Evaluation of Genetic Diversity Among Kongpo Monkshood (*Aconitum kongboense* L.) Germplasm Accessions Revealed by Inter Simple Sequence Repeat Markers

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Abstract. Inter simple sequence repeat (ISSR) were used to evaluate the genetic diversity of Kongpo Monkshood (*Aconitum kongboense* L.) in Motuo, Tibet Plateau. From 70 accessions of three populations, 10 out of 100 informative ISSR primers were chosen for polymorphism analysis. Percentage of polymorphic bands was 50% to 66.67% with a mean of 58.42%. The effective number of alleles (N_e) was between 1.545 (population 3) and 1.586 (population 2), and the mean value was 1.564; the Nei's gene diversity (h) ranged from 0.315 to 0.327 with the average value of 0.320; the value of Shannon's information index (I) ranged from 0.459 to 0.478, with the mean of 0.469. Based on molecular data, cluster analysis classified the 70 cultivars into three groups. Most accessions were related to the geographical origin and their genetic backgrounds. Bayesian structure and PCoA analysis were consistent with the dendrogram result. Based on the analysis, it will provide a reference for Kongpo Monkshood breeding purposes and contribute to identification, rational exploitation, and conservation of germplasms.

Kongpo Monkshood (*Aconitum kongboense* L.), native to China, are mostly used as folk medicine to treat arthritis pain (Meng et al., 2014; Xiao et al., 2006). It has a large genus with ≈ 300 species that widespread distribute in the temperate regions of the Northern Hemisphere. China, particularly in Hengduan Mountains region, is the most important center of diversity and speciation of this genus, such as Yunnan, Sichuan, and Xizang. The root tuber of this perennial plant possesses many kinds of diterpenoid alkaloids including vilmorrianine A, kongboenine, chasmaconitine, talatisamine (Wang, 2001). Now, mostly studies in Kongpo Monkshood were focus on its taxonomic (Wang et al., 2009), pharmacophylogenetic study (Xiao et al., 2006), and aconitine extraction (Beike et al., 2004).

ISSR marker is considered as a simple and quick technique that combines most of the

advantages of simple sequence repeats (SSRs) and randomly amplified polymorphic DNA (RAPD) (Linos et al., 2014; Reddy et al., 2002). To date, ISSR marker was highly polymorphism and widely used in genetic diversity, genome fingerprinting, phylogenetic, and evolution biology of different species (Mao and Fang, 2014; Zhang et al., 2014). Recently, studies on genetic diversity of *Aconitum carmichaeli* Debx (*Aconitum* L. genus) have been reported using RAPD (Zhang et al., 2009), random amplified microsatellite polymorphism (RAMP) (Hu et al., 2014) and amplified fragment length polymorphism (AFLP) (Meng et al., 2014). However, no report has been assessed for analyzing genetic diversity of Kongpo Monkshood germplasm using molecular markers.

The present investigation addresses to estimate the genetic diversity and variability among and within Kongpo Monkshood collected from the Tibet Plateau, China, using ISSR markers. Based on the analysis, it will provide a reference for Kongpo Monkshood breeding purposes and contribute to identification, rational exploitation, and conservation of germplasms.

Materials and Methods

Plant materials. A panel of 70 Kongpo Monkshood accessions from three different sites was collected in the Tibet Autonomous Region, China. The detailed description of

samples used in the present studies was shown in Table 1.

DNA extraction. For the all samples, fresh leaf tissues were randomly harvested from healthy and mature plant, and stored in sealed plastic bags. Then the sampled tissues were frozen in -80°C . Genomic DNA was isolated from 0.1 g leaf tissue using CTAB method of Doyle (1990). DNA was checked by electrophoresis on 1.0% agarose gels, and detected quantitatively by a spectrophotometer (ultraviolet-1800, Japan). Finally, DNA samples were diluted to 50 ng/ μL and then stored at -20°C for polymerized chain reaction (PCR) amplification.

ISSR markers and PCR amplification. ISSR reaction was performed as previously described by Wang et al. (2013) with minor modifications. The ISSR-PCR optimized reaction conditions included a total volume of 25 μL containing 50 ng template DNA, 10 pmol each primer, 1x PCR Buffer, 0.25 mM of each dNTP, 2.5 mM Mg^{2+} , and 0.2 U Taq DNA polymerase (TakaRa, Japan). All amplification reactions were conducted with a PCR Thermal Cycler Dice (TakaRa, Japan). The cycling conditions were as follows: 5 min at 94°C for an initiation step, 40 cycles of 1 min at 94°C , 1 min at a primer-appropriate temperature, 1 min at 72°C , and a final cycle of 10 min at 72°C . PCR products were detected by 2.0% agarose gel with 0.5 $\mu\text{L}/\text{mL}$ GelRed and photographed by Bio-image Systems (GG/D2-GeneGnius2).

Analysis of amplification profiles. Each bands that were amplified with ISSR primers, were scored manually as present (1) or absent (0) in each primer to produce a set of binary code for ISSR analysis. Only those fragments that were reproducible and clear were considered for scoring. To estimate the genetic diversity of Kongpo Monkshood, genetic parameters including observed number of alleles (N_a), effective number of alleles (N_e), Nei's gene diversity (h), Shannon's information index (I), the number of polymorphic loci (NPL), and the percentage of polymorphic loci (PPL) of populations, Nei's genetic differentiation index (G_{st}), and gene flow (N_m) were calculated using PopGene32 software (version 1.32). The molecular marker data were analyzed at both individual and population levels. Genetic variances among and within populations were analyzed to assign components of genetic variation to hierarchical sets of populations using analysis of molecular variance (AMOVA) 1.55 software (Yeh et al., 1999). To assess the genetic relationships among individuals, unweighted pair group method with arithmetic mean (UPGMA) and principle coordinate analysis (PCoA) were performed based on Nei's genetic distance (Nei, 1973) using the NTSYS-pc version 2.10s software (Rohlf, 2000) to generate three-dimensional (3D) scatter plots. Correlation analysis was carried out with the SPSS statistics software 17.0 (Nie et al., 1975).

Results

ISSR amplification. In this work, 10 out of 100 ISSR primers were used here, which could product clear, identifiable, reproducible, and relatively high polymorphism. One hundred and one clearly identifiable bands in a size

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Table 1. Locations and sample size of 70 accessions in the study.

Population no.	Code	Sample size	Location	Longitude (E)	Latitude (N)	Altitude (m)
1	s	15	Jiazha village, Cuijiu country, Tibet, China	29.29	92.72	3,260
2	k	26	Langkazi village, Kare country, Tibet, China	29.26	90.42	4,500
3	m	29	Zhaxue village, Mozu country, Tibet, China	30.08	91.77	4,000

Table 2. Information on inter simple sequence repeat primers among three populations used in this study.

Primer code	Primer sequence (5'-3')	Annealing temp (°C)	No. of total amplified bands	No. of total polymorphic amplified bands	Percentage of polymorphic bands (%)	Range of the bands size (bp)
UBC 808	(AG) ₈ -C	52	12	7	58.33	1,900~550
UBC 811	(GA) ₈ -C	52	10	6	60.00	1,750~500
UBC 825	(AC) ₈ -T	52	7	4	57.14	1,950~600
UBC 834	(AG) ₈ -YT	52	15	10	66.67	2,000~500
UBC 835	(AG) ₈ -YC	52	10	6	60.00	1,900~750
UBC 841	(GA) ₈ -YC	53.5	11	6	54.55	1,900~600
UBC 847	(CA) ₈ -RC	54	7	4	57.14	1,800~600
UBC 848	(CA) ₈ -RG	53.5	13	8	61.54	2,000~550
UBC 850	(GT) ₈ -YC	54	8	4	50.00	1,800~650
UBC 855	(AC) ₈ -YT	53.5	8	4	50.00	1,900~700
Total	—	—	101.00	59.00	—	2,000~500
Mean	—	—	10.10	5.90	58.42	—

N = (A, G, C, T), R = (A, G), Y = (C, T).

range from 500 to 2000 base pair (bp) were obtained using the 10 primers (Table 2). Among the amplified fragments, 59 bands were polymorphic ranged from 4 to 10 with an average of 5.9 per primers, and the percentage of polymorphism were 50% to 66.67% with a mean of 58.42%. From the 10 ISSR primers, no population-specific fragments were observed.

The genetic diversity analysis was tested according to geographic area, and its results were shown in Table 3. All parameters showed that there were no significant differences among three areas. The observed number of alleles (N_a) of the three geographical groups was ranked as follows: population 3 > population 2 > population 1. The effective number of alleles (N_e) was between 1.545 (population 3) and 1.586 (population 2); the Nei's gene diversity (h) ranged from 0.315 to 0.327 with the average value of 0.320; the value of Shannon's information index (I) ranged from 0.459 to 0.478; the percentage of polymorphic loci of 70 accessions had a higher value, ranging from 77.59% to 87.93%, with an average of 82.76%. In addition, Nei's genetic differentiation index (G_{st}) was 0.1942, and the estimate of gene flow was more than 1 (N_m , 2.0750).

Correlation analysis showed that the differences in Nei's genetic diversity among the three populations were highly significant negatively related to their geographical distances ($r = -1.00$, $P < 0.01$) (Fig. 1A), whereas it was significantly correlated with the location altitude ($r = 0.95$, $P < 0.05$) (Fig. 1B).

Genetic variation and genetic relatedness assay of *Aconitum kongboense*. To illustrate the genetic variation among and within populations, AMOVA was carried out, and the results were presented in Table 4. Of the total genetic diversity, 22.25% of genetic variation resided among populations, whereas 77.75% existed within populations. Consequently, genetic variation of *A. kongboense* mainly existed in within populations.

Genetic relationship assays of the 70 *A. kongboense* accessions were conducted using UPGMA dendrogram based on Jaccard's similarity coefficients (Fig. 2). The similarity

Table 3. the genetic parameters of tested populations from different geographic areas in the study.

Population name	N_a	N_e	h	I	NPL	PPL (%)
Population 1	1.776 ± 0.421	1.560 ± 0.368	0.315 ± 0.191	0.459 ± 0.268	45	77.59
Population 2	1.828 ± 0.381	1.586 ± 0.373	0.327 ± 0.185	0.478 ± 0.253	48	82.76
Population 3	1.879 ± 0.329	1.545 ± 0.356	0.319 ± 0.170	0.470 ± 0.227	51	87.93
Mean	1.828	1.564	0.320	0.469	48	82.76

N_a : observed number of alleles, N_e : effective number of alleles, h : Nei's gene diversity, I : Shannon's information index, NPL: the number of polymorphic loci, PPL: the percentage of polymorphic loci.

Fig. 1. Correlation analysis between Nei's genetic diversity with samples collected at geographical distance (A) and site's altitude (B).

Fig. 2. Dendrogram based on Jaccard's coefficient. s1-s15: samples from Jiazha village, k1-k29: samples from Langkazi village, m1-m26: samples from Zhaxue village.

coefficients among all populations ranged from 0.77 to 0.97, with an average value of 0.87. According to UPGMA dendrogram analysis, 70 samples were clustered into three groups in a common node at similarity coefficient of 0.80. Group I included 29 accessions, with 5 from Jiazha village, 6 from Langkazi village, and 18 from Zhaxue village. Twenty cultivars from Langkazi village belonged to Group II. Group III consisted of 21 samples, 10 from Jiazha village and 11 from Langkazi village. Furthermore, Group III was further subdivided into two subgroups. Ten *A. kongboense* cultivars were in the subgroup A together. Subgroup B included 11 populations from Langkazi village. There appeared to be significant sampling localities clustering overall.

To analyze hierarchical population structure of all samples, a Bayesian structure was performed by structure 2.1 based on a mathematical model (Fig. 3). The results demonstrated that model with $K = 3$ explained the data satisfactorily, suggesting that the most likely number of accessions was three, which calculated by the log probability of data for the value of K . Red, blue, and green color vertical bars represented the genotypes. Additionally, in the PCoA analysis, 70 individuals were clearly separated into three distinct groups, which in was accordant with the UPGMA cluster analysis (Fig. 4).

Discussions

ISSR is known as its effectiveness and practicability for identifying genetic diversity and assessing genetic variations in many plant species (Wang et al., 2013; Zhao et al., 2014). Therefore, in the present study, ISSR markers were the firstly used to analyze the genetic diversity of Kongpo Monkhood cultivars, and our results showed that high polymorphism existed in these germplasm.

Genetic diversity plays an important role in plant evolution; both adaptation and speciation depend upon genetic diversity (Amos and Harwood, 1998; Jiang et al., 2012). In this study, based on ISSR data, a higher percentage of polymorphism (58.42%) was found in the 70 individuals, suggesting that accessions possessed a relatively high degree of genetic diversity (Table 2). However, the degree of diversity was lower than the values previously reported in other species with the same family, such as *A. carmichaeli* Debx (83.5%) (Tian, 2007), *A. leucostomum* (88.1%) (Gao et al., 2014). The possible reason was due to long-term hostile environmental conditions and disturbance by human activities (immoderate collection). As we know, Qinghai-Tibet plateau, averaging over 4000 m above sea level and covering an area of 200,000 km², has the complex geological features with vagaries of climate. Although grown in this area, the same species, however, possessed significant difference in genetic diversity (Wang and Ding, 2007).

At the population level (Table 3), in population 1, the lowest indices, including N_a (1.776), h (0.315), I (0.459), NPL (45),

Table 4. Analysis of molecular variance for 70 individuals used in this study.

Source of variance	df	Sum of squared deviations	Mean squares	Variance component	Percentage total (%)	<i>P</i> value*
Among populations	2	794.77	397.39	2.77	22.25	<0.001
Within populations	69	650.12	9.42	9.70	77.75	<0.001

df = degrees of freedom.

Fig. 3. Bayesian analysis of population structure for $K = 3$ based on ISSR data. Each vertical bar represents a genotype. 1–15: samples from Jiazha village, 16–41: samples from Langkazi village, 42–70: samples from Zhaxue village.

Fig. 4. Principal coordinates analysis for the inter simple sequence repeat evolution of Kongpo Monkhood accessions.

PPL (77.59), were detected in this group while some parameters were the highest in population 3 with N_a (1.879), NPL (0.319), PPL (87.93%). Actually, population 2 also had some high values, such as N_e , h , I . Compared with the lower polymorphisms at RAPD loci reported in *A. carmichaeli* Debx accessions, Zhang et al. (2009) found polymorphic proportion with 24.95%, 50.63%, and 50.46% in different populations. Hu et al. (2014) estimated that this species possessed percentage of polymorphism with a value of 78.2%. Therefore, our study results had a higher value of polymorphism, implying that the diversity of each population was possibly affected by its location. Furthermore, the higher value could be attributed to a wide range of geographical distribution, which has been shown to effectively change in a large population (Hamrick and Godt, 1996).

Correlation analysis showed significantly related to Nei's genetic diversity of all populations and their corresponding location altitudes ($r = 0.95$, $P < 0.05$) (Fig. 1), which further confirmed the higher value caused by geographical distribution. Guan et al. (2014) concluded that sites with higher altitudes (4000–5000 m) were much more evolution and genetic variability than those from lower altitudes. In this study, both population 2 and population 3 collected from higher altitudes that tolerate by strongly intensity of ultra-violet radiation and could be induced to produce a variety of genetic variations. Therefore, these two populations possessed higher genetic diversities. On the contrary, population 3 is distributed in a lower altitude, which could be largely affected by human activities. Because geographical distances is a vital factor for driving genetic diversity of populations, the correlation analysis was also detected in our

work ($r = -1.00$, $P < 0.01$). A highly significant negative correlation was obtained in the populations (Fig. 1A). The same point could be found in Wang et al. (2013) and Guan et al. (2014). The significant result suggested that samples collected in different places have a large influence on the population structure of Kongpo Monkshood.

A high level of genetic variability is seen as health indicator and confers the ability to respond to environmental changes (Amos and Harwood, 1998; Fielder et al., 2015). Estimates within population variation based on the ISSR markers were higher than the values with dominant markers (AFLP) (Meng et al., 2014), which was 52.77% of genetic variation resided among populations and 47.23% existed within populations in AFLP, whereas 22.25% and 77.75% in corresponding parts with ISSR markers, respectively (Table 4), and our results were consistent with a lower *Gst* values (0.1942). Additionally, *Nm* was higher than 1 and suggested strong gene flow among widely distributed populations. As the high variability of mostly molecular markers (RAPD, ISSR, AFLP, or SSR), the values of within population variation were often much higher than those in among population variation (Fang et al., 2012; Nybom, 2004).

Aconitum L. is difficult to classify even though at level of genus, because of different classification standard, species traits' variation, and lacking of correlation among them. Therefore, researches have conducted many works in taxonomical study of *Aconitum* L., including classic taxonomy, morphology, cytology, molecular systematic, and biochemistry in traditionally (Luo et al., 2005; Song et al., 2012; Xiao et al., 2006). However, this species classification has not reached a consensus so far. Now, cluster analysis was considered as an effective tool for cultivar classification (Fang et al., 2012). Some results were obtained the cultivar classification based on molecular markers (Tian et al., 2007). Based on ISSR data, three clustering methods used in this study were generated for revealing clear relationship among populations. The analysis results suggested groups significantly related to their geographical area, which reflected the geographic distribution patterns in Kongpo Monkshood (Fig. 2). Group I, II, III populations, in the UPGMA dendrogram, cultivated on the high altitudes area ≈ 4000 m, 4500 m, 3260 m, respectively. For Bayesian clustering (Fig. 3) and PCoA analysis (Fig. 4), the results of relationship were consistent with the dendrogram. Interestingly, the phylogenetic analysis of ISSR was agreement with known pedigrees and previous marker evolution (Luo et al., 2005; Tian et al., 2007). The genetic relationship revealed by ISSR markers should thus be beneficial to Kongpo Monkshood breeding programs in the future.

This study, the first report on Kongpo Monkshood from Tibet Plateau, can contribute to our knowledge of genetic diversity and relationship among this species. Using ISSR markers, we efficiently and successfully detected genetic variation among Kongpo Monkshood individuals. Finally, our findings will help for breeding and conservation of Kongpo Monkshood.

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