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Genetic variation in wild populations of the tuber crop *Amorphophallus konjac* (Araceae) in central China as revealed by AFLP markers

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ABSTRACT. *Amorphophallus konjac* is an economically important crop. In order to provide baseline information for sustainable development and conservation of the wild plant resources of *A. konjac*, we studied the genetic diversity and population structure of this species using amplified fragment length polymorphism (AFLP) molecular markers. We sampled 139 individuals from 10 wild populations of *A. konjac* in central China. Using five AFLP primer combinations, we scored a total of 270 DNA fragments, most of which were polymorphic (98.2%). Percentage of polymorphic loci, Nei's genetic diversity index, and Shannon's information index showed high levels of genetic variation within *A. konjac* populations. Analysis of molecular variance indicated that most of the variance (68%) resided within populations. The coefficient of genetic differentiation between populations was 0.348 and the estimated gene flow was 0.469, indicating that there was limited gene flow among the populations. Unweighted pair group method with arithmetic mean cluster analysis and principal coordinates analysis indicated that geographically close populations were more likely

to cluster together. The Mantel test revealed a significant correlation between geographic and genetic distances (R^2 = 0.2521, P < 0.05). The special insect-pollination system of *A*. *konjac* and the complex geography of central China are likely to have contributed to the current pattern of genetic variation of this species. In the present study, we provide several suggestions on the future protection of the wild plant genetic resources of *A. konjac*.

Key words: AFLP; *Amorphophallus konjac*; Crop; Genetic diversity; Central China

INTRODUCTION

Amorphophallus konjac K. Koch ex N. E. Br., belonging to the family Araceae, is an herbaceous perennial species. This species has an underground stem in the form of a tuber, which contains abundant water-soluble dietary fiber, commonly known as konjac glucomannan; therefore, it has long been used as a traditional medicine and food source (Arvill and Bodin, 1995; Chua et al., 2010). *A. konjac* has been recognized as one of the most important crops and has tremendous potential for commercial development. It has been widely domesticated and utilized in China, Japan, and Southeast Asia for thousands of years (Chua et al., 2010). In Japan, *A*. *konjac* was recorded to have been introduced in the sixth century by a religious delegation from North Korea (Liu, 2004). In China, *A. konjac* is a native species and is mainly domesticated in the Sichuan, Hubei, Yunnan, Guizhou, Guangdong, Guangxi, and Fujian provinces.

Wild relatives of domesticated plants are important gene reservoirs for improving commercial varieties (Oyama et al., 2006). However, human activities, including digging up tubers and collecting leaves as a source of food and medicine for the local people, and special regional development strategies, have led the extant wild populations of *A*. *konjac* to decline in abundance. Our field investigations showed that the extant wild populations of *A*. *konjac* are mainly distributed in the Wuling Mountain region of central China and therefore, effective conservation of *A*. *konjac* is urgently needed to preserve the remaining populations.

Evaluation and conservation of genetic diversity for wild plant resources is essential to guarantee sustainable development of crop materials (Wang et al., 2007). Currently, only a few studies have been reported regarding the genetic diversity of *A. konjac* (Zhang et al., 2001; Teng et al., 2006a, b; Xuan, 2010; Ren and Pan, 2013; Zheng et al., 2013). Moreover, all of these studies attempted to identify species taxonomically and resolve the phylogenetic relationships of germplasm resources of *Amorphophallus* species, including *A. konjac*. To date, no study has been undertaken to reveal the level and pattern of genetic variation among wild populations of *A. konjac*. Amplified fragment length polymorphisms (AFLPs) have been widely applied to assess the genetic diversity of various plants, mainly owing to their capability to detect a large number of polymorphisms in different regions of the target genome and good repeatability (Vos et al., 1995; Nybom, 2004; Hasbún et al., 2012). AFLP markers have been successfully used to assess genetic variation among populations of another important tuber crop in the same genus, *Amorphophallus variabilis*, in Indonesia (Santosa et al., 2012).

In the present study, we use AFLP markers to reveal the genetic diversity and population structure of 10 wild populations of *A. konjac* from central China as a first step towards gaining enhanced knowledge of genome diversity in *A. konjac*. Based on the genetic variation analysis, we draw recommendations on its conservation.

MATERIAL AND METHODS

Plant sampling

A total of 139 individuals from 10 wild populations of *A. konjac* in central China were sampled (Figure 1). Leaf material from 6 to 19 randomly selected individuals was collected from each population at intervals of at least 5 m. Table 1 provides details of the sampling localities and sample sizes. Leaf samples were stored in silica gel until DNA extraction. Vouchers of all sampled populations are deposited in the Herbarium of Wuhan University, Wuhan, China (WU).

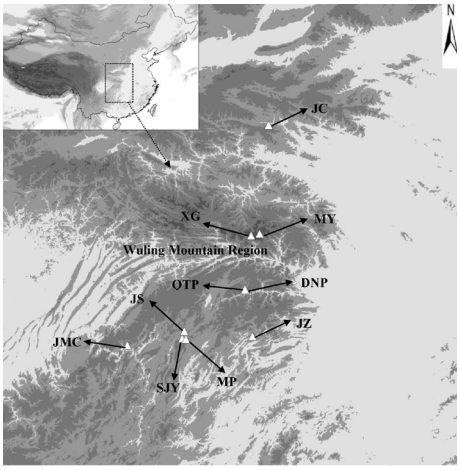

Figure 1. Locations of the 10 sampled populations of *Amorphophallus konjac* in the Wuling Mountain region, central China (Population codes as in Table 1).

Table 1. Location and sample sizes of 10 populations of *Amorphophallus konjac* from central China.

Population Code	Locality	Longitude (E)	Latitude (N)	Altitude (m)	Sample size
XG	Xiagu, Shengnongjia, Hubei	110°14.521'	31°26.608'	560	15
JC	Jiuchong, Shengnongjia, Hubei	110°33.280'	33°25.075'	800	12
MY	Muyu, Shengnongjia, Hubei	110°23.501'	31°28.144'	1060	19
JMC	Jiamachi, Xianfeng, Enshi, Hubei	108°58.519'	29°28.068'	1100	6
SJY	Sanjiaoyan, Xianfeng, Enshi, Hubei	109°00.293'	29°35.653'	882	18
MP	Maoping, Xianfeng, Enshi, Hubei	109°04.281'	29°35.285'	997	16
JS	Jianshan, Xianfeng, Enshi, Hubei	109°02.381'	29°43.263'	587	17
QTP	Qingtaiping, Badong, Enshi, Hubei	110°08.086'	30°29.452'	512	8
DNP	Dananping, Badong, Enshi, Hubei	110°08.076'	30°28.410'	362	12
JZ	Jinzang, Sangzhi, Zhangjiajie, Hunan	110°15.711'	29°37.632'	1025	16

DNA extraction and AFLP analyses

Total genomic DNA was extracted from dry leaf material using the Plant Genomic DNA kit (Tiangen, Beijing, China) following the manufacturer protocol. DNA concentrations were estimated and standardized on 2.0% (w/v) agarose gels. The AFLP procedure followed established protocols (Vos et al., 1995) with slight modifications. Approximately 300 to 500 ng of DNA was digested with 10 U each *Eco*RI and *Mse*I restriction enzymes at 37°C for 1 h and 65°C for 1 h.

An initial screening of selective primers using 20 primer combinations with three selective nucleotides was performed on 14 individuals from seven populations (two individuals per population). Fourteen individuals were replicated in order to exclude non-reproducible bands. Five primer combinations were chosen for the full analysis because they resulted in clear and reproducible bands (Table 2). Polymerase chain reactions (PCR) were carried out in a volume of 25 µL containing 0.25 mM each dNTP, 2.5 mL 10X Taq buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 50 mM KCl), 1 mM each primer, 1U Taq polymerase (Tianyuan Biotech, Wuhan, China), and 40 ng DNA template. Amplification was conducted on a BIO-RAD thermocycler (Bio-Rad, Hercules, CA, USA), and commenced with 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s annealing at 56°C, and 5 min extension at 72°C, with a final extension step of 16 min at 72°C. PCR products were separated using 6.0% denaturing polyacrylamide gel electrophoresis (PAGE) for 2.5 h at 50°C on a JY-EPC3000 system (Junyi, China), and detected using silver staining techniques.

Table 2. Primer sequences used in amplified fragment length polymorphism (AFLP) analysis of 10 populations of *Amorphophallus konjac* from central China.

Primer combinations	Primer sequence
MC01 + EA01	MC01: 5'-GATGAGTCCTGAGTAACAG-3' EA01: 5'-GACTGCGTACCAATTCATC-3'
MC02 + E02	MC02: 5'-GATGAGTCCTGAGTAACCA-3' E02: 5'-GACTGCGTACCAATTCACC-3'
MC02 + EA05	MC02: 5'-GATGAGTCCTGAGTAACCA-3' EA05: 5'-GACTGCGTACCAATTCAAG-3'
MC02 + EA06	MC02: 5'-GATGAGTCCTGAGTAACCA-3' EA06: 5'-GACTGCGTACCAATTCAAC-3'
MC10 + EA05	MC10: 5'-GATGAGTCCTGAGTAACGT-3' EA05: 5'-GACTGCGTACCAATTCAAG-3'

Data analyses

Reliable AFLP bands were scored as either present (1) or absent (0), transformed into a 0/1 binary character matrix, and ambiguous bands were discarded. At the species level and within each population, the genetic diversity parameters, including the percentage of polymorphic loci (*PPL*), Nei's gene diversity (*H*), and Shannon information index (*I*), were calculated using GenAlEx 6 (Peakall and Smouse, 2006). Private AFLP bands in each population were also calculated using GenAlEx 6.

Genetic variation was evaluated using analysis of molecular variance (AMOVA) in GenAlEx 6. Variance was apportioned into the following components, among individuals within a population and among populations. The significance of differentiation between populations was tested by examining 9999 permutations.

The program POPGENE 1.32 (Yeh et al., 1999) was used to estimate the genetic differentiation coefficient (G_{ST}), Nei's (1972) genetic distance, and gene flow (N_m) (McDermott and McDonald, 1993) between the sampled populations, where:

$$N_m = (1/G_{ST}\text{-}1)/4$$

The genetic distance matrix was then used to construct an unweighted pair group method with arithmetic mean (UPGMA) tree of the 10 populations. To illustrate populations grouped according to the AFLP fragment similarity pattern, a principal coordinates analysis (PCoA) was performed in GenAlEx 6 based on Nei's (1972) genetic distance.

In order to test for a correlation between genetic (Nei's genetic distance; Nei, 1972) and geographical distances (in km) among populations, a Mantel test was performed using GenAlEx 6 (9999 permutations).

RESULTS

Genetic diversity

From 139 samples from 10 populations of *A. konjac*, a total of 270 bands were generated using five primer combinations. Two hundred and sixty-five (98.1%) of these were polymorphic at the species level. The number of polymorphic loci for each population ranged from 79 (29.3%, in population Jianshan, JS) to 181 (67.0%, in population Xiagu, XG). Based on the values of *H* in a single population, the highest diversity was observed in population XG (0.202) and the lowest diversity was in population Dananping (DNP; 0.066), with a total of 0.205 at the species level (Table 3). The values of *I* showed a similar trend to those of *H*, with a total of 0.330 at the species level (Table 3). No private AFLP bands were observed in any population.

Table 3. Genetic diversity of 10 populations of *Amorphophallus konjac* from central China (Population codes as in Table 1).

Population	Npl	PPL	H	I
XG	181	67.0	0.202	0.313
JC	126	46.7	0.161	0.241
MY	125	46.3	0.140	0.216
JMC	132	48.9	0.201	0.293
SJY	155	57.4	0.164	0.255
MP	112	41.5	0.114	0.180
JS	79	29.3	0.074	0.119
QTP	123	45.6	0.110	0.184
DNP	86	31.9	0.066	0.113
JZ	157	58.2	0.137	0.224
Total	265	98.2	0.205	0.330

Npl = Number of polymorphic loci; *PPL* = Percentage of polymorphic loci; *H* = Nei's gene diversity; *I* = Shannon's information index.

Genetic differentiation

G_{ST} among populations was 0.348 and gene flow was estimated to be low (N_m = 0.469). AMOVA showed that 32.0% of the total variance was attributed to differences among populations, while 68.0% was attributed to differences within populations (P < 0.001) (Table 4).

Table 4. Analysis of molecular variance (AMOVA) for 10 populations of *Amorphophallus konjac* from central China.

Source of variation	d.f.	SSD	Variance components	Percentage of variation (%)
Among populations	9	1313.02	9.17	0.32
Within populations	129	2527.57	19.59	0.68
Total	138	3840.59	28.77	

d.f. = degrees of freedom; SSD = sum of squares.

Genetic relationship among populations

Nei's genetic identities and genetic distance were calculated between all pairs of the 10 populations (Table 5). The smallest genetic distance (0.014) was between populations Qingtaiping (QTP) and DNP, and the largest genetic distance (0.191) occurred between DNP and XG.

The UPGMA dendrogram shows that the 10 populations of *A. konjac* form three major groups (groups I, II, and III; Figure 2), with populations Sanjiaoyan (SJY), Maoping (MP), and JS from Xianfeng, Hubei province in Group I, populations QTP, DNP from Badong and Jinzang (JZ) from Sangzhi in Group II, and populations XG, Jiuchong (JC), and Muyu (MY) from Shennongjia, Hubei province in Group III. The population from Jiamachi (JMC), Xianfeng in Hubei province was genetically distinct from the other populations. The UPGMA dendrogram showed a correlation between geographic and genetic distances.

Table 5. Pairwise population matrix of Nei's (1972) genetic distance (below diagonal) and genetic identity (above diagonal) for 10 populations of *Amorphophallus konjac* from central China (Population codes as in Table 1).

Population	XG	JC	MY	JMC	SJY	MP	JS	QTP	DNP	JZ
XG		0.962	0.929	0.871	0.881	0.861	0.837	0.847	0.826	0.837
JC	0.039		0.951	0.869	0.901	0.884	0.860	0.860	0.841	0.853
MY	0.074	0.050		0.923	0.951	0.929	0.901	0.902	0.884	0.894
JMC	0.138	0.140	0.080		0.926	0.908	0.871	0.900	0.875	0.884
SJY	0.127	0.104	0.050	0.077		0.971	0.944	0.928	0.916	0.928
MP	0.149	0.124	0.074	0.097	0.029		0.965	0.923	0.913	0.914
JS	0.178	0.151	0.104	0.138	0.057	0.036		0.946	0.939	0.930
QTP	0.166	0.151	0.103	0.106	0.075	0.080	0.056		0.986	0.974
DNP	0.191	0.173	0.124	0.133	0.087	0.091	0.063	0.014		0.981
JZ	0.178	0.159	0.112	0.123	0.074	0.090	0.073	0.026	0.019	

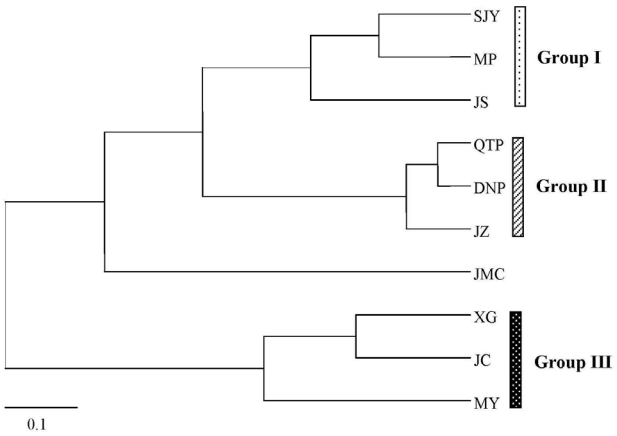

Figure 2. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram illustrating the genetic relationships among 10 populations of *Amorphophallus konjac* from central China (Population codes as in Table 1).

PCoA analysis (Figure 3) indicated significant genetic structure among the current populations of *A. konjac*: populations from geographically close locations frequently clustered together. The first (PC1) and second (PC2) principal component axes accounted for 46.75 and 18.49% of the total variation, respectively.

The Mantel test showed that genetic distances were significantly correlated with geographic distances among all sampled populations of *A. konjac* (R^2 = 0.2521, P < 0.05) (Figure 4).

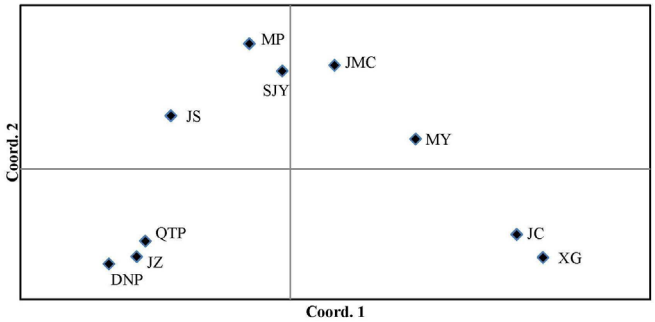

Figure 3. Principal coordinates analysis (PCoA) of 10 populations of *Amorphophallus konjac* from central China based on amplified fragment length polymorphisms (AFLPs) (Population codes as in Table 1). The variance explained by Coord. 1 and Coord. 2 is 46.75 and 18.49%, respectively.

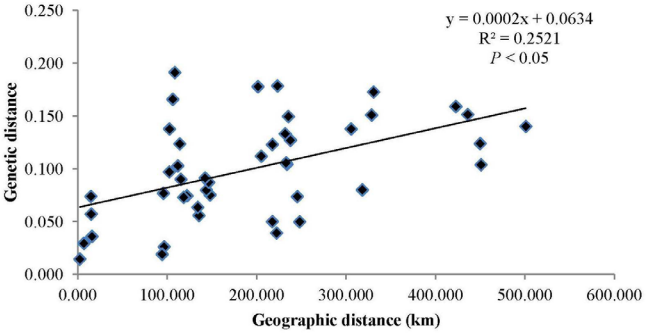

Figure 4. Relationship between pairwise Nei's (1972) genetic distance and geographic distance in the 10 studied populations of *Amorphophallus konjac* from central China, examined using Mantel's test.

DISCUSSION

In this study, using five AFLP primer combinations, we generated a total of 270 bands from 139 individuals from 10 wild populations of *A. konjac* in China. Among these bands, 98.1% (265) were polymorphic, indicating that AFLPs can supply rich genetic information for *A. konjac* and are a good technique to evaluate its genetic variation.

Within most of the studied populations, we detected a relatively high level of genetic diversity (*PPL* values ranged from 29.3 to 67%, *H* ranged from 0.066 to 0.202, and *I* ranged from 0.113 to 0.313) compared to the results obtained for other endangered plant species in the same geographic region (e.g., Wuling Mountain region) using AFLP markers. For example, using five AFLP primer combinations, Yang et al. (2007) studied the genetic variation of eight populations of *Psilopeganum sinensis*, a rare plant species endemic to central China, and found a low level of within-population genetic diversity (*PPL*: 3.3 to 16.7%; *H*: 0.01987 to 0.06987; *I*: 0.0197 to 0.0816). The levels of genetic diversity within populations detected in *A. konjac* were similar to an AFLP genetic

variation study on the endangered plant *Cercidiphyllum japonicum* from the Shennongjia region of Hubei province (*PPL*: 39.19 to 71.62%; *H*: 0.122 to 0.191; *I*: 0.186 to 0.295; Yuan et al., 2012).

Genetic diversity of a plant species is determined by factors such as mating systems, life history, evolutionary history, geographic distribution range, and environmental factors (Loveless and Hamrick, 1984; Hamrick and Godt, 1990, 1996). The high levels of genetic diversity found in populations of *A*. *konjac* in this study may be explained firstly by its mating systems. Although *A*. *konjac* is a clonal plant and reproduces asexually through tubers, it can also reproduce sexually by out-crossed seeds. Similar to *Amorphophallus henryi* and *Amorphophallus virosus*, the pollinators of *A*. *konjac* are beetles (e.g., *Onthophagus* spp and *Staphyinidae* spp) and/or ants (Sivadasan and Sabu, 1989; Beath, 1996; Zhong, 2006; Pan et al., 2013; Pan C, personal observations). The transfer of pollen by insect pollinators between different plants may increase the possibility of outcrossing and, subsequently, would increase the genetic diversity in the studied populations of *A*. *konjac*.

Currently, the wild populations of *A*. *konjac* are mainly distributed in the Wuling Mountain region (i.e., East Sichuan province, West Hubei province, Northwest Hunan province, and East Guizhou province) of central China. This region has been presumed to be a long-term glacial refuge for plant species because of its high diversity and endemism (Ying et al., 1993; Wu and Wu, 1998; Ying, 2001; Wang et al., 2009). A large number of studies have shown that plant populations in putative refugia commonly harbor higher levels of genetic diversity relative to their likely descendant population (Lewis and Crawford, 1995; Comes and Kadereit, 1998). The stable and suitable climate conditions in this refuge since historical glacial periods may have preserved genetic diversity of the studied populations of *A*. *konjac*. The clonal habit of this species may also have fixed some of the genetic variation. In addition, although the population number and size of *A*. *konjac* in its other distribution regions in China has greatly reduced due to human activities, most of the sampled populations in the current study were located in natural reserves (e.g., Shennongjia National Natural Reserve), which may have protected the populations from suffering heavy destruction and loss of genetic diversity in this region.

In the current study, AMOVA revealed a moderate level of genetic differentiation among wild populations of *A*. *konjac* (32.0% of the total genetic variation resided among populations; F_{ST} = 0.32). The coefficient of genetic differentiation (G_{ST} = 0.348) of the studied *A*. *konjac* populations was higher than that in studies on outbreeding species using RAPD markers (average G_{ST} = 0.220), but similar to that using ISSR markers (average G_{ST} = 0.340; Nybom, 2004). Different G_{ST} values have been revealed from different studied plant species, sampled mainly from the Wuling Mountain region using AFLP markers. For example, Yuan et al. (2012) revealed a very low level of population differentiation in *C*. *japonicum* (G_{ST} = 0.135), whereas Yang et al. (2007) found high G_{ST} (0.5069) among populations of *P*. *sinensis*. Factors, such as different evolutionary histories and mating systems of different species, and different sampling ranges in different studies, should account for the different patterns of population genetic differentiation revealed in these studies. For *C*. *japonicum*, Yuan et al. (2012) only sampled four populations in a narrow geographic region (Shennongjia). In addition, pollen and seeds of this species are mainly dispersed by wind (Yuan et al., 2012). Long-distance dispersal of pollen and seeds by wind may have promoted gene flow among close populations of *C*. *japonicum* and reduced genetic differentiation (Yuan et al., 2012). However, *P*. *sinensis* is an insect-pollinated species and the pollinators are mainly small insects, such as ants and hover flies (Zhang and Ye, 2011). In a previous population genetic study, Yang et al. (2007) sampled a relative large and complex geographic region (e.g., several mountains in the Three Gorge Reservoir area). The small insect pollination mating system and the complex geography may have limited gene flow among the *P*. *sinensis* populations, and therefore promoted genetic differentiation (Yang et al., 2007).

In the current study, the Mantel test revealed that genetic distance was significantly correlated with geographic distance for the *A. konjac* populations. UPGMA and PCoA analyses also showed that geographically close populations are more likely to cluster together. Thus, "isolation by distance" (Wright, 1943) may be the main mechanism explaining genetic differentiation among populations of *A*. *konjac*. Aside from geographic distance, the special pollination system of *A*. *Konjac* and the complex geography of the studied region could also be factors contributing to genetic differentiation. As stated above, *A*. *konjac* is an insect-pollinated species mainly pollinated by beetles and ants (Pan C, personal observations). Field observations have suggested poor long-distance gene flow caused by such pollinators among populations of *Amorphophallus* species (Sivadasan and Sabu, 1989; Beath, 1996; Zhong, 2006; Pan et al., 2013; Pan C, personal observations). Estimated gene flow in this study was shown to be low (N_m = 0.469), indicating gene flow among populations of *A*. *konjac* is limited. In addition, our sampled populations were located in different mountains, such as Shennongjia, Badong, Xianfeng, and Zhangjiajie. The geographic barrier of these mountains may also have prevented genetic exchange among populations of *A*. *konjac* to some extent. Restricted gene flow could account for the substantial genetic differentiation found in *A*. *konjac*.

In conclusion, with AFLP markers, high levels of genetic diversity within populations and moderate levels of genetic differentiation between 10 wild populations of *A*. *konjac* have been revealed. The high genetic variability of this economically important wild plant genetic resource could be used as an important breeding source for the development of commercially valuable traits in *A*. *konjac*. Despite the fact that the current studied wild populations of *A*. *konjac* maintain high genetic variability, most of the distribution range of this species is suffering losses in population numbers, size, and genetic diversity; therefore, the first priority should be to take measures to conserve this species. Our current population genetic study could provide baseline information for the conservation of this species. For example, we first suggest conservation of the wild *A*. *konjac* populations *in situ* considering that genetic resources have declined greatly, even for those populations distributed in national natural reserves, because of human activities. In this study, we revealed significant genetic structure, with the 10 populations clustered into three major population groups, and each group included geographically close populations. Therefore, in the conservation of *A*. *konjac* plants, we should treat these population groups as distinct "evolutionary significant units". In *ex-situ* conservation, we suggest sampling of more population groups, which would boost conservation of genetic variation to a large extent.

Conflicts of interest

The authors declare no conflict of interest.

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