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Full Length Article

Genetic Diversity and Genetic Relationships of *Amomum tsao-ko* Based on Random Amplified Polymorphic DNA Markers

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Abstract

Amomum tsao-ko is a perennial herb of the ginger family. Fruit is used as a medicine and is often added to foods as a spice, but little information on its genetic features is currently available. In this study, 96 *A. tsao-ko* samples from eight populations were analyzed using RAPD molecular markers. Twelve RAPD primers produced 492 loci, 98.98% (487) of which were polymorphic. Marker index (MI) values and mean polymorphism information content (PIC) represented 8.095 and 0.197, respectively. At the population level, the percentage of polymorphic bands (PPB) was 57.18%, Nei's gene diversity (H) was 0.121, and Shannon's information index (I) was 0.202. The genetic differentiation coefficient among populations was Gst=0.084, which was similar to the 6% genetic variation among populations analyzed by AMOVA, indicating that there was no genetic differentiation among the populations of *A. tsao-ko*. A Mantel test (r = 0.153, P >0.05) further indicated that the geographic distance was not related to estimates of genetic distance. UMPGA cluster and principal coordinate analysis (PCoA) all divided 96 *A. tsao-ko* samples into three major groups.

Keywords: Genetic diversity; *A. tsao-ko*; RAPD marker; Polymorphic

Introduction

Amomum tsao-ko Crevostet Lemaire is a perennial herb of the genus Zingiberaceae, stem tufted, plant height up to 3 meters, leaf blade narrowly elliptic or oblong, yellow flower, and the fruit is red when ripe, the dry fruit is a traditional Chinese herbal medicine (Fig. 1). It is mainly distributed in southwest China and Vietnam at altitudes ranging from 1300 to 1800 m (Wu and Larsen, 2000; Xia *et al*., 2004). Previous research showed the major active compounds in *A. tsao-ko* were essential oils (EOs), which can be separated and extracted from the dried fruit using steam distillation, microwave assisted extraction, and ultrasonic extraction (Feng *et al*., 2010; Li *et al*., 2011). The EOs mainly consisted of monoterpene hydrocarbons and oxygenated monoterpenes such as1, 8-cineole, α-pinene, β-pinene, terpineol, geraniol and geranial (Liu *et al*., 2011). The fruit of *A. tsao-ko* has the effects of eliminating phlegm and dampness as well as warming stomach and cold-dispelling, and it is also used as a spice in cooking (Lim, 2013; Shi *et al*., 2014). Since the edible fruits of *A. tsao-ko* have been overharvested for trade, it is listed as 'Near Threatened' by IUCN (Leong-Skornickova *et al*., 2012). Yunnan province (SW China) is the main production area of *A. tsao-ko*, and the planting area accounts for approximately 90% of China.

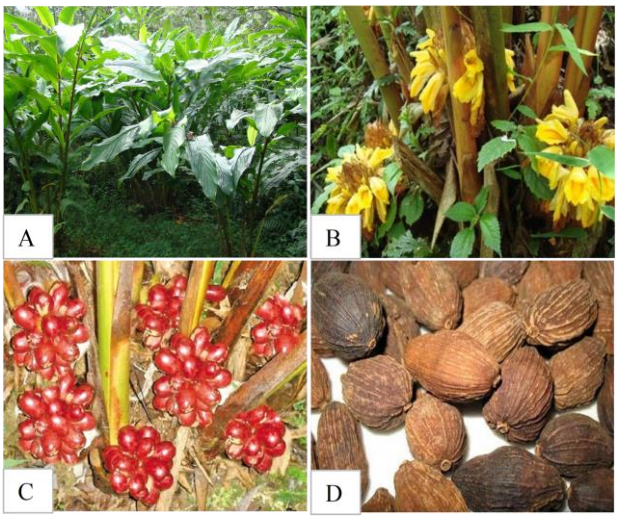

Fig. 1: Plant, flower and fruit morphology of *A. tsao-ko*. A, plant morphology; B, flower; C, fresh fruit; D, dry fruit

Traditionally, the fruit shape is the main basis for *A. tsao-ko* classification can be classified as round, oval, long and shuttle type. Due to multivariate environmental factors and ecological plasticity, it is hard to figure out the genetic

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relationships of *A. tsao-ko* populations according to morphological traits. At present, the molecular genetics research of *A. tsao-ko* germplasm resources is still not perfect. Insensitive to environmental factors relative to morphological data, molecular markers are remarkably efficient in providing abundant information. Several molecular marker types have been applied to evaluate the genetic diversity of *Amomum* L. species including random amplified polymorphic DNA (RAPD) in *A. villosum* (Wang *et al*., 2000; Xu and Ding, 2005), amplified fragment length polymorphisms (AFLPs) in Thai *Amomum* species (Kaewsri *et al*., 2007), plus inter-simple sequence repeats (ISSRs) in *Amomi Fructus* (Zhang *et al*., 2011).

The survival and development of species relies largely on genetic diversity (Booy *et al*., 2000). Quantification of the genetic diversity of populations contributes to the analysis of its evolutionary potential and the prediction of its future fate and is of great importance to preservation and utilization of germplasm resources. RAPD is an efficient tool for exploring genetic variation and assessing diversity, and it has been widely employed for germplasm recognition in many plants (Nagl *et al*., 2011; Shafi *et al*., 2016; Bakhsh *et al*., 2017; Baruah *et al*., 2017; Kumari *et al*., 2017).

As a traditional Chinese medicine and spice, research on *A. tsao-ko* has mainly concentrated on its chemical composition (Yang *et al*., 2008; Liu *et al*., 2011; Zhang *et al*., 2012). Whereas, the genetic diversity of *A. tsao-ko* has been the subject of little research so far, this severely limits the preservation and use of the spice. Microsatellite (SSR) markers have recently been employed in genetic analysis of 60 *A. tsao-ko* individuals from three populations (Baoshan population, Maguan population and Fadou population) and low genetic diversity was detected using few markers (nine SSR markers) (Yang *et al*., 2014). Lately, Ma *et al*. (2017a, b) used five pairs of SSRs to analyze 44 *A. tsao-ko*. The results showed that genetic diversity of *A. tsao-ko* in Jinping County was higher than that of Yang *et al*. (2014).

As an effective and instrument approach to identify genetic variation, RAPD can produce markers which covering the genome without prior knowledge of their sequence (Williams *et al*., 1990). A RAPD marker system was suitable for the determination of genetic differentiation and diversity in *A. tsao-ko* germplasm in this study, serving three purposes, that was, to estimate genetic diversity of different geographically cultivated populations in Yunnan Province, reveal the distribution pattern of genetic variability within/among populations, and provides suggestions for the preservation and utilization of such important herbal medicine.

Materials and Methods

The experiments were carried out from October 2016 to May 2017. *A. tsao-ko* resources survey and leaf collection were conducted in October–November 2016, which have a wide range of representative, and cover the traditional *A. tsao-ko* production area in Yunnan Province (N22°53'24"-N 24°49'48", E 98°18'36"-E 103°31'48"). The genomic DNA extraction, RAPD reaction system optimization and primer screening were carried out between December 2016 and February 2017. Then PAPD-PCR amplification of *A. tsao-ko* samples and data analysis were conducted from March 2017 to May 2017.

Plant Materials

Plant materials, totaled 96 individuals of *A. tsao-ko*, were collected from Yunnan Province, China (Fig. 2 and Table 1). There were eight geographic populations, each comprising 12 individuals that were distributed at a distance of over 50 m. Young leaf materials were collected and later reserved in the lab at the temperature of -80°C before DNA extraction.

DNA Extraction

A modified cetyltrimethyl ammonium bromide (CTAB) was employed to extract genomic DNA (Doyle, 1987). After extraction, the purity of DNA was estimated by NanoDrop-2000 version 1.0 spectrophotometer, the quality of DNA was determined by running on one percent agarose gel electrophoresis. Finally, the DNA was adjusted to 20 ng/μL for PCR amplification.

RAPD Amplification

Individual RAPD-PCRs were conducted in a 25 μL reaction volume, which contained 40 ng DNA, 4.0 mmol/L $MgCl_2$, and 2.0U Taq DNA polymerase, 0.4 μmol/L per primer, 0.2 mmol/L dNTPs and 2.5 μL of 10 × PCR reaction buffer (Mg^{2+}-free). ETC-811 PCR Thermocycler was used in all amplifications (Eastwin, Beijing, China). PCR program was carried at initial denaturing at 95°C for 5 min (one cycle); followed by 95°C for 1 min, 36°C for 1 min, 72°C for 1.5 min (40 cycle); a final extension at 72°C for 8 min. Fragments were parted on 5% denaturing polyacrylamide gels in 10×TBE buffer, followed by visualization of silver nitrate staining.

Statistical Analyses

Data procured were input into a binary matrix for analysis. A missing or ambiguous band was scored as 0 (absent) while distinct and visible bands were marked as 1 (present). The population was assumed to be in Hardy-Weinberg equilibrium, genetic polymorphism parameters for each population were obtained using POPGENE 1.32 (Yeh *et al*., 1999). PIC values was calculated by formula $PIC_i=2f_i(1-f_i)$ (Roldàn-Ruiz *et al*., 2000). MI (marker index) was calculated using the formula given by Prevost and Wilkinson (1999).

Table 1: Population information and number of *A. tsao-ko* samples used in this study

Code	Population ID	No. of samples	Voucher numbers	Latitude (N)	Longitude (E)	Altitude (m)	Location
J	Pop1	12	J22, J24, J32, J43, J46, J50, J54, J70, J90, J99, J101, J102	22°54'36"	103°13'12"	1665	Jingping, Yunnan
Y	Pop2	12	Y8, Y12, Y23, Y25, Y32, Y35, Y38, Y39, Y43, Y44, Y46, Y50	23°3'0"	102°55'12"	2108	Yuanyang, Yunnan
L	Pop3	12	L4, L7, L9, L11, L15, L16, L20, L27, L33, L39, L43, L50	22°53'24"	102°24'43"	1880	Lvchun, Yunnan
P	Pop4	12	P3, P4, P7, P23, P26, P31, P33, P35, P38, P44, P48, P49	23°2'24"	103°31'48"	1721	Pingbian, Yunnan
LC	Pop5	12	LC4, LC10, LC12, LC17, LC18, LC20, LC25, LC34, LC35, LC38, LC41, LC48	22°54'36"	99°49'12"	1924	Lancang, Yunnan
YX	Pop6	12	YX2, YX3, YX5, YX6, YX8, YX12, YX13, YX16, YX18, YX19, YX23	24°17'24"	100°6'36"	1811	Yunxian, Yunnan
B	Pop7	12	B3, B6, B7, B11, B15, B19, B21, B24, B34, B38, B40, B42	24°49'48"	98°46'48"	1873	Baoshan, Yunnan
D	Pop8	12	D2, D3, D5, D6, D8, D14, D15, D27, D28, D35, D57, D58	24°45'0"	98°18'36"	1822	Lianghe, Yunnan

Table 2: Primer sequences, number of amplified and polymorphic bands, percentage of polymorphic bands, and PIC and MI values of RAPD primers used for genotyping *A. tsao-ko*

Primer name	Primer (5'-3') Sequence	TNB[a]	NPB[b]	PPB[c]	PIC[d]	MI[e]
OPA4	AATCGGGCTG	37.000	36.000	97.297	0.187	7.088
OPA7	GAAACGGGTG	26.000	26.000	100.000	0.184	4.776
OPA8	GTGACGTAGG	42.000	41.000	97.619	0.191	7.842
OPA9	GGGTAACGCC	52.000	52.000	100.000	0.173	9.006
OPA19	CAAACGTCGG	46.000	46.000	100.000	0.175	8.070
OPA20	GTTGCGATCC	41.000	41.000	100.000	0.179	7.330
OPB1	GTTTCGCTCC	50.000	50.000	100.000	0.212	10.622
OPB5	TGCGCCCTTC	32.000	31.000	96.875	0.157	5.018
OPB7	GGTGACGCAG	33.000	32.000	96.970	0.231	7.860
OPB18	CCACAGCAGT	43.000	43.000	100.000	0.239	10.275
OPB19	ACCCCCGAAG	45.000	44.000	97.778	0.212	9.315
OPC4	CCGCATCTAC	45.000	45.000	100.000	0.221	9.935
Mean		41.000	40.583	98.878	0.197	8.095
Total		492.000	487.000	98.984		

[a]TNB: total number of bands; [b]NPB: number of polymorphic bands; [c]PPB: percentage of polymorphic bands; [d]PIC: polymorphism information content; [e]MI: marker index

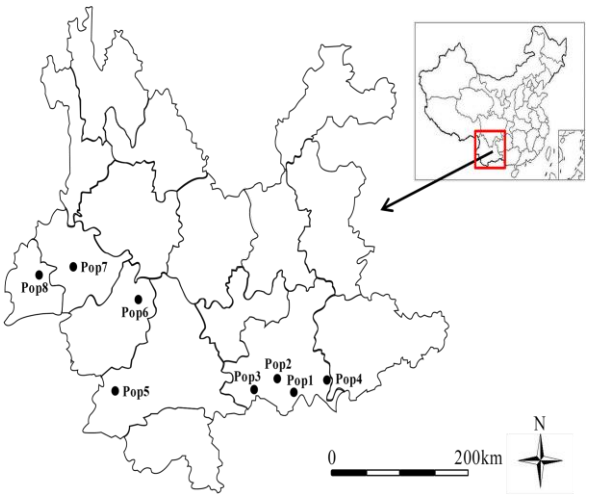

Fig. 2: Geographic distribution of eight *A. tsao-ko* populations in China. Further details of the locations are provided in Table 1

A clustering analysis was carried out with NTSYS-pc software version 2.10 (Rohlf, 2000). An equation on account of the similarity matrix of simple matching (SM) coefficients (Nei and Li, 1979) was constructed using UPGMA (the unweighted pair-wise method and the arithmetic average method). The modules DCENTER and EIGEN were implemented in NTSYS-pc for a principal coordinate analysis (PCoA) and the dispersion of accessions was visualized by two principal coordinates in a bi-dimensional array of eigenvectors. GenALEx software was used to perform analysis of molecular variance (AMOVA) (Peakall and Smouse, 2006). The correlation between geographic and genetic distance was estimated by Mantel test using NTSYS program, version 2.1 (Nei, 1972).

Results

Characteristics of RAPD Markers

Of total 120 RAPD primers were used for screening, 12 were able to produce consistent and distinguishable bands (Fig. 3). Table 2 showed characteristics of the 12 RAPD primers. 492 bands were amplified for 96 *A. tsao-ko* individuals,487 (98.98%) of them were polymorphic. The highest number of amplification bands was 52 (OPA9), and the lowest with 26 (OPA7) averaging 41 fragments per primer. PIC values ranged from 0.157 (OPB5) to 0.239 (OPB18) averaging 0.197. The highest MI was observed for the primer OPB1 (10.622) and the lowest for the primer OPA7 (4.776), with an average of 8.095.

Analysis of Genetic Diversity

Within populations, the percentage of polymorphic bands was 51.21 ~ 64.92% with an average of 57.18%, and H, 0.114 to 0.140 with an average of 0.121, and I, 0.189 to 0.232 with an average of 0.202. Average Na per locus was 1.572, and average Ne per locus was 1.180.Ne and polymorphic content were found highest in Pop3, and lowest in Pop4 (Table 3). These results revealed that *A. tsao-ko* had relatively low genetic diversity both within and among populations.

Genetic Differentiation in Populations

A. tsao-ko genetic differentiation analysis among eight populations exposed that Ht was 0.133, Hs was 0.121, Gst was 0.084, and Nm was 5.425 (Table 3). Most of the genetic variation (91.6%) appeared within the population.

Table 3: Mean values genetic diversity parameters for eight *A. tsao-ko* populations

Population ID	PPB[a]	Na[b]	Ne[c]	H[d]	I[e]	Ht[f]	Hs[g]	Gst[h]	Nm[i]	Hs/Ht
Pop1	61.90%	1.619	1.189	0.129	0.216					
Pop2	57.66%	1.577	1.172	0.119	0.199					
Pop3	64.92%	1.649	1.206	0.140	0.232					
Pop4	56.25%	1.563	1.168	0.115	0.193					
Pop5	51.21%	1.512	1.185	0.118	0.190					
Pop6	53.23%	1.532	1.169	0.114	0.189					
Pop7	54.23%	1.542	1.171	0.116	0.193					
Pop8	58.06%	1.581	1.178	0.121	0.202					
Average	57.18%	1.572	1.180	0.121	0.202					
Total	98.98%	1.990	1.187	0.133	0.235	0.133	0.121	0.084	5.425	0.916

[a]PPB:percentage of polymorphic bands; [b]Na:observed number of alleles; [c]Ne:effective number of alleles; [d]H:Nei's gene diversity; [e]I:Shannon's Information index; [f]Ht:total genetic diversity; [g]Hs:genetic diversity within populations; [h]Gst:genetic differentiation among populations; [i]Nm:gene flow estimated from Gst

Table 4: Analysis of molecular variance (AMOVA) based on RAPD markers in *A. tsao-ko*

Source of variation	Degrees of freedom (df)	Sum of squares (SS)	Estimate of variance components	Percentageof total variance (%)	P-value
Among Populations	7	863.573	4.506	6%	<0.001
Within Populations	88	6098.167	69.297	94%	<0.001
Total	95	6961.740	73.803	100%	

This can be further backed up by another molecular variance analysis (AMOVA), which revealed that variation within populations accounted for 94% of the entire variation ($p<0.001$) (Table 4). The genetic distance among populations of *A. tsao-ko* varied from 0.0094 to 0.0239 (Table 5). Pop4 and Pop7 had the shortest genetic distance, demonstrating a closer relationship between them. In contrast, Pop2 and Pop5 had the longest genetic distance, signifying a more distant relationship between them. Furthermore, the genetic distance and geographic distance did not show significant correlations by a Mantel test ($r = 0.153$, $P > 0.05$).

Cluster Analysis and Principal Coordinates Analysis (PCoA)

Revealed by the data from RAPD marker, the simple matching coefficient varied from 0.78 to 0.96 with an average of 0.8175. Fig. 4 depicts a dendrogram based on the similarity matrix. In this dendrogram, 96 individuals of *A. tsao-ko* were clustered into three major groups. Cluster I included 17 samples of *A. tsao-ko* from seven different populations (all except Pop7); Cluster II consisted of 48 samples; and cluster III comprised the remaining 31 samples. On the strength of the genetic similarity matrix, PCoA was conducted to further comprehend the genetic relationships among *A. tsao-ko* samples (Fig. 5). The first two principal components represented 10.52 and 5.82% of the entire molecular variation correspondingly. The PCoA plot revealed a similar grouping of samples to the dendrogram analysis.

Discussion

A. tsao-ko is an important crop with drug and diet attributes. It has been cultivated in China historically, especially in

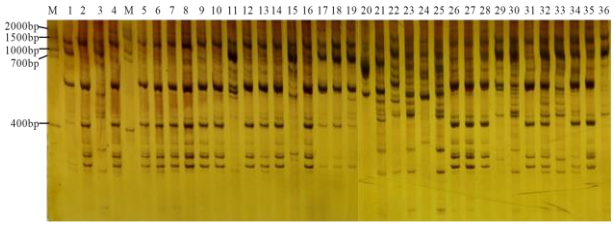

Fig. 3: RAPD profiles of *A. tsao-ko* generated by the primer OPA19. Note: Lanes from left to right: 1–12: J22, J24, J32, J43, J46, J50, J54, J70, J90, J99, J101, and J102;13–24:Y8, Y12, Y23, Y25, Y32, Y35, Y38, Y39, Y43, Y44, Y46, and Y50;15–36:L4, L7, L9, L11, L15, L16, L20, L27, L33, L39, L43, and L50. Lane M was for a DL2000 DNA marker

Yunnan Province (Yang *et al*., 2014). To date, few studies have applied molecular marker technology to study the genetic diversity of *A. tsao-ko*. In the present study, the genetic diversity of eight *A. tsao-ko* populations was found to be significantly different, but the level of diversity was lower than the average (0.22 or 0.23) genetic diversity of various other plant populations based on RAPD, AFLP, and ISSR dominant markers (Nybom, 2004). The results revealed that *A. tsao-ko* had relatively low genetic diversity, which is in accordance with that reported by Yang *et al*. (2014). In addition, low genetic diversity has also been detected in *A. Fructus* (Zhang *et al*., 2011). *A. tsao-ko* thrives in moist, well-drained, organic matter-rich soil in shade or partial shade. Owing to its strict environmental requirements, this plant is mainly distributed in Yunnan province, southwestern China (Wu and Larsen, 2000), and this narrow distribution may be the main reason for the low level of genetic diversity (Hamrick and Godt, 1996).

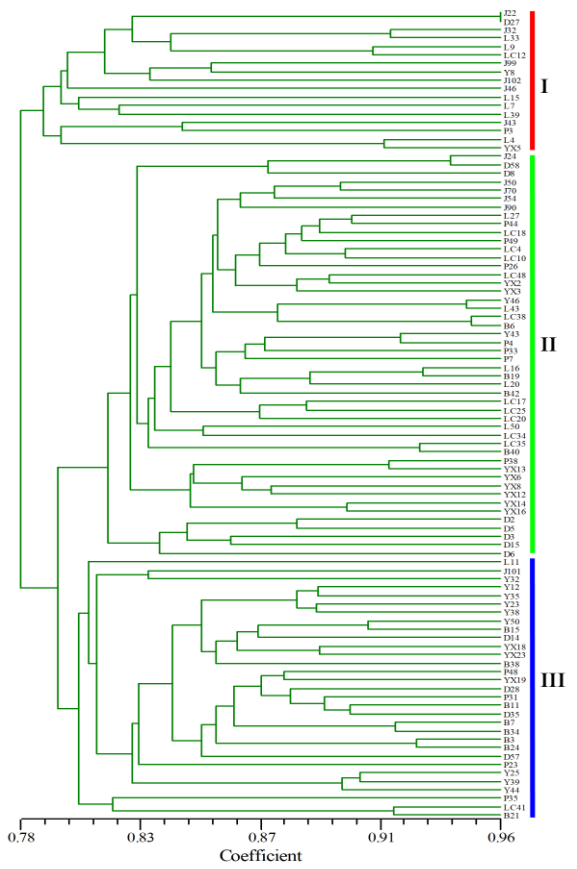

Fig. 4: UPGMA dendrogram of 96 *A. tsao-ko* samples based on RAPD molecular markers

Table 5: Nei's genetic identity (above the diagonal) and genetic distance (below diagonal) among populations of *A. tsao-ko*

Population	Pop1	Pop2	Pop3	Pop4	Pop5	Pop6	Pop7	Pop8
Pop1	****	0.9838	0.9899	0.9891	0.9821	0.9853	0.9856	0.9863
Pop2	0.0163	****	0.9834	0.9844	0.9764	0.9793	0.9856	0.9838
Pop3	0.0101	0.0167	****	0.9901	0.9849	0.9837	0.9879	0.9861
Pop4	0.0109	0.0158	0.0099	****	0.9858	0.9868	0.9906	0.9880
Pop5	0.0181	0.0239	0.0152	0.0143	****	0.9846	0.9856	0.9826
Pop6	0.0148	0.0210	0.0164	0.0133	0.0155	****	0.9866	0.9860
Pop7	0.0145	0.0145	0.0121	0.0094	0.0145	0.0135	****	0.9890
Pop8	0.0138	0.0163	0.0140	0.0121	0.0176	0.0141	0.0111	****

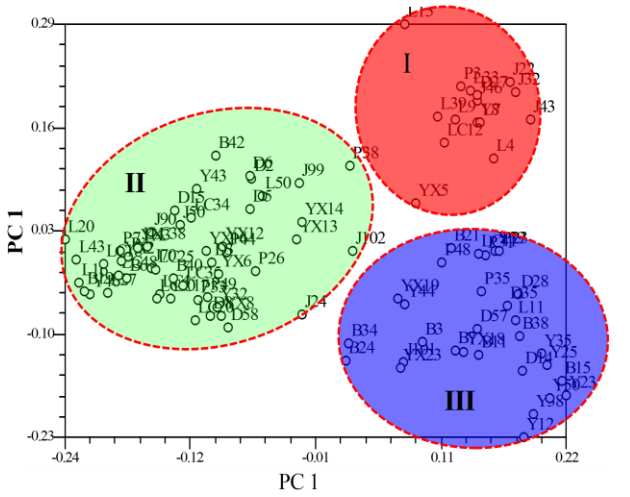

Fig. 5: Two-dimensional principal coordinates analysis created from RAPD-based genetic similarity estimates for 96 samples of *A. tsao-ko*. PC 1 and PC 2 represent the first and second principal coordinates, respectively

The current results reveal a low level of genetic diversity at the species level (H=0.133). Nei's gene diversity (H) was relatively low in Pop6 (Yunxian population) at 0.114, whereas maximum H (0.140) was observed in Pop3 (Lvchun population). *A. tsao-ko* cultivation (Pop1 and Pop3) has existed for more than 300 years in Yunnan, of which Honghe state has the longest cultivation history. Relatively strong artificial selection occurred during the process of introduction of the *A. tsao-ko* into other states, with individuals exhibiting high yield, high germination rate, and good disease resistance being more likely to be introduced, leading to reduced genetic diversity in other areas.

It is genetic variation available within a population that bestows a plant the evolutionary potential and ability to survive in tough environmental conditions. Both POPGENE (91.6%) and AMOVA (94%) analyses revealed that the majority of *A. tsao-ko* genetic differentiation existed within populations, which also indicated that this species is heterologous (Hamrick and Godt, 1996; Bussell, 1999). Hamrick (1987) reported 16 species of cross-pollinated plants with high gene flow (Nm). The Nm of *A. tsao-ko* among populations was nearly 4-fold greater than that of the 16 species, and this level of migration would prevent divisions among populations. Hartl and Clark (1997) noted that the influence of genetic drift exerted on genetic differentiation could be hindered by high gene flow. The two significant factors in genetic differentiation among populations are genetic drift and selection. The genetic drift can be prevented from causing genetic differentiation when Nm is above 1. The relatively low genetic differentiation and high gene flow in *A. tsao-ko* indicated both random mating among populations and that the species does not face genetic drift or population decline. Habitat heterogeneity is the major cause for the genetic differentiation of *A. tsao-ko* among populations. Wang (1994) noted the genetic identity from the same species but in different populations it was approximately 0.90 in spermatophytes. In this study, the genetic identity of *A. tsao-ko* between populations (0.9855) was higher than 0.90, which indicated that the genetic differentiation between populations was due to different growth environments. This result coincides with the hypothesis of high gene flow among *A. tsao-ko* populations.

Mantel test results showed no significant correlation

between geographical distance and genetic distance. This may be attributed to *A. tsao-ko* originating in Jinping County (Honghe State) and Maguan County (Wenshan State) gradually being introduced to other parts of Yunnan Province, which may have resulted in high levels of gene flow among populations over long geographic distance. Thus, the geographic distance was not a major factor in contributing to the degree of genetic differentiation among *A. tsao-ko* populations. Results of the Mantel test were also supported by genetic identity and cluster analysis. As indicated in the cluster analysis, eight populations of *A. tsao-ko* were divided into three major groups (Fig. 4). We noticed that it was a phylogeographic structure where populations near each other, such as Pop1 and Pop2, did not cluster together. There are two possible reasons for this phenomenon, one is due to the small sample size of each population and the other is a high level of gene flow and gene exchange (Wang *et al*., 2016).

In this study, the cluster analysis unequivocally divided 96 *A. tsao-ko* samples into three main groups. According to dendrograms, little or no location specificity existed among the *A. tsao-ko* genotypes, as reported for *Galega officinalis* (Wang *et al*., 2012) and *Ricinus communis* (Bhaveshb *et al*., 2010; Pecina-Quintero *et al*., 2013). Opportunities to migrate *A. tsao-ko* seedlings from growers in one area to the neighboring region or from growers in different agro-climates areas for the procurement of high-yielding plant material for *A. tsao-ko* farming are possible explanations for the above results.

The longstanding survival of a species largely counts on a high level of genetic diversity. As it stands, economic activities and artificial selection (seedling breeding process) are posing considerable threats to *A. tsao-ko* populations, which may face imminent extinction in lack of effective conservation measures. According to the results herein, populations with higher levels of genetic diversity, such as Pop1 and Pop3, should be protected first via *in situ* conservation. Further, it is necessary to establish a germplasm nursery in a native area such as Jinping County.

Conclusion

This study, first of its kind, has demonstrated that the RAPD is a reliable and powerful tool to evaluate genetic diversity and relationships among *A. tsao-ko* genotypes. At the population level, the percentage of polymorphic bands (51.21~64.92%) and short genetic distance (0.0094 to 0.0239) revealed the low genetic diversity in *A. tsao-ko*, which suggested that there is a need to bolster the conservation of *A. tsao-ko* resources.

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