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Full Length Article

Molecular Phylogeny and Population Structure of *Aconitum carmichaelii* (Fuzi) in Western China

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Abstract

Aconitum carmichaelii Debx. is a traditional Chinese medicinal herb. Its lateral root, named as Fuzi, has been cultivated manually for thousands of years. Recently, Fuzi agriculture is more and more bustling, and large amount of germplasm resources are needed. However it remains unclear to what extent the genetic diversity and population phylogeny for this species. In this paper, we used the nuclear gene (*Serine-threonine kinase*) and chloroplast fragments (*psb*A-*trn*H) to study 87 wild *A. carmichaelii* samples from 8 populations in China. Results showed that 118 variable sites existed in 1325 bp combined fragment, accounting for 8.91% of the total number of sites. Their average genetic distance was 0.014. The intra-population genetic distance was 0.003 (CQ) to 0.018 (YN), while the inter-population genetic distance was 0.007 (between SD and CQ) to 0.024 (between GZ and NW). Through geographical population structure analysis, significant divergence was observed among all samples and partial populations. Bayesian inference (BI), neighbor-joining (NJ) and unweighted pair group method with arithmetic mean (UPGMA) trees were constructed to reveal the molecular phylogeny and population structure of *A. carmichaelii* in western China. Results indicated that the geographical topography might control its genetic diversity. In the end, the suitable germplasm provenances were recommended for Daodi agricultural farms in the future. © 2018 Friends Science Publishers

Keywords: *Aconitum carmichaelii*; Molecular phylogeny; Population structure; Daodi herbs; Germplasm provenances

Introduction

Aconitum carmichaelii Debx. is a long used medicinal herb, whose lateral roots (LRs) and tap roots (TRs) are designated as "Fuzi" and "Chuanwu" respectively in Chinese Pharmacopoeia (Kang *et al.*, 2012; Yang *et al.*, 2016). Its soak solution has also been used as hunting anesthetic, insect repellants and so on. Since the Tang Dynasty, Fuzi has been cultivated manually. As an important Daodi drug (i.e., genuine medicinal materials) and economic crop in China, it has now been mainly cultivated in Sichuan, Shaanxi and Yunnan provinces. The products are also exported to some Eastern Asian countries, such as Japan, Korea, Mongolia, India and so on (Zhou *et al.*, 2015).

Although agronomic farming technologies have been improved for more than 1400 years, it is ignored about the genetic diversity study and protection of *A. carmichaelii* species in China. Then in the 1950s – 1970s, an administrative herb-introduction movement wept across the country (Qin, 2012). The germplasm geographic isolation was broke extensively. In the last decade, Fuzi industries attract great investment again. Standardized and concentrated agricultural farm bases emerge one after another in the genuine (Daodi) areas. For instance, Sichuan province had more than two thousand hectares in 2015 (Wen *et al.*, 2016). However, some bottleneck questions appear mortal threats, such as the genetic characterization decline, fungus infection and so on. Thus, it becomes again an inevitable task to breed the good germplasm and protect the genetic diversity of *A. carmichaelii* species.

For genus *Aconitum*, the greatest center of present diversity is in the mountains of eastern Asia, especially in China (Jiang *et al.*, 2014; Hong *et al.*, 2017), where *A. carmichaelii* is distributed most widely among the congeneric plants. According to the Flora of China and the census of Chinese herbal medicine resources, *A. carmichaelii* and the four variants growin 15 provinces, from the eastern edge of the Sichuan-Tibet Plateau, eastward to the middle/lower reaches of the Yangtze River and the upper reaches of the Pearl River, and from Jiangsu and Shandong provinces, northward to southern areas of Liaoning province. The vastness of distribution region and the polytropic transitive morphology are the main obstructives to launch the genetic diversity study for *A. carmichaelii* plants. Unlike other congeneric species (*A. delavayi*, *A. nemorum* and so on) (Zhang *et al.*, 2003;

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Jiang *et al*., 2014), the molecular phylogeny and population structure of *A. carmichaelii* were reported rarely. Scholars usually aim at its morphological and molecular identification (Park *et al.*, 2017), population classification among the whole genus plants (Hong *et al*., 2017; Rajabloo *et al.*, 2017), even among the Ranunculaceae plants (Xie and Li, 2012).

In the present study, we spent years to collect the wild *A. carmichaelii* samples in its main distribution regions. The combined sequence of nuclear gene (*Serine-threonine kinase*) and chloroplast regions (*psb*A-*trn*H) was used to perform phylogenetic analyses on this species. The objectives of this study were to (a) clarify the present molecular diversity and population structures for this species, and (b) recommend suitable germplasm-introduction resources for Daodi regions. This study is likely to supplementa better understanding of speciation and evolution for *A. carmichaelii*, and facilitate a better industry development for Daodi Fuzi.

Materials and Methods

Materials

During 2013–2016, our research team spent four years to collect wild Fuzi plants around China and established the State Bank of Chinese Drug Germplam Resources. The bank included a nursery with the most abundant *Aconitum carmichaelii* germplasm resources. In this study, a total of 87 wild *A. carmichaelii* samples from 25 counties were collected (Fig. 1). They cover the whole western distribution regions in China. Of which, 4 samples were from Shandong province. Based on their geographic distribution, these samples were divided into eight populations: Shaanxi (SX), Sichuan (SC), Yunnan (YN), Guizhou (GZ), Henan (HN), Chongqing (CQ), Shanxi (Ningwu County, NW) and Shandong (SD). The sample information of each population was shown in Table S1.

The living plants were brought back and cultivated in the greenhouse of The State Bank of Chinese Drug Germplasm Resources of Chengdu university of Traditional Chinese Medicine. After parallel cultivation, their fresh leaves were collected into tubes and conserved at -20^{o}C for genomic DNA extraction.

DNA Extraction and PCR Amplification

The genomic DNA of *A. carmichaelii* was extracted from leaves using Tiangen plant DNA extraction kit (Tiangen Biotech, Beijing, China). The phylogenetic relationships and population structure of *A. carmichaelii* were analyzed using the modified barcoding methods (Xie and Li, 2012; Hong *et al*., 2017). In this study, a nuclear gene (*Serine-threonine kinase*) fragment and chloroplast non-coding region *psb*A-*trn*H were amplified, respectively and combined together. *Serine-threonine kinase* gene was found to have the highest variation among the most nuclear genes in transcriptome database of *A. carmichaelii*. Their amplification primers were 5'-AGAAGAAGGTGTTTTGAGAATGGAC-3' and 5'-CCAGACGCACCCGCAGTCGTATCCG-3' for *Serine-threonine kinase*, and 5'-CGCGCATGGTGGATTCACAATCC- 3' and 5'-GTTATGCATGAACGTAATGCTC - 3' for *psb*A-*trn*H. The 25 μL PCR amplification system included 9.5 μL of ddH_2O, 12.5 μL of Taq Mix, 1 μL of extracted DNA and 1 μL of each of the two 5 nmol/μL primers. The amplification condition was as follow: initial denaturation at 94^{o}C for 3 min, 35 cycles of denaturation at 94^{o}C for 1 min, annealing at 53^{o}C for 40 s, extension at 72^{o}C for 1 min 30 s, followed by a 10 min extension at 72^{o}C. After sequencing and clipping, the two fragments jointed together for the following analysis.

Sequence Analysis

The *Serine-threonine kinase* gene and *psb*A-*trn*H region of 87 *A. carmichaelii* samples were aligned by Clustal X 1.83 software, respectively, and adjusted manually. After removing the primer sequence, the *Serine-threonine kinase* gene and *psb*A-*trn*H region of the same individual were spliced together, and then aligned using Clustal X 1.83 software. The average intra and inter-population genetic distances were calculated by MEGA v7 software based on Kimura 2 parameter (K2P) model(Srivathsan and Meier, 2012). Genetic structure was evaluated through the analysis of molecular variance (AMOVA) in Arlequinv 3.1 software (Excoffier *et al.*, 2007). The overall and pairwise genetic divergence using the fixation index Fst were also evaluated with Arlequin software.

Phylogenetic analyses of *A. carmichaelii* were performed using three methods: Bayesian inference (BI), neighbor-joining (NJ) and unweighted pair group method with arithmetic mean (UPGMA). BI trees were constructed in MyBayes v3.1.2. The best-fit nucleotide substitution model was assessed using jModelTest v1.1 (Posada) with the Akaike information criterion (AIC). Four Markov Chain Monte Carlo (MCMC) chains including one cold chain and three heated chains were run for 10000000 generations and sampled every 1000 generations. The first 25% of trees were discarded as burn-in, and the remaining trees with a stable likelihood score were used to construct a single 50% majority rule consensus tree. NJ and UPGMA trees were constructed in MEGA v7 based on the K2P-distance matrices from with 1000 bootstrap replicates.

Two different methods were used to evaluate the population demography of all samples and every population. First, the population demography was analyzed through comparing mismatch distributions in each geographic sample with those expected in stationary and expanding populations using DnaSP v4.10.7 (Rozas *et al.*, 2003). Second, under the model of recent population expansion, we calculated Tajima's D and Fu's FS for each population in Arlequin program.

Table S1: The information of *A. carmichaelii* used in this study

Population	Samples number	Collecting locations	Longitude	Latitude
NW	NW1	Ningwu county, Shanxi	112.30	39.00
	NW2			
	NW3			
	NW4			
	NW5			
SX	NZ1	Nanzheng county, Shaanxi	106.38	32.48
	NZ2			
	NZ3			
	NZ4		106.52	33.39
	NZ5			
	NZ6			
	NZ7		106.55	32.54
	NZ8			
	NZ9			
	LB1	Liuba county, Shaanxi	106.88	33.66
	LB2		106.86	33.69
	LB3			
	ZB1	Zhenba county, Shaanxi	107.57	32.29
	ZB2		107.53	32.31
	TB1	Taibai county, Shaanxi	107.71	34.09
	TB2			
	TB3			
	TB4		107.25	33.98
	TB5			
	TB6			
	ZZ1	Zhouzhi county, Shaanxi	108.14	33.97
SC	BC1	Beichuan county, Sichuan	104.44	31.78
	BC2			
	BC3			
	BX1	Baoxing county, Sichuan	102.81	30.37
	BX2			
	BX3		102.71	30.47
	JY1	Jiangyou city, Sichuan	105.08	32.10
	BT1	Butuo county, Sichuan	102.91	27.65
	BT2			
	BT3			
	BT4			
	BT5			
	BT6		102.94	27.71
	BT7			
	BT8			
	BT9			
	BT10			
	ML1	Muli county, Sichuan	101.25	27.90
	EM1	Emei city, Sichuan	103.40	29.50
	EM2			
	EM3			
CQ	CK1	Chengkou county, Chongqing	108.63	31.82
	CK2			
	CK3		108.66	31.95
	YY1	Youyang county, Chongqing	108.82	28.75
	YY2			
	YY3			
YN	LJ1	Lijiang city, Yunnan	100.28	26.78
	LJ2			
	LJ3			
	LJ4			
	LJ5			
	LJ6		101.15	26.46
	LJ7			
	LJ8			
	LJ9			
	DQ1	Diqing city, Yunnan	99.64	27.90
	DQ2			
	ZT1	Zhaotong city, Yunnan	103.71	27.34
	ZT2			
	ZT3			
HN	LS1	Lushi county, Henan	111.05	34.05
	LS2			
	LS3			
	LS4		110.85	34.16
	LS5			
	LS6			
	NX1	Neixiang county, Henan	111.92	33.50
	NX2			
	NX3		112.02	33.36
GZ	GY1	Guiyang city, Guizhou	106.74	26.68
	DS1	Dushan county, Guizhou	107.56	25.84
	DS2			
	DS3			
	LPS1	Liupanshui city, Guizhou	104.83	26.62
	LPS2			
	LPS3		104.84	26.61
SD	YT1	Yantai city, Shandong	121.75	37.25
	TS1	Taishan, Shandong	117.10	36.25
	TS2			
	TS3			

Results

Genetic Variability

After alignment, the sequence length of *Serine-threonine kinase* gene and *psb*A-*trn*H of 87*A. carmichaelii* samples ranged from 996 to 999bp and from 304 to 318bp, respectively. Followed clipping process, the final spliced sequences contained 1325 bp. There were 118 variable sites in 87 samples, which accounted for 8.91% of the total number of sites, including 38 singleton variable sites and 80 parsimony informative sites. The numbers of variable sites of SX, SC, YN, GZ, HN, CQ, NW and SD populations were 54, 41, 62, 48, 37, 9, 30 and 27, respectively. The average base content of all samples was T=31.8%, C=16.8%, A= 25.1% and G=26.3%.

The average genetic distance among all samples was 0.014 based on the K2P model. Among the eight populations, the CQ population had the smallest intra-population genetic distance (0.003) followed by SC population (0.007), and the YN population had the largest (0.018).

The smallest inter-population genetic distance existed between SD and CQ populations (0.007) followed by SC and SX (0.009), and the highest occurred between the GZ and NW populations (0.024) (Table 1).

Population Structure and Demography

The AMOVA analysis indicated that 59.96% of molecular variation contained within populations and 21.64% occurred among populations. As given in Table 1, significant divergence across all samples was observed (*F*st=0.400, P<0.001), which suggested a high level of geographical population structure.

Table 1: Average genetic distances intra-population (on the diagonal) and inter-population (below the diagonal), and *F*st values (above the diagonal) of *A. carmichaelii*

Populations	SC	SX	CQ	GZ	YN	HN	NW	SD
SC	0.007	0.072*	0.562*	0.441*	0.209*	0.279*	0.396*	0.618*
SX	0.009	0.010	0.305*	0.280*	0.145*	0.216*	0.384*	0.121
CQ	0.013	0.010	0.003	0.131	0.228*	0.540*	0.719*	0.177*
GZ	0.017	0.016	0.010	0.015	0.068	0.410*	0.469*	0.015
YN	0.015	0.016	0.015	0.018	0.018	0.257*	0.323*	0.079
HN	0.012	0.013	0.016	0.022	0.020	0.011	0.427*	0.350*
NW	0.012	0.015	0.021	0.024	0.022	0.018	0.010	0.413*
SD	0.012	0.011	0.007	0.013	0.016	0.017	0.017	0.010

*$P<0.05$

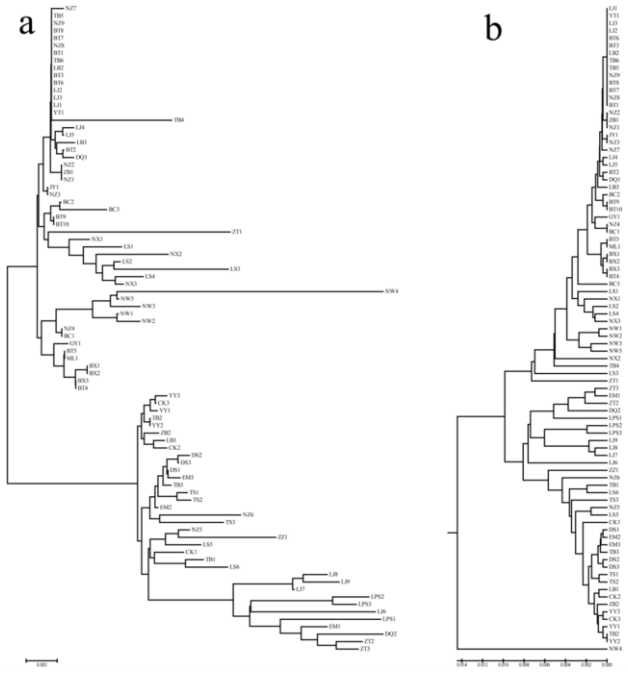

Fig. S1: NJ tree (a) and UPGMA tree (b) of *A. carmichaelii* samples

It had been revealed by pairwise *F*st values that there was significant divergence between any populations (P<0.05), except between CQ and GZ populations, GZ and YN populations, SD and SX, GZ, YN populations (P>0.05).

By population demography analysis of all samples and each geographical population, neither the mismatch distribution test nor the selective neutrality test supported the hypothesis that *A. carmichaelii* had passed a population bottleneck or expansion. Firstly, the shape of the mismatch distribution was ragged and multimodal for all samples and each geographical population, which indicated the size of *A. carmichaelii* population was very stable (Fig. 2). Secondly, the values of Fu's and Tajima's D were not significant (P>0.05) for all samples and each geographical population (Fig. 2).

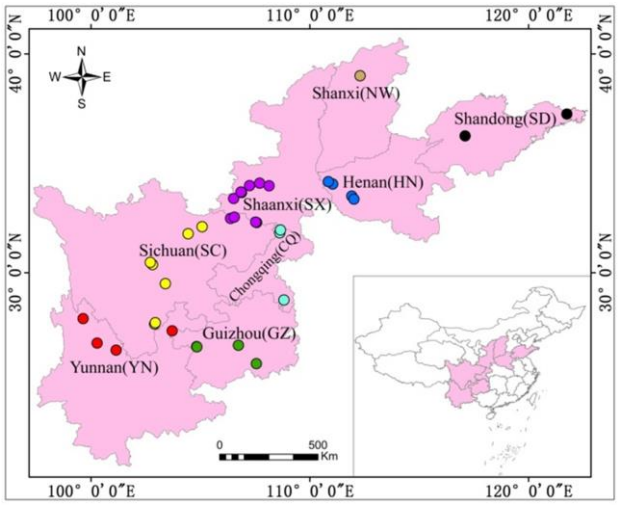

Fig. 1: The distribution of *A. carmichaelii* samples used in this study

Phylogenetic Analysis

BI tree verified the data results and analysis conclusion: (1) most populations (except for SD) could gather in one or near clades like NW, CQ, SC and HN; (2) some populations, like YN and GZ, SX and SC, SX and HN with CQ, had small divergence (Table 1)and close to each other (Fig. 3).

Furthermore, the phylogenetic tree displayed some new clues. Firstly, the YN population samples most clustered in the upstream clades and seemed to be more genetically ancient than others. Secondly, Shandong samples (TS_n and YT_n) were clustered in different population clades. TS1 and TS2 plants were close to SX samples in the tree, and YT1 was genetically close to HN. Thirdly, there were large amounts of *A. carmichaelii* plants were intersecting in the phylogenetic tree, indicating complex gene flow among all *A. carmichaelii* populations in China.

The NJ and UPGMA trees had similar topologies: *A. carmichaelii* samples mainly clustered into two clades, generally of the northern and southern groups (Supplement Fig. S1). Then their population structures had parallel cluster patterns with BI tree (Fig. 3).

Discussion

China is the diversity and endemism center of the genus *Aconitum* (Jabbour and Renner, 2012; Jabeen *et al*., 2013; Hong *et al*., 2017). Therefore, it is very hard to collect the whole representative wild Fuzi samples. Moreover, academic debate still exists on the *A. carmichaelii* morphological identification and species classification. Some morphology transition plants are divided into different systematic taxa by different scholars.

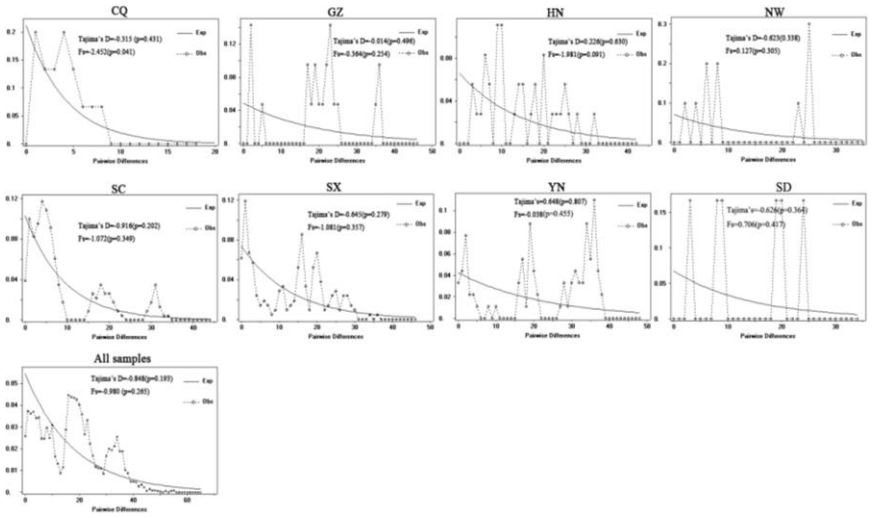

Fig. 2: The population demography analysis for *A. carmichaelii*

In the TCM system, confused names, "Cao Wu", "Wu Tou", "Wu Yao" and so on, are widespread for Fuzi (Sun *et al*., 2012; Liu *et al*., 2015; Liu *et al*., 2017). They are often distinct *Aconitum* species in different regions. Then the intermittent discoveries of new morphological variability further challenge traditional classification experiences (Luo *et al*., 2005; Hong *et al*., 2017). Thus, the hardness of collecting samples might result in study deficiency of Fuzi diversity and population in this paper.

The complex geographical topography, especially the mountains, reflects importantly the genetic diversity and revolution of *A. carmichaelii* plants. For genetic differences within independent populations, Yunnan province is located in the Hengduan Mountains of Himalaya. Then the complicated geographical topography and climatic environment might be reasons why the majority of *A. carmichaelii* plants had high genetic difference in this population. The results could be verified by GZ population samples, which were also distributed on the edge of the Hengduan Mountains and had high genetic variation.

For genetic diversities among populations, they were affected by the geographical topography too. On one hand, these samples from Shaanxi, Henan, Chongqing and Shaanxi, displayed prominent population independence. The Qinling Mountains and its extension mountains (Funiu Mountain, Lu Mountain, Tongbai Mountain, Que Mountain and so on) must space their gene flow and separate them into different populations. The results are similar to other *Aconitum* species and plants. However, these mountains stretch from north to south, along the direction of latitudes, while Wu Mountain connects them in Chongqing City, also with the north-south direction, which weakens the extinction effect in the ice age (Zhang, 2003). Thus, the data in our study showed that the population divergence between Shaanxi, Guizhou and Yunnan were not significant.

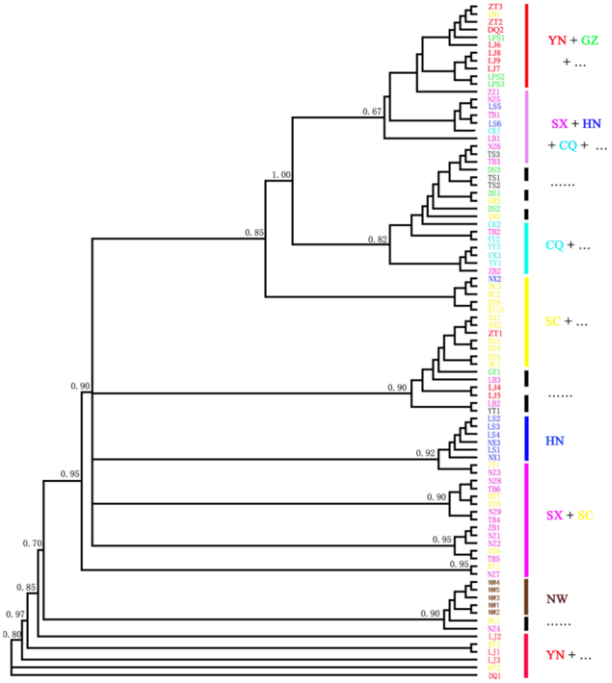

Fig. 3: Phylogenetic tree of *A. carmichaelii* constructed with Bayesian inference. Numbers at nodes represent Bayesian posterior probabilities

On the second hand, the population divergence between Yunnan and Guizhou was small. They together contain the Xuefeng Mountain and Wuling Mountain. An overlapping phenomenon might exist in the process of *Aconitum* species evolution. Then the small population divergence between Yunnan and Guizhou provinces was consistent with their small genetic distance data (0.018). It should be related to their shared habitats, the edge of Hengduan Mountains and Yunnan-Guizhou Plateau.

On the third hand, *A. carmichaelii* was fond of high altitude habitats, mainly distributing on mountain hillsides (about 800–2500 m). It is a bold guess that *Aconitum* plants might specially perform continuity evolution along with coherent mountain ranges. However, further studies are needed on this aspect.

As analyzed above, Fuzi is cultivated manually for more than one thousand years. Its tuberous root resources were introduced several times from Tang Dynasty. And the agriculture and human factors should severely affect the genetic diversity of *A. carmichaelii* species in China. Particularly worth mentioning is the administrative herb-introduction movement in the 1950s – 1970s, while the germplasm geographic isolation was broke extensively (Qin, 2012). Sichuan province is the traditional Daodi agricultural region for Fuzi herbal crop, which is bred in higher mountains and cultivated in lower flatlands in long history (Kang *et al*., 2012). Long time self-introduction might reduce the genetic difference of SC population samples. However, those introduction actions were random, not with scientific guidance (Luo *et al*., 2006). In recent years, Fuzi industry is bustling and prospering again but fair amount of good "seeds" is deficient.

In this paper, one important aim is to recommend suitable germplasm-introduction resources for Daodi regions (Jiangyou City and Butuo County of Sichuan province). The results showed that Yunnan and Ningwu population samples seemed situated on the headstream of gene flow. Compared to Shaanxi province, Lijiang and Diqing of Yunnan province and Ningwu County of Shanxi Province are more suitable for allopatric germplasm-introduction regions.

It's worth noting that Butuo County is near to Yunnan province, their *A. carmichaelii* samples and population being genetically homeologous. For Butuo agricultural farms, the northern regions (like Ningwu County) had more advantages than the southern regions (Yunnan province) as the provenances in the future.

Conclusion

It was found that there existed distinct population structures for this species. Geographical and manual factors both functioned importantly to affect its biodiversity and genetic population structures. Then suitable germplasm provenances were recommended for Daodi agricultural farms in the future. Our findings will supplement a better understanding of speciation and evolution for *A. carmichaelii*, as well as it will facilitate a better industry development for Daodi Fuzi.

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