all samples was extracted using a Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China). The DNA quality was evaluated by 1.0% agarose gel electrophoresis. DNA quantification was conducted with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States) and the DNA concentration was adjusted to 20 ng/ $\mu\text{L}$ .

In total, 497 SSR markers from eight genera of Lamiaceae were used to perform cross-amplification tests on 16 randomly selected *E. stauntonii* samples from different populations (Table 2). Primers were synthesized by RuiBiotech (Beijing, China). To allow fluorescent labeling, the universal M13 sequence was added to the 5' end of all forward primers. The PCR mixture was prepared in accordance with the method of Zhang et al. (2015) with modifications. Briefly, the PCR mixture (20  $\mu\text{l}$ ) contained 10  $\mu\text{l}$  of 2  $\times$  Taq PCR Mix, 2  $\mu\text{l}$  (40 ng) genomic DNA, 4  $\mu\text{l}$  (4 pmol) fluorescent-dye-labeled M13 primer, and 4  $\mu\text{l}$  (4 pmol) mixed complementary forward and reverse primers. The temperature profile used was as follows: an initial step of 5 min at  $94^{\circ}\text{C}$ , followed by 35 cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $54^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$ , then eight cycles of 30 s at  $94^{\circ}\text{C}$ , 30 s at  $53^{\circ}\text{C}$ , and 30 s at  $72^{\circ}\text{C}$ , and a final step of 10 min at  $72^{\circ}\text{C}$ . Capillary electrophoresis of the PCR products was performed using an ABI 3730xl DNA Analyzer (Tiangen Biotech, Beijing, China). GeneMarker version 2.2.0 software (Holland and Parson,

**TABLE 2** | SSR markers from eight genera in the Lamiaceae used to evaluate cross-genera transferability to *Elytholzia stauntonii*.

Genera used for cross amplification	Number of SSR markers tested	References
<i>Salvia</i> L.	297	Radosavljević et al., 2011, 2012; Jiao et al., 2020; Krak et al., 2020
<i>Perilla</i> L.	128	Kwon et al., 2005; Sa et al., 2015, 2018, 2019; Ma et al., 2017; Wang et al., 2017; Park et al., 2019; Ha et al., 2021
<i>Mentha</i> L.	25	Vining et al., 2019
<i>Hyptis</i> Jacq.	15	Blank et al., 2014
<i>Rosmarinus</i> L.	12	Segarra-Moragues and Gleiser, 2009
<i>Scutellaria</i> L.	10	Zhang et al., 2014
<i>Pogostemon</i> Desf.	6	Sandes et al., 2013
<i>Leonurus</i> L.	4	Ren, 2012

2011) was used to analyze the fragment size of the products and determine whether the primers detected polymorphisms.

## Data Analysis

The SSR markers that successfully amplified single and specific bands within the expected product size range were considered to be transferable. The percentage transferability for each donor genus was calculated as (number of SSRs transferred/total number of SSRs screened)  $\times$  100. Similarly, the percentage transferability of SSR markers classified by repeat motifs (di-/tri-/tetra-/penta-/hexa-/complex-nucleotide) was estimated. Population diversity and population structure of *E. stauntonii* were assessed based on the transferred microsatellite loci.

For each SSR locus, important indices of genetic diversity were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012) and Microsatellite-Toolkit (Park, 2001) software. The indices comprised number of alleles ( $N_A$ ), number of effective alleles ( $N_E$ ), Shannon's information index ( $I$ ), observed heterozygosity ( $H_O$ ), expected heterozygosity ( $H_E$ ), inbreeding coefficient ( $F_{IS}$ ), gene flow ( $N_m$ ), and genetic differentiation index ( $F_{ST}$ ). The polymorphic information content (PIC) value and Hardy-Weinberg equilibrium (HWE) for the loci were calculated using PowerMarker version 3.25 (Liu and Muse, 2005).

Analysis of molecular variance (AMOVA) was conducted using Arlequin version 3.5 (Excoffier and Lischer, 2010) to estimate the variance components of genetic variation among and within populations. Bayesian analysis was performed to evaluate the population genetic structure and detect the most likely number of population genetic clusters of *E. stauntonii* using STRUCTURE version 2.3.4 (Pritchard et al., 2000). For each simulated value of  $K$  (range from 1 to 18), 15 independent runs were performed with a burn-in period of 200,000 iterations followed by 1,000,000 Markov chain Monte Carlo repetitions. The  $\Delta K$  method (Evanno et al., 2005) was implemented in the Structure Harvester (Earl and VonHoldt, 2012) program based on the STRUCTURE results to determine the optimum  $K$  value. The percentage membership of each individual in every cluster ( $Q$  value) was determined; an individual with a  $Q$  value higher than 0.80 was considered to have a single genetic component (a pure individual). Nei's genetic distance (DA) (Nei et al., 1983) among the 825 individuals and 18 populations was calculated using PowerMarker version 3.25 (Liu and Muse, 2005). To explore the genetic relationships among all samples, the DA matrix was used to construct a dendrogram by hierarchical clustering with the unweighted pair group method with arithmetic mean (UPGMA) using PowerMarker version 3.25 (Liu and Muse, 2005). The dendrograms were visualized, manipulated, and annotated with

the Interactive Tree of Life online tool (Letunic and Bork, 2007). Based on the standardized covariance of genetic distance, a principal coordinate analysis (PCoA) was conducted with GenAlEx version 6.5 (Peakall and Smouse, 2012). A clustering heatmap was prepared with Microsoft Excel based on the pairwise Nei's genetic distance between populations and the  $F_{ST}$  values.

The geographic distance between the sampling locations was calculated with Geographic Distance Matrix Generator version 1.2.3 software<sup>1</sup>. The Mantel test was performed to analyze the correlation between geographic and genetic distance of different populations using the "ggpubr"<sup>2</sup> and "ggplot2" (Ginestet, 2011) packages in R version 4.1.1 (Ihaka and Gentleman, 1996). The map was created using the "sf" (Pebesma, 2018), "ggplot2" (Ginestet, 2011), and "ggspatial"<sup>3</sup> packages in R version 4.1.1.

## RESULTS

### Transferability of Simple Sequence Repeats Markers

Of the 497 SSRs mined, 45 primers (9.05%) successfully amplified genomic DNA of *E. stauntonii* and produced PCR products of the expected size (Supplementary Table 1). Transferability of the SSR markers varied among eight genera of Lamiaceae: 26.67% of the SSR markers of *Hyptis* Jacq., 25.00% of *Leonurus* L., 16.67% of *Pogostemon* Desf., 13.28% of *Perilla* L., 8.33% of *Rosmarinus* L., 6.73% of *Salvia* L., and 4.00% of *Mentha* L. were transferable to *E. stauntonii*, whereas no SSR marker was transferable from *Scutellaria* L. (Figure 1). The transferability of SSR markers based on simple di-/tri-nucleotide repeat motifs (7.89–9.65%) was

<sup>1</sup>[http://biodiversityinformatics.amnh.org/open\\_source/gdmg/index.php](http://biodiversityinformatics.amnh.org/open_source/gdmg/index.php)

<sup>2</sup><https://cran.r-project.org/package=ggpubr>

<sup>3</sup><https://cran.r-project.org/package=ggspatial>

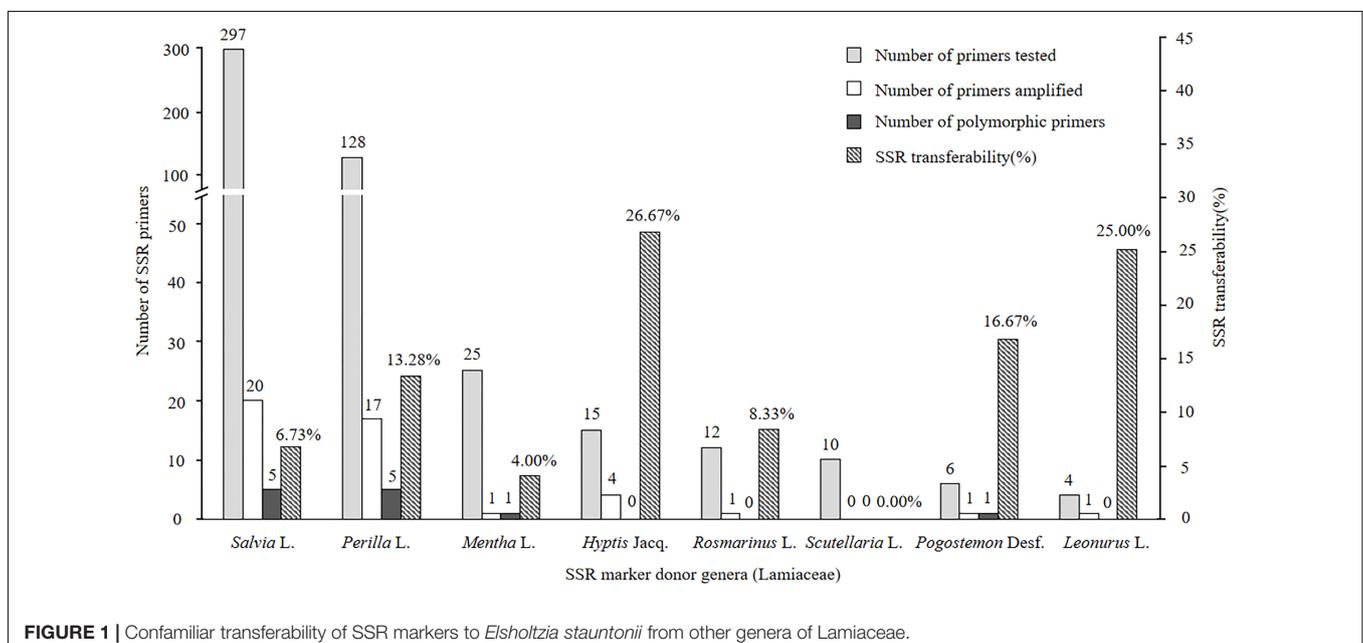
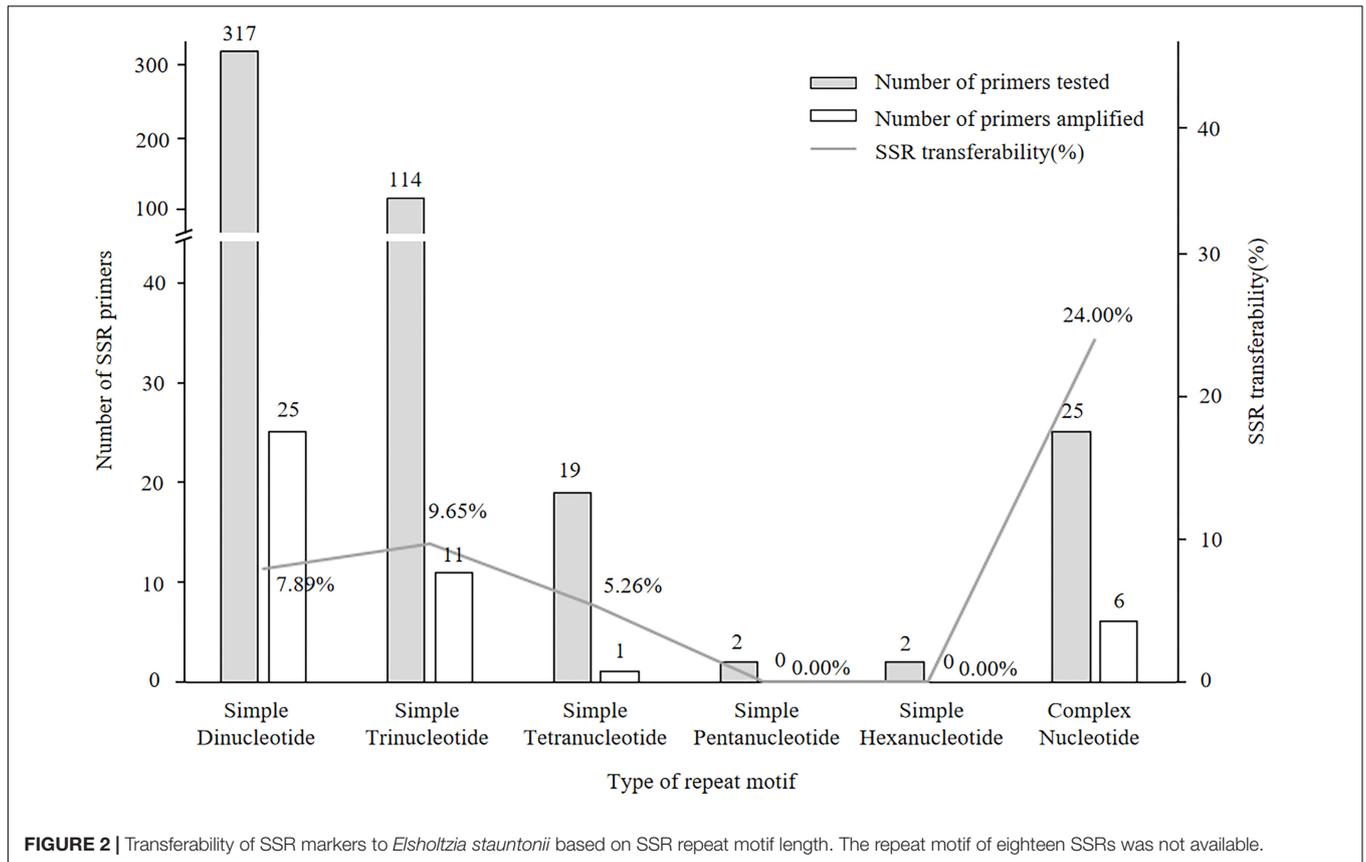


FIGURE 1 | Confamilial transferability of SSR markers to *Elythia stauntonii* from other genera of Lamiaceae.

**TABLE 3 |** Characterization of 10 polymorphic SSR primer pairs.

SSR primer	Primer sequence (5' to 3')	Repeat motif	Allele size (bp)	$N_A$	$H_O$	$H_E$	PIC	$F_{IS}$	HWE
KNUPF15 <sup>a</sup>	F:CCACACGTAAACCTCATAAACCC R:TTATCTCTAAAGAAATCGGGCA	(CT) <sub>16</sub>	168–186	10	0.611	0.750	0.708	0.185	***
KNUPF67 <sup>b</sup>	F:ATTGATTCTCTATCAACCTGGC R:CTCATCATCGGATCAACCTAGT	(GCT) <sub>7</sub>	189–201	4	0.194	0.264	0.230	0.265	***
ssps251 <sup>c</sup>	F:GACGCTCAAATGGTGAATCC R:ACGCTGATCTGGAAGATGG	(TC) <sub>11</sub>	231–277	24	0.469	0.879	0.868	0.466	***
ssps321 <sup>c</sup>	F:ACGTGAACACACACCACCAT R:ACAGCATCAGCAACAGCAAC	(GCT) <sub>10</sub>	220–235	8	0.336	0.425	0.390	0.209	***
ssps344 <sup>c</sup>	F:CCTTCACCTGGGATGGAGTA R:GCCCTTTCAACCAAAACAAA	(CT) <sub>10</sub>	177–235	2	0.241	0.214	0.191	-0.126	***
ssps512 <sup>c</sup>	F:GGCTCCTCGTTTTATGGTGA R:GCATTAGTTGATGCCGAGGT	(GA) <sub>11</sub>	163–195	3	0.236	0.499	0.376	0.527	***
ssps711 <sup>c</sup>	F:CCGACGTGAACATACACCAC R:TTATGCAGCAGCAGTTTTTG	(GCT) <sub>10</sub>	335–350	6	0.327	0.414	0.379	0.210	***
Pca6 <sup>d</sup>	F:ACAAAGGGTTGACGATTG R:GTGATGAAACTGTCTCTCTG	(TG) <sub>4</sub> (TC) (TG) <sub>5</sub> (AG) <sub>4</sub> (TGTT) <sub>3</sub>	232–248	9	0.391	0.584	0.545	0.330	***
GBPF203 <sup>e</sup>	F:GTTTTGTTGCAGCTCGATTT R:TGGGTTTGAAAGTATTGATG	(GA) <sub>5</sub> (TAA) (AG) <sub>26</sub>	132–162	2	0.439	0.352	0.290	-0.247	***
C3787 <sup>f</sup>	F:GAGAGTACGGCGAGTAATTG R:TATCAACGTGAAGGAGACTTG	(ATTT) <sub>4</sub>	145–157	2	0.356	0.396	0.317	0.101	**
Mean				7.000	0.360	0.478	0.429	0.192	-

$N_A$ , number of alleles;  $H_O$ , observed heterozygosity;  $H_E$ , expected heterozygosity; PIC, polymorphic information content;  $F_{IS}$ , inbreeding coefficient; HWE, Hardy–Weinberg equilibrium; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . <sup>a,b,c,d,e,f</sup>, Previously published microsatellite markers (<sup>a</sup>: Sa et al., 2018; <sup>b</sup>: Sa et al., 2019; <sup>c</sup>: Jiao et al., 2020; <sup>d</sup>: Sandes et al., 2013; <sup>e</sup>: Sa et al., 2015; <sup>f</sup>: Vining et al., 2019).

higher than that based on tetra-/penta-/hexa-nucleotide repeat motifs (Figure 2). The SSR markers based on complex (simple imperfect, compound perfect/imperfect) nucleotide repeats also showed high transferability (24.00%).

## Polymorphism Analysis Based on Transferable Simple Sequence Repeats Markers

Among the 45 transferable SSR markers, 35 markers were monomorphic (33 primers) or showed very low levels of polymorphism (two primers) based on capillary electrophoresis results (Supplementary Table 1). Ten (2.01%) primer pairs showed higher polymorphism and were used for analysis of genetic diversity in *E. stauntonii* (Table 3).

In total, 70 alleles were amplified by the 10 polymorphic SSR markers among 825 *E. stauntonii* individuals, with a mean of 7.000 observed alleles per locus, and ranging in length from 132 to 350 bp (Table 3). The number of alleles per marker varied from 2 (ssps344, GBPF203, and C3787) to 24 (ssps251). Among the 10 markers, the observed heterozygosity ( $H_O$ ) of eight markers was lower than the expected heterozygosity ( $H_E$ ), and the average inbreeding coefficient ( $F_{is}$ ) was positive. The PIC values ranged from 0.191 to 0.868, with a mean of 0.429. Eight SSR markers showed moderate or high polymorphism levels (PIC > 0.25) and two markers (ssps344 and KNUPF67) showed low polymorphism levels (PIC < 0.25) among the tested *E. stauntonii* populations. All SSR genotyping data for the 10 loci showed strongly significant deviation from the HWE ( $p < 0.01$ ) (Table 3).

## Population Genetic Diversity and Differentiation

In general, the difference in genetic diversity among the 18 geographical populations of *E. stauntonii* was observed (Table 4). The genetic diversity indices  $N_A$  and  $N_E$  of individual populations ranged from 2.400 (XW) to 3.900 (XM), and from 1.406 (ZQ) to 2.532 (CX), with means of 3.267 and 1.971, respectively. The average  $H_O$  and  $H_E$  were 0.359 and 0.388, ranging from 0.253 (XW) to 0.478 (CX), and from 0.260 (ZQ) to 0.506 (CX), respectively. The  $I$  value ranged from 0.450 (ZQ) to 0.887 (CX), with an average of 0.688. The highest level of genetic diversity was detected in CX, whereas XW and ZQ showed the lowest genetic diversity (Table 4).

The average  $F_{st}$ , a measure of the degree of genetic differentiation among populations, was 0.181 and the average  $N_m$  was 1.252 (Table 4). These results were consistent with those of AMOVA, which revealed that 18.53% of the genetic variation occurred among the populations and 81.47% was present within the populations (Table 5).

The pairwise  $F_{st}$  ranged from 0.020 (between populations CY and ZC) to 0.199 (between populations CX and ZQ) (Figure 3). The pairwise Nei's genetic distance ranged from 0.034 to 0.359, with the highest value (0.359) between the CX and ZQ populations and the second highest (0.328) between the CX and XW populations (Figure 3). In general, populations that were geographically closer had lower genetic distances

**TABLE 4** | Genetic diversity for 10 polymorphic SSR markers in 18 natural populations of *Elythia stauntonii* (total of 825 individuals).

Population	$N_A$	$N_E$	$I$	$H_O$	$H_E$	$F_{st}$	$N_m$
HS	2.600	1.674	0.520	0.263	0.311		
XW	2.400	1.545	0.477	0.253	0.284		
XM	3.900	2.331	0.699	0.351	0.361		
SZ	3.700	2.030	0.675	0.291	0.358		
SP	3.600	1.969	0.704	0.419	0.393		
BF	3.600	2.066	0.691	0.324	0.353		
BZ	3.600	2.205	0.745	0.377	0.405		
ZQ	2.600	1.406	0.450	0.273	0.260		
ZF	3.700	1.943	0.757	0.417	0.415		
BL	3.100	1.888	0.695	0.416	0.406		
BY	3.300	2.275	0.790	0.438	0.444		
ZC	3.700	2.005	0.775	0.372	0.447		
CF	3.200	1.726	0.624	0.319	0.352		
CY	3.200	2.227	0.848	0.409	0.497		
CL	3.500	1.816	0.608	0.291	0.317		
CX	3.500	2.532	0.887	0.478	0.506		
CC	2.800	2.065	0.782	0.430	0.478		
QD	2.800	1.771	0.664	0.348	0.401		
Mean	3.267	1.971	0.688	0.359	0.388	0.181	1.252

$N_E$ , number of effective alleles;  $I$ , Shannon's Information Index;  $F_{st}$ , genetic differentiation index;  $N_m$ , gene flow.

(SZ–XM, 52.43 km, 0.034), whereas higher genetic distances were observed for populations that were farther apart (CX–XW, 490.81 km, 0.328). Mantel test results revealed a strongly significant correlation between genetic and geographic distance among the 18 natural populations ( $r = 0.56$ ,  $p < 0.001$ ) (Figure 4).

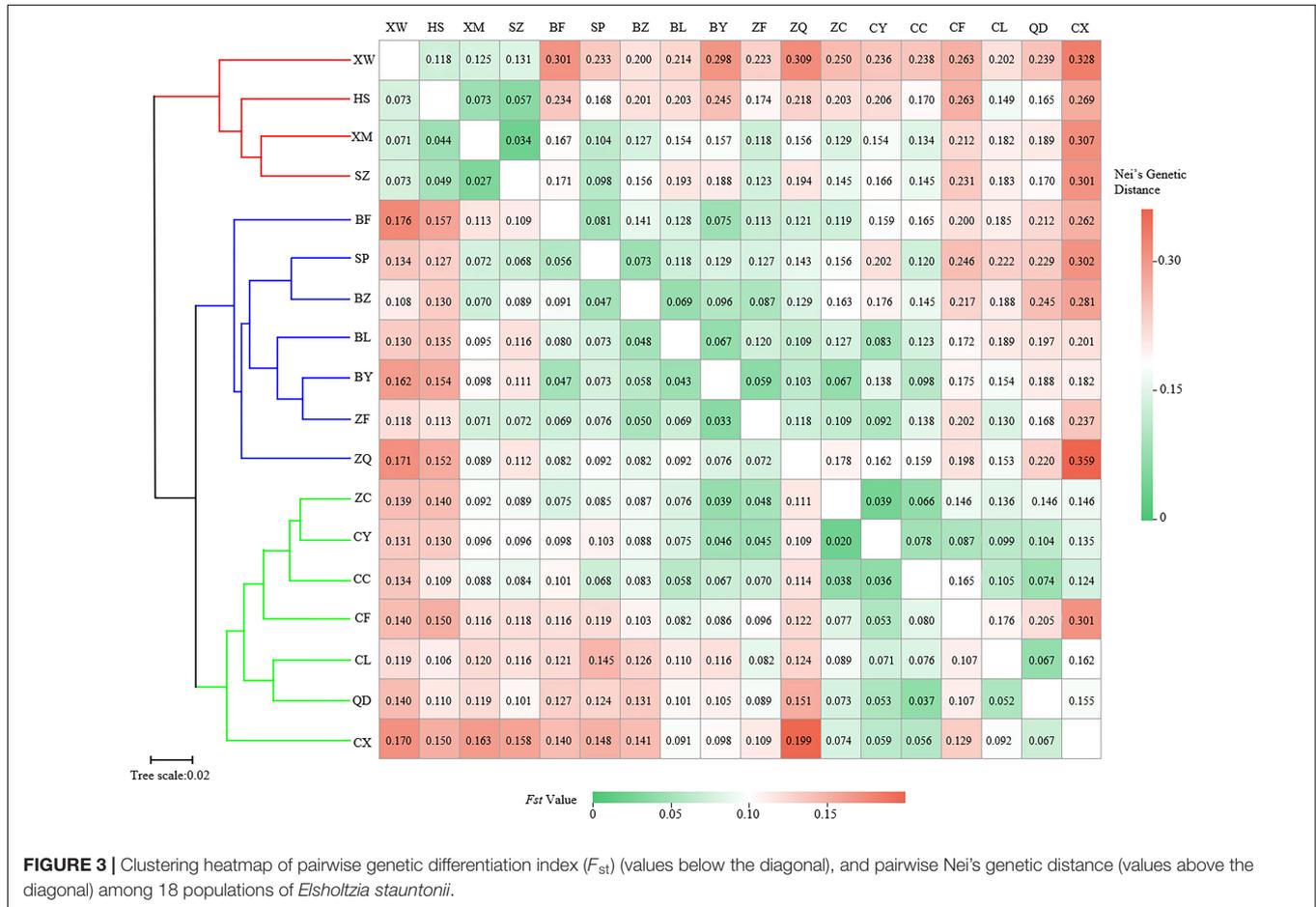
## Genetic Relationship and Population Structure

To determine the genetic structure of *E. stauntonii* populations, the co-ancestral relationship among populations was analyzed based on a Bayesian assignment model. The  $Q$  value of 680 individuals, accounting for 82.42% of the total samples, was greater than 0.8, indicating that most individuals had a relatively simple genetic background (Supplementary Table 2). STRUCTURE analysis revealed a distinct peak for  $\Delta K$  at  $K = 2$ , indicating that the *E. stauntonii* samples could be grouped into two major clusters; these two subpopulations roughly corresponded to the southwestern and northeastern regions of Hebei Province (Figures 5A,B, 6). The four populations (ZQ, ZF, BY, and BL) situated at the junction of the two regions contained abundant genetic variation and the lineages were obviously mixed (Figure 6). These populations contained genetic information of dual origins, with an average assignment of 49.7% to Cluster I (red) and 50.3% to Cluster II (green), whereas the other populations were relatively independent (Figure 6 and Supplementary Table 3).

The UPGMA dendrogram grouped the 18 *E. stauntonii* populations into three major clusters. The populations XW, HS, XM, and SZ formed one cluster, the populations BF, SP, BZ, BL, BY, ZF, and ZQ formed a separate cluster, and the

**TABLE 5** | Analysis of molecular variance (AMOVA) for 825 *Elsholtzia stauntonii* individuals clustered in 18 populations.

Source of variation	Degrees of freedom	Sum of squares	Percentage of variation (%)	P-value
Among populations	17	727.551	18.53%	<0.001
Within populations	807	3178.441	81.47%	<0.001
Total	824	3905.992	100.00%	



remaining populations (ZC, CY, CC, CF, CL, QD, and CX) formed a third group (Figure 3). However, the UPGMA tree of 825 individuals contained clusters of individuals from different populations and the samples were grouped into two major clades (Figure 7A). Consistent with the population dendrogram, the individuals of seven populations (ZC, CY, CC, CF, CL, QD, and CX) in the northeastern region were merged into a cluster and showed a broadly mixed ancestry, whereas the majority of individuals from the 11 populations in the southwestern region were grouped in the same cluster. In general, genetic proximity was observed between neighboring populations, whereas few genotypes shared an ancestry with individuals in geographically distant populations (Figure 7A).

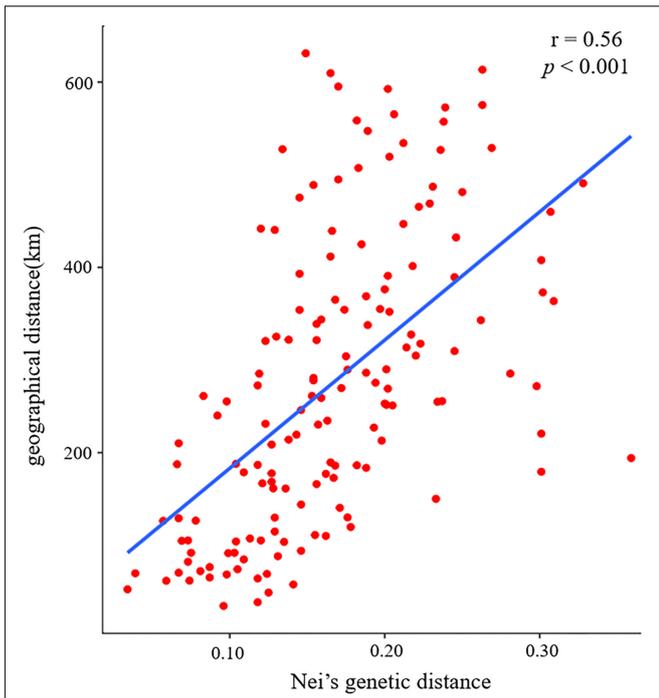
Principal coordinate analysis was performed to visualize the multidimensional relationships among individuals based on genetic distance (Figure 7B). The closer they were on the PCoA graph, the less genetically differentiated they were, such as the

HS, XM and SZ populations. Principal coordinates (PC) 1 and 2 explained 25.51% and 23.83% of the variance, respectively, and a combined 49.34% of the total variation. The PCoA analysis separated the populations into two clusters, which further confirmed the presence of genetic structure among *E. stauntonii* populations (Figure 7B).

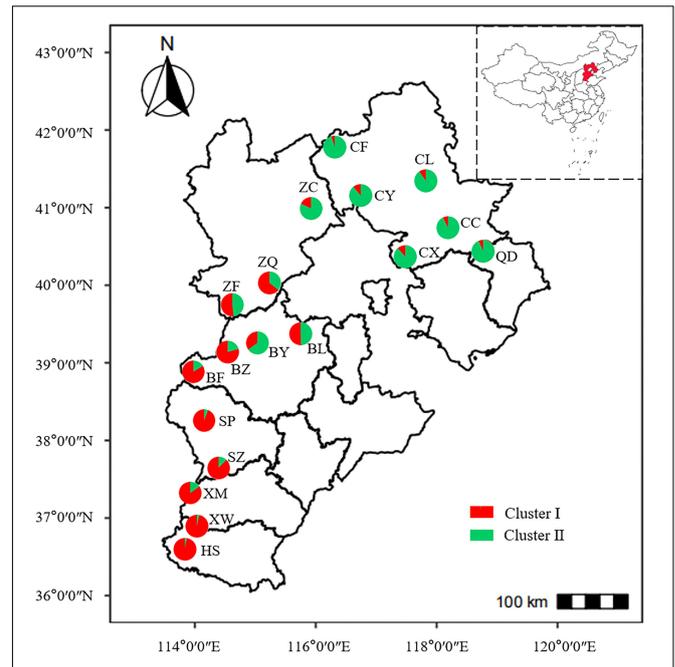
## DISCUSSION

### Cross-Genera Transferability of Microsatellite Markers

Cross-taxa transferability enables the development of microsatellite markers, based on markers from related species, at low cost (Kalia et al., 2011; Bernardes et al., 2021). In the present study, the transferability observed (9.05%) was similar to the average of approximately 10% reported in cross-genera



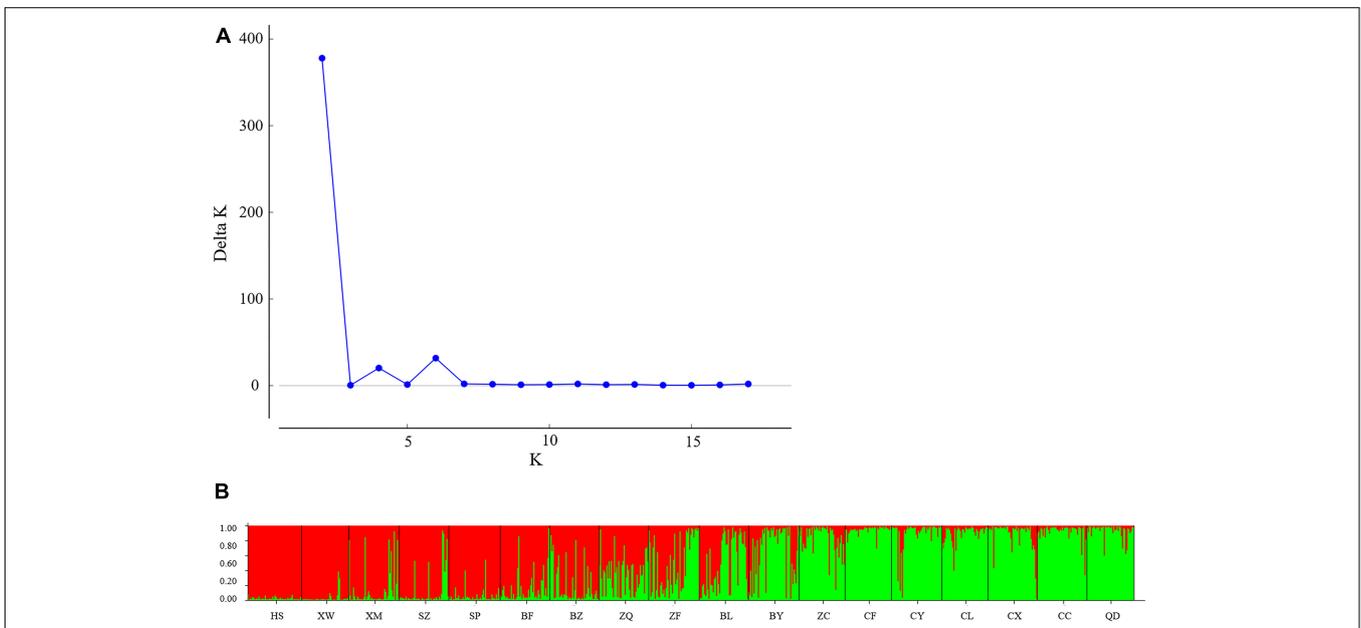
**FIGURE 4 |** Mantel test results of the correlation between Nei's genetic distance and geographical distance among 18 populations of *Elsholtzia stauntonii*.



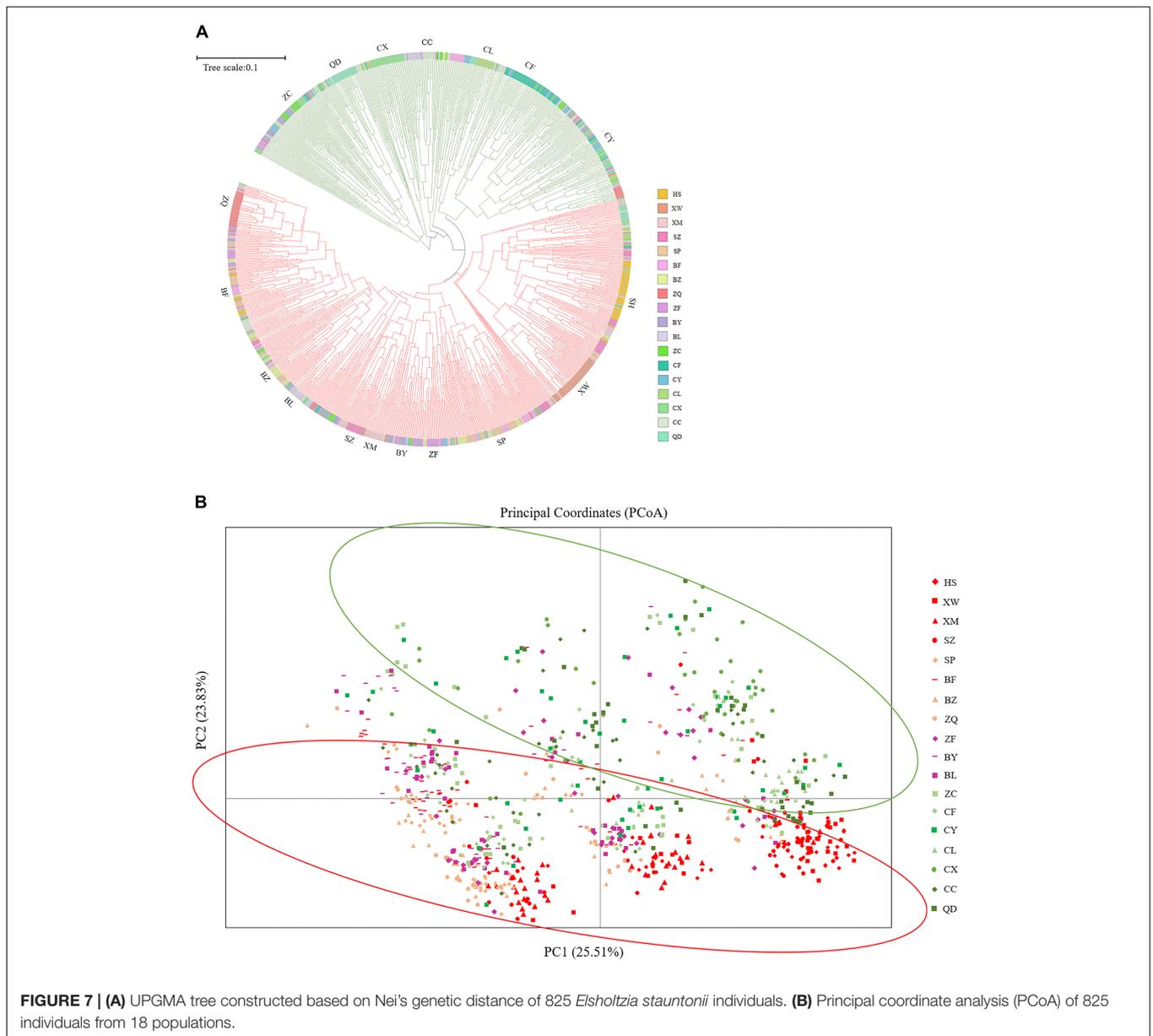
**FIGURE 6 |** Geographical distribution of 18 populations of *Elsholtzia stauntonii* and lineage contribution to gene pools. The pie diagram was drawn based on the results of STRUCTURE analysis with  $K = 2$  and represents the average proportion of cluster membership of all individuals in each population.

transferability studies of eudicots between 1997 and mid-2006 (Barbará et al., 2007). However, the percentage transferability determined here was lower than the cross-genera amplification

percentage observed in some families, such as Bignoniaceae (40.58%) (Kalia et al., 2020) and Cactaceae (35.16%) (Bombonato et al., 2019), and much lower than that between species within the same genus (Miranda et al., 2020; Pern et al., 2020; Li et al., 2021).



**FIGURE 5 |** Population structure of 825 *Elsholtzia stauntonii* individuals based on 10 SSR markers. **(A)** Delta  $K$  values from STRUCTURE analysis of the *E. stauntonii* individuals. **(B)** Histogram from STRUCTURE analysis for the model with  $K = 2$ .



The transferability of SSR markers between species or genera is determined by the conservation of DNA sequences and the stability of primer binding sites in flanking regions of SSRs during evolution (Ellegren, 2000; Saeed et al., 2016). A higher cross-taxa amplification percentage of SSR markers is expected between species with a close phylogenetic relationship, especially when both donor and target species are congeneric (Lesser et al., 2012; Karaca et al., 2013; Bharti et al., 2018). Therefore, it was speculated that *E. stauntonii* might have a relatively distant genetic relationship with its donor species (Jarne and Lagoda, 1996; Li et al., 2017). Nevertheless, although the primary contributing factor is phylogenetic proximity, transferability may also be influenced by other factors, such as the size and complexity of the relevant genome and whether the microsatellite is sited within a coding region (Oliveira et al., 2006).

The SSR transferability is also affected by the base repeat type of SSR core units (Gao et al., 2013). In general, SSR markers with a trinucleotide repeat motif show higher transferability than those with dinucleotide-type repeat motifs (Ellis and Burke, 2007; Harijan et al., 2017). A similar trend in heterologous SSR marker transferability has been reported for Euphorbiaceae, with the transfer rate of simple trinucleotide repeats being 11.44% higher than that of dinucleotide repeats (Dharajiya et al., 2020). One reason might be that the selection of frameshift mutations limits the fixation of microsatellites whose motif lengths are not divisible by three (e.g., di-/tetra-/pentanucleotide repeats), whereas fixation of mutant microsatellite whose motif lengths are divisible by three (tri-/hexanucleotide repeats) is not affected by differential selection pressures in coding and non-coding regions (Metzgar et al., 2000; Dutta et al., 2011;

Shivakumar et al., 2017). Moreover, microsatellites based on a complex nucleotide sequence show a lower mutation rate and higher transferability than simple repeats (Jin et al., 1996; Kalia et al., 2020). Given that more than 63% of the SSR markers used in the present study were di-nucleotide motif repeats, this may be an additional reason for the low transferability. Regardless, the 10 polymorphic transferable SSR markers identified in this study could be used to accelerate analysis of the genetic diversity of *E. stauntonii* and related species.

## Genetic Diversity and Population Structure Analysis

All 10 transferable SSR primers used in the present study deviated significantly from the HWE ( $p < 0.01$ ), which might reflect that the samples were collected from natural populations affected by complex environmental factors and human activities (Wang and Shete, 2012; Peng et al., 2021). The investigation found that *E. stauntonii* mainly resided beside rivers and valleys at lower altitudes, and its living environment was easily disturbed by tourism, grazing, and farmland planting. The mean PIC was 0.429, indicating that the genetic diversity of *E. stauntonii* germplasm in Hebei Province was generally moderate ( $0.25 < \text{PIC} < 0.5$ ) (Botstein et al., 1980), which was lower than that reported for *Scutellaria baicalensis* (0.72) (Qi et al., 2015) and *Perilla frutescens* (0.582) (Park et al., 2019). The genetic diversity possessed by a species is determined by its evolutionary and ecological history (Aboukhalid et al., 2017). For *E. stauntonii*, the wide original geographic distribution theoretically contributes to the high level of genetic diversity detected. The moderate genetic diversity observed in this study might be limited by the number of markers, the test method, or the regions of sample collection (Budak et al., 2004; Serrote et al., 2020). Furthermore, it is generally accepted that markers of high transferability are less polymorphic among closely related species (Ni et al., 2015).

Genetic variation of a population can be measured by the number of alleles and heterozygosity (Huang et al., 2020; Savić et al., 2021). The mean  $N_A$  (7.000) and  $N_E$  (1.971) observed in the present study were slightly higher than the values (6.45 and 1.67, respectively) for 12 *Plectranthus edulis* populations evaluated using 20 SSR markers (Gadissa et al., 2018), but lower than the values reported for *Salvia splendens* ( $N_A = 11.829$ ,  $N_E = 3.261$ ) (Jiao et al., 2020) and *Juglans nigra* ( $N_A = 9.99$ ,  $N_E = 7.51$ ) (Schneider et al., 2019). Compared with previous studies on Lamiaceae species, *E. stauntonii* ( $H_O = 0.360$ ) has higher genetic diversity than *Salvia fruticosa* ( $H_O = 0.307$ ) (Radosavljević et al., 2015) and *Hedeoma piperita* ( $H_O = 0.3$ ) (Herrera-Arroyo et al., 2020), but a higher level of genetic diversity has been observed in *Rosmarinus officinalis* ( $H_O = 0.63$ ) (Segarra-Moragues et al., 2015). These results also indicated that the tested *E. stauntonii* samples showed moderate genetic diversity. The observed heterozygosity in the present study was lower than the expected heterozygosity ( $H_E = 0.478$ ), indicating that the number of heterozygotes was lower than the predicted value (García-Arias et al., 2018). The average

inbreeding coefficient ( $F_{is} = 0.192$ ) in the population was positive and high, which similarly suggested there was an overabundance of homozygotes (Liu et al., 2019; Bairu et al., 2021). The reproductive characteristics of species might be an important factor in heterozygote deficiency in populations; for example, the combination of hermaphroditic flowers and profuse flowering of *E. stauntonii* may lead to improved levels of inbreeding which can reduce the genetic diversity of this widely distributed species (Zigene et al., 2021).

Genetic differentiation is an important index of the genetic structure of populations within a species (Wang, 2020). In the current study, gene flow of *E. stauntonii* ( $N_m = 1.252$ ) was moderately low, lower than that reported for *Eragrostis tef* ( $N_m = 4.742$ ) (Tadesse et al., 2021) and *Mucuna pruriens* ( $N_m = 7.48$ ) (Lepcha and Sathyanarayana, 2021), but it was consistent with the high genetic differentiation ( $F_{st} = 0.181$ ) (Balloux and Lugon-Moulin, 2002). The low gene flow, which might be caused by the small sampling range and habitat fragmentation, increased the within-population genetic variation and promoted population differentiation (Young et al., 1996; Li et al., 2014; Peng et al., 2021). Notably, among the 18 populations, the greatest genetic differentiation was observed between the CX and ZQ populations, which may be considered as potential parents for hybrid breeding. Furthermore, AMOVA revealed that the variation among *E. stauntonii* populations (18.53%) was higher than that of many other plant species, such as *Origanum vulgare* subsp. *hirtum* (8%) (Aleksieva et al., 2021) and *Allium sativum* L. (12.29%) (Poljuha et al., 2021), suggesting that disruption of gene flow between the populations played a role in determining the genetic differentiation of populations. Similarly, the results of the Mantel test ( $r = 0.56$ ,  $p < 0.001$ ) showed that increase in geographic distance reduced gene flow. Therefore, geographic distance may be the principal factor for genetic differentiation in *E. stauntonii*.

In the UPGMA clustering analysis, a certain deviation was observed in the clustering of individuals and of populations, which might be due to the high degree of gene exchange between populations. Overall, the clustering results observed with STRUCTURE, PCoA, and UPGMA were essentially similar, indicating that there might be two gene pools of *E. stauntonii*. Examination of the topography and geomorphology in Hebei Province indicated that the genetic structure of *E. stauntonii* was delimited according to geographical location and topographic barriers: one genealogical lineage each was located near the Taihang Mountains and the Yanshan Mountains. Previous studies have shown that geographical barriers play a crucial role in preventing plant dispersal (Fan et al., 2013; Zhao and Gong, 2015; Zeng et al., 2018), for example, the Tanaka Line in southwest China has a marked effect on the divergence and evolution of plant species (Ju et al., 2018). In this study, populations ZQ and CX on both sides of the mountain ranges presented the greatest  $F_{st}$  and genetic distance values, although their geographic distance was relatively close (194.14 km apart from each other), which also indicated the role of geographical barriers in genetic differentiation. The Taihang and Yanshan mountains may be responsible for maintaining the major southwest–northeast split of *E. stauntonii* populations by hindering animal-mediated gene

exchange. Thus, natural adaptation and geographical barriers may explain the differentiation of the two regions. Moreover, in the present analyses, each group was more or less intermingled with lineages of the other group, indicating that there was a certain level of gene flow between the populations, which has also been reported in *Tectona grandis* (Balakrishnan et al., 2021). The levels of genetic admixture differed substantially from population to population and seemed to be associated with the geographic location of the population, which indicated that interbreeding was an ongoing process, especially for the ZF, ZQ, BY, and BL populations (Mangaravite et al., 2016). These four populations had relatively high gene introgression, indicating that these populations had relatively rich genetic backgrounds and might be potential centers of diversification in this species. From the perspective of cultivar development and conservation, these populations may be valuable because they may contain novel alleles.

## CONCLUSION

In this study, SSR markers from seven genera of Lamiaceae were transferable to *E. stauntonii*. Analysis based on 10 transferable SSR markers showed that, although the genetic diversity in 18 geographical populations of *E. stauntonii* was slightly lower than that of certain other species in the Lamiaceae, the genetic diversity was generally rich and the populations showed a high degree of genetic differentiation. STRUCTURE, PCoA, and UPGMA clustering analyses of all individuals resolved two genetic clusters, which might reflect geographical barriers to gene flow. By screening the study materials with superior traits and rich genetic variation, some individuals in the present experiment are expected to be utilized in the future improvement programmes of *E. stauntonii* to meet the increasing demand

from the floriculture, essential oil, pharmaceutical, and other industries. The information obtained from the analysis of genetic diversity and population structure provides a foundation for the conservation, management, and utilization of *E. stauntonii*.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

YL and BC conceived and designed the experiments and obtained the funding. CZ, XL, and HZ conducted the experiments. CJ and BC contributed to the investigation. CZ, LH, and ML collected and analyzed the data. CZ wrote the manuscript. YL provided suggestions and comments for the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2022.903674/full#supplementary-material>

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