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# Influences of harvesting on genetic diversity and population structure of *Anemone altaica* (Ranunculaceae), a traditional Chinese medicinal herb

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ABSTRACT

The genetic diversity and population structure of *Anemone altaica*, an important medicinal herb used in traditional Chinese medicine, were estimated using ISSR markers within eight populations in Qinling Mountains, Northwestern China. The results showed that *A. altaica* has a relatively high genetic diversity ($PPB = 89.9\%$, $H_e = 0.293$) at species level, and within populations ($PPB = 61.7\%$, $H_e = 0.215$). Based on analyses of $G_{ST}$, AMOVA and gene flow a significant genetic differentiation was revealed. Influences of human harvesting activities on genetic diversity and population structures of *A. altaica* were determined by comparing their difference between over harvesting populations and low harvesting populations. At last, operative management policies for sustainable use of this species is suggested in this study.


## 1. Introduction

*Anemone altaica* Fisch. ex C. A. Mey (Ranunculaceae), an important medicinal herb, is widely found in deciduous forests and shrubs at elevations between 1200 and 1800 m crossing South-East Europe, Russia to North Asia (Wang, 1980). The rhizome of *A. altaica* is commonly used to treat epilepsy, neurasthenia and rheumatoid arthritis in the traditional Chinese medicine (Wu et al., 1988). Pharmacological and phytochemical studies reveal that *A. altaica* has a great potential in developing new medicines for cardiovascular and cerebral vascular diseases (Zou et al., 2005; Shi et al., 2008; Wang, 2008). Due to the rapidly increasing demands for this medical material, over-harvesting of wild resources has led to a remarkable decrease in the population size of *A. altaica* in field (Wang, 2007; Yuan, 2009; Zhu, 2009).

As plant populations become small, they are at increased risk of extinction as a result of population demographic shifts, increased inbreeding, and genetic isolation and drift. Economically important plants extracted from the wild are at additional risk, because harvest further reduces effective population size and compounds loss of genetic diversity and the threat of extinction (Cruse-Sanders et al., 2005). Populations of harvested plants may go extinct more rapidly due to over-harvesting than they do from population fragmentation and habitat destruction alone (Vance, 2002). Assessment of genetic diversity within populations of harvested plants contributes vital information regarding its evolutionary history and is critical to the development of effective conservation and management practice. In this study, we conducted an assessment of genetic diversity among populations of *A. altaica* in the Qinling Mountains, China, using the internal simple sequence repeat (ISSR) markers which were broadly developed and applied in the genetic diversity investigation at population level

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(Chen et al., 2009; Zhang and Dai, 2010). Results here provide critical information regarding the level and distribution of genetic diversity among populations and the development of effective conservation and management practice for *A. altaica*.

## 2. Materials and methods

### 2.1. Anemone altaica

*A. altaica* is a perennial spring ephemeral with prostrate rhizome, solitary and ternate leaf. In early spring (about end March to early April), each apical bud of a rhizome develops a single sprout, some with one terminal flower and a whorl of three palmately dissected stem leaves. The above-ground parts will shrivel after seed maturation about end May to early June. Both vegetative and sexual reproductions play a crucial role in established populations. Human harvesting activities always create more opportunities to its vegetative reproduction.

The Qinling Mountains is presently serving as one of the main geographical areas of *A. altaica* supply. It is located between 104°30′-112°52′ E and 32°50′-34°45′ N. It is the geographic boundary of subtropics and temperate zone in China (Zhu, 1958; Zhou et al., 2011). Deciduous broad-leaved forests are the main vegetation on the northern slope whereas the evergreen broad-leaved forests cover the southern slope. *A. altaica* is principally found on the northern slope. Deforestation and habitat fragmentation have also occurred in the study area.

### 2.2. Samples collection

The leaflets of *A. altaica* were collected from 160 individuals covering 8 populations (two in Longtangou valley, two in Geqigou valley, and four in Shanxigou valley) on the northern slope of the Qinling Mountains, China. In each population, 20 individuals were randomly selected for sample collection. To avoid resampling from the same clone, we ensure that the distance between individuals was more than 5 m. The leaf tissues were dried with silica gel and stored at room temperature till DNA extraction.

To compare the influence of human harvesting activities on genetic diversity of *A. altaica* at the population level, four populations considered as “over-harvesting group” (including populations LH, GH, SH1, and SH2, see Table 1) because they frequently were over-collected and four populations considered as “low-harvesting group” (including populations LL, GL, SL1, and SL2, see Table 1) because they scarcely affected by harvesting activities.

### 2.3. DNA isolation and PCR-ISSR amplification

Total genomic DNA was extracted form dried leaves following a modified CTAB method (see Doyle, 1991). One hundred ISSR primer sequences were obtained from the University of British Columbia (UBC Primer Set #9, Biotechnology Laboratory). Primers were initially screened using 16 samples (2 randomly selected from each population) to test their efficiency of amplification, and primers which can generate clear and reproducible banding patterns were selected for further analyses. PCR reaction were carried out in 20 ul volume of 1 × Promega (Madison, Wisconson, USA) Taq buffers and 1 unit of Taq polymerase, 50–100 ng template DNA, 2.5 mM $MgCl_2$, 0.4 uM of both forward and reverse primers, and 0.2 mM dNTPs, with the following cycle program: initial denaturation at 95 °C for 3 min, followed by 30 s at 95 °C, 45s annealing at 50–53 °C, and a 2 min extension step at 72 °C for 40 cycles, then a final extension step for 10 min at 72 °C. Amplification products were electrophoretically separated in 1.6% agarose gels buffered with 0.5% TBE. A 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used as a size marker.

### 2.4. Data analysis

For each of the ISSR primers, only distinct, reproducible and well-resolved fragments were scored as the presence (1) and absence (0) with same molecular weight (bp) to form a binary matrix. Genetic diversity parameters, including the percentage of polymorphic loci (*PPB*), observed number of alleles per locus ($A_n$), the effective number of alleles per locus

**Table 1**
Information on the eight populations sampled for ISSR analysis.

| Site | Population | Sample size | Altitude (m) | Latitude (N) | Longitude (E) | Collecting intensity |
|---|---|---|---|---|---|---|
| Longtan valley | LH | 20 | 1470 | 34°20′ | 106°29′ | Over-harvesting |
| | LL | 20 | 1740 | 34°18′ | 106°20′ | Low-harvesting |
| Geqigou valley | GH | 20 | 1539 | 34°18′ | 106°37′ | Over-harvesting |
| | GL | 20 | 1625 | 34°18′ | 106°37′ | Low-harvesting |
| Shanxigou valley | SH1 | 20 | 1460 | 34°19′ | 106°37′ | Over-harvesting |
| | SL1 | 20 | 1460 | 34°19′ | 106°37′ | Low-harvesting |
| | SH2 | 20 | 1250 | 34°20′ | 106°37′ | Over-harvesting |
| | SL2 | 20 | 1250 | 34°20′ | 106°37′ | Low-harvesting |

($A_e$), Nei's (1973) gene diversity ($H_e$, namely expected heterozygosity) were estimated and analyzed using a computer program POPGENE 1.31 (Yeh et al., 1999). The Shannon's diversity index ($H_o$) was also calculated for ISSR diploid data according to $H_o = -\sum pilog_2(pi)$, in which $pi$ is the frequency of a given ISSR fragment (Lewinton, 1972). To further analyze population genetic structure, the Nei's coefficient of genetic differentiation ($G_{ST}$) was also calculated and the average gene flow was estimated from the formula: $Nm = 0.25(1 - G_{ST})/G_{ST}$ among the populations. Genetic distance between populations (Nei, 1973) was computed using this program. In addition, an analysis of molecular variance (AMOVA) was applied to estimate variance within and among eight populations based on ISSR phenotypes using GenALEX 6.0 version (Peakall and Smouse, 2006). Unpaired two-tailed Student $t$ test was used to determine the differences of genetic diversity between populations.

## 3. Results

### 3.1. Genetic diversity

In total, fifteen primer pairs (UBC # 808, 811, 812, 815, 824, 834, 835, 836, 840, 841, 843, 844, 847, 853 and 857) which can generate clear and reproducible banding patterns were selected for the final analysis. The fifteen primer pairs produced a total of 109 bands (loci) ranging from 180 bp to 1500 bp in size throughout eight populations. Mean for per primer pair was 7.3 ± 2.890 (SD), with a range from 3 to 14 bands. The *PPB* value was 89.9% and the $A_n$ and $A_e$ values were 1.889 and 1.492, respectively, at species level (Table 2). Assuming Hardy–Weinberg equilibrium is applicable, the $H_o$ and $H_e$ values were 0.444 and 0.293, respectively.

At population level, the average *PPB* value was 61.7%, ranging from 54.1% (within the population LH) to 67.9% (within the population SL1). The $A_n$ values ranged from 1.440 to 1.578 (mean = 1.524). The average $A_e$ per locus was 1.369 ranging from 1.308 to 1.412. Among the eight populations, the population LL exhibited the highest genetic diversity ($H_o = 0.355$; $H_e = 0.239$), whereas the population LH exhibited the lowest genetic diversity ($H_o = 0.270$; $H_e = 0.180$) (see Table 3).

### 3.2. Genetic differentiation

The $G_{ST}$ value was 0.266 across the all polymorphic loci, indicating 26.6% of total genetic variation was distributed among populations and 73.4% of total genetic variation was distributed within populations (see Table 2). According to the classification proposed by Wright (1951) the genetic variation estimate of *A. altaica* among populations was high, indicating a high genetic differentiation at species level. The AMOVA analysis showed that the significant variations were identified both within and among populations with 28% of the variation was within populations (72%, $p < 0.001$)(see Table 3), in accordance with $G_{ST}$ analysis. The overall gene flow (Nm) was 0.69 among populations, showing a low migration rate between populations each generation. Nei's Genetic distance (D) between populations varied from 0.054 (between SL1 and SH1) to 0.208 (between LL and SL2), with an average of 0.121.

### 3.3. Influence of harvesting on genetic diversity

To investigate whether human harvesting activity has resulted in the genetic differentiation of *A. altaica*, we compared the differences of genetic diversity between the "low harvesting group" and the "over harvesting group" by an unpaired two-tailed Student $t$ test using SPSS 16.0. Results showed that there was no a significant difference between the "low harvesting group" and the "over harvesting group", but all diversity indexes *PPB*, $A_n$, $A_e$, $H_o$ and $H_e$ were lower in "over harvesting group" compared to the "low harvesting group". Further, we analyzed the genetic differentiation ($G_{ST}$) within two groups the "low harvesting group" and the "over harvesting group". The $G_{ST}$ values were 0.232, 0.259, respectively, suggesting that genetic differentiation within the over harvesting group is higher compared to low harvesting group (see Table 2). The AMOVA analysis within two groups (the "low harvesting group" and the "over harvesting group") showed that the major variations were distributed within populations (79%, $p < 0.001$; 73%, $p < 0.001$, respectively) and the genetic differentiation among populations of over harvesting group was slightly higher (see Table 4).

**Table 2**
Summary of mean genetic diversity at the species and population levels for *A. Altaica*. Genetic diversity statistics are percent polymorphic loci (*PPB*); number of alleles ($A_n$); effective number of alleles ($A_e$); Shannon's diversity index ($H_o$); expected heterozygosity ($H_e$). Genetic structure, $G_{ST}$, is the Nei's coefficient of genetic differentiation. The estimated number of migrants per generation, Nm, is calculated as $Nm = (1\text{-}G_{ST})/4G_{ST}$.

| | *PPB* | $A_n$ | $A_e$ | $H_o$ | $H_e$ | $G_{ST}$ | Nm |
|---|---|---|---|---|---|---|---|
| Species level | 89.91 | 1.899 | 1.492 | 0.444 | 0.293 | 0.266 | 0.69 |
| Population level (mean) | 61.70 | 1.524 | 1.369 | 0.322 | 0.215 | – | – |
| Low harvesting (mean) | 65.83 | 1.569 | 1.401 | 0.348 | 0.233 | 0.232 | – |
| Over harvesting (mean) | 57.57 | 1.479 | 1.334 | 0.296 | 0.198 | 0.259 | – |

**Table 3**
Summary of within-population genetic diversity. Genetic diversity statistics are described in the text and in Table 2.

| Population | *PPB* | $A_n$ | $A_e$ | $H_o$ (SD) | $H_e$ (SD) |
|---|---|---|---|---|---|
| LL | 66.06 | 1.569 | 1.412 | 0.355 (0.027) | 0.239 (0.019) |
| GL | 64.22 | 1.578 | 1.395 | 0.341 (0.027) | 0.229 (0.019) |
| SL1 | 67.89 | 1.578 | 1.405 | 0.354 (0.027) | 0.237 (0.019) |
| SL2 | 65.14 | 1.550 | 1.392 | 0.341 (0.027) | 0.228 (0.019) |
| LH | 54.13 | 1.440 | 1.308 | 0.270 (0.027) | 0.180 (0.019) |
| GH | 60.55 | 1.532 | 1.365 | 0.317 (0.028) | 0.213 (0.019) |
| SH1 | 56.88 | 1.431 | 1.327 | 0.293 (0.027) | 0.194 (0.019) |
| SH2 | 58.72 | 1.514 | 1.346 | 0.305 (0.027) | 0.203 (0.019) |
| Average | 61.70 | 1.524 | 1.369 | 0.322 (0.010) | 0.215 (0.007) |

## 4. Discussion

### 4.1. Genetic diversity and structure

Due to affordable cost, technical easiness and high reproducibility (Gupta et al., 1994), ISSR has broadly been applied in studies of population genetic diversity (Hu et al., 2010; Li et al., 2011) and germplasm identification (Meng et al., 2011). Our current ISSR assay revealed that *A. altaica* harbored rich genetic diversity with *PPB* = 89.9%, which was slightly higher than other species in genus *Anemone* such as *Anemone coronaria* (*PPB* = 80.0%, see Yonash et al., 2004) and *Anemonenemorosa* (*PPB* = 73.4%, see Rusterholz et al., 2009). Based on morphological characters of *A. altaica* flowers (including yellow petals and nectar glands) and our field observation, out-breeding might be the main breeding system under natural condition. Generally, maintaining a high genetic diversity in natural populations is an adaptation strategy of a species to environmental heterogeneity (Hedrick, 1976). In ecological sense, the high genetic diversity of *A. altaica* may help it with adapting to capricious weather in early spring in these areas. The high genetic diversity of *A. altaica* may result from its evolutionary history.

Many factors, such as habitat fragmentation, genetic draft, mating systems, gene flow, and selection contribute greatly to the genetic differentiation of a plant species (Schaal et al., 1998; Templeton, 2006; Zhao et al., 2007). In this study, our results from both $G_{ST}$ and AMOVA analyses revealed that the significant genetic differentiation occurred in *A. altaica*. Accordingly, the gene flow (Nm) between populations was low (0.69), which could result in a great genetic differentiation between populations. Based on our field observation, most individuals were annually harvested before setting seeds for many years. Human harvesting activity may be the main factor which causes the genetic differentiation of *A. altaica* in this area.

### 4.2. Influences of harvesting on genetic diversity and conservational implication

Populations of over-harvested plants may go extinct more rapidly than those suffer from population fragmentation and habitat destruction alone (Vance, 2002; Cruse-Sanders et al., 2005). In this study, all genetic diversity indices were lower in the over-harvesting group compared to the low-harvesting group. The genetic differentiation is greater in the over-harvesting group ($G_{ST} = 0.259$) than the over-harvesting group ($G_{ST} = 0.232$). These results reflected that human harvesting pressure had, to certain degree, if not too much, influenced the genetic diversity of *A. altaica*.

It is necessary to maintain as much genetic diversity of *A. altaica* as possible for the conservation and sustainable use of this species in practice. Setting collecting quota restrictions and operating rotation harvesting system among the main valleys may

**Table 4**
Analysis of molecular variance (AMOVA) within/among populations.

| Source of variation | *d.f.* | SSD | MSD | Variance component | % total | $p^*$ |
|---|---|---|---|---|---|---|
| All populations | | | | | | |
| Among populations | 7 | 748.21 | 106.887 | 4.727 | 28% | <0.001 |
| Within populations | 152 | 1875.65 | 12.340 | 12.340 | 72% | <0.001 |
| Low vs. over | 1 | 83.069 | 83.069 | 0.000 | 0% | <0.001 |
| Among populations | 6 | 665.138 | 110.856 | 4.926 | 29% | <0.001 |
| Within populations | 152 | 1875.650 | 12.340 | 12.340 | 71% | <0.001 |
| Low | | | | | | |
| Among populations | 3 | 321.188 | 107.063 | 4.707 | 27% | <0.001 |
| Within populations | 76 | 982.550 | 12.928 | 12.928 | 73% | <0.001 |
| Over | | | | | | |
| Among populations | 3 | 343.950 | 114.650 | 5.145 | 30% | <0.001 |
| Within populations | 76 | 893.100 | 11.751 | 11.751 | 70% | <0.001 |

Low: low-harvesting group; Over: over-harvesting group.
*d.f.*: degrees of freedom; SSD: sum of squares; MSD: mean squared deviation.
* Significance tests after 1000 permutation.

be efficient to decrease the influences of over-harvesting in some areas. Operative management policies should focus on regulating the collecting intensity and switching harvesting time for sustainable use of *A. altaica*.

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