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# Conservation and population genetic diversity of *Curcuma wenyujin* (Zingiberaceae), a multifunctional medicinal herb

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**ABSTRACT.** *Curcuma wenyujin* is an important multifunctional medicinal herb in China. Currently, populations of *C. wenyujin* are decreasing, and wild individuals have almost disappeared from their natural habitats. Moreover, little is known regarding the molecular characteristics of this plant. In this study, we investigated the genetic diversity and variation of five populations of *C. wenyujin*, using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. We found that the percentages of polymorphic loci (*PPL*) at the species level (98.25% by RAPD and 100% by ISSR) were significantly higher than those at the population level (66.32% by RAPD and 67.14% by ISSR). The highest values of *PPL*, expected heterozygosity, and Shannon's information index were in Pop1, while the lowest values were in Pop2. Both DNA markers revealed a short

 

genetic distance between Pop1 and Pop2 (0.1424 by RAPD and 0.1904 by ISSR). Phylogenetic trees produced similar results, with Pop1, Pop2, and Pop5 in one group and Pop3 and Pop4 in another. There were no significant correlations between their genetic distances and their geographical distances. The highest genetic diversity was in Pop1 and the lowest was in Pop2, and genetic diversity at the species level was relatively low, but much higher than that at the population level. We recommended the establishment of a germplasm bank, *in situ* conservation, and propagation of wild individuals. The present study will improve the evaluation, protection, and utilization of the population resources of *C. wenyujin*.

**Key words:** *Curcuma wenyujin*; Population; Phylogenetic analysis; Inter simple sequence repeat (ISSR); Genetic diversity; Random amplified polymorphic DNA (RAPD)

## INTRODUCTION

*Curcuma,* a plant genus of the family Zingiberaceae, consists of about 70 species of rhizomatous herbs that are distributed worldwide, including at least 20 species in China, a few of which have been characterized as herbal medicines or food (Xiao et al., 2008). *Curcuma wenyujin* Y.H. Chen et C. Ling has been used as an important medicinal plant in China for over 100 years (The State Pharmacopoeia Commission of P. R. China, 2010). *C. wenyujin* rhizome extract contains a variety of bioactive ingredients, such as curdione (Xia et al., 2012), aurantiamide (Ma et al., 2009; Xia et al., 2012), and sesquiterpene (Sun et al., 2009; Dong et al., 2013; Zhu et al., 2013a). These substances can not only induce apoptosis (Xiao et al., 2008; Lim et al., 2010), inhibit platelet aggregation (Xia et al., 2012), and tumor growth (Sun et al., 2009; Lim et al., 2010), but also serve as clinical agents of anti-oxidation, anti-microbial action (Dong et al., 2013; Zhu et al., 2013b), and anti-inflammation (Tohda et al., 2006).

*C. wenyujin* grows in Wenzhou, which is a coastal city in southeast China (latitude 27°03'-28°36'N, longitude 119°37'-121°18'E). Thousands of hectares of *C. wenyujin* are cultivated annually, and over 10,000 tons are harvested each year to meet the growing market demand. Recently, such phenomena as population degradation, the disappearance of wild individuals, and environmental deterioration (e.g., industrial pollution, insecticides, etc.) have occurred in its natural habitats, which directly jeopardizes its population diversity and survival, and may eventually result in a decline in yield and product quality (Smithson and Lenne, 1996; Zhu et al., 2000; Tao et al., 2007).

DNA markers are effective tools in evaluating genetic diversity (Fernandez et al., 2002; Phong et al., 2011), constructing genetic maps (Gupta et al., 2012), and molecular identification and classification (Fernandez et al., 2002). Random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994) markers are polymerase chain reaction (PCR)-amplified approaches to acquire the genetic information of populations or individuals. RAPD primers are of approximately 10 nucleotides size with random sequence, and can bind to complementary regions in the genome, whereas ISSR primers are designed to only bind to SSRs; therefore, the two markers in the same genome obtain different information (Das et al., 2011; Phong et al., 2011; Singh et al.,

 

2012). RAPD markers have similar advantages as ISSR markers: there is no need for prior sequence information, they are easy to conduct, they are inexpensive, there is a versatile set of primers, and they provide highly reproducible amplifications (Fernandez et al., 2002; Phong et al., 2011; Tripathi et al., 2012).

RAPD and ISSR markers have been successfully applied in many plant and animal species: singly, together, or combined with other molecular markers (Tripathi et al., 2012; Ding et al., 2013). In Zingiberaceae plants, Singh et al. (2012) reported that the average percentage of polymorphism among 60 turmeric (*Curcuma longa* L.) was 91.4% by RAPD and 95.4% by ISSR, and there was a 62% correlation between their genetic similarity and their geographical locations. Mohanty et al. (2011) assessed the genetic stability of micropropagated *Zingiber rubens* by using RAPD and ISSR markers, and found that the duration of the culture period (more than two years) did not affect the plants' genetic integrity; Das et al. (2011) determined the genetic diversity and molecular classification of nine *Curcuma* species from northeast India using DNA fingerprinting (ISSR, RAPD, and amplified fragment length polymorphism). However, in the case of *C. wenyujin*, previous studies have mainly focused on the isolation and characterization of the plant's bioactive ingredients (Xia et al., 2012; Zhu et al., 2013a), as well as their functions (Sun et al., 2009; Lim et al., 2010; Zhu et al., 2013b). Only three studies have been conducted on the genetic diversity (or variation) of *C. wenyujin* using single DNA markers (RAPD or ISSR) (Tao et al., 2007, 2009; Wang et al., 2008). In the present study, we used RAPD and ISSR markers to investigate the genetic diversity (and variation) of *C. wenyujin* populations in Wenzhou in order to improve the evaluation, conservation, sensible exploitation, and sustainable development of populations of this species.

## MATERIAL AND METHODS

### Plant material collection and genome DNA extraction

Young leaves from 100 individual plants from five populations of *C. wenyujin* (20 individuals from each population) in Wenzhou, China, were randomly sampled (Figure 1). The populations were named YueQS (Pop1), PingYM (Pop2) (Figure 2), RuiAM (Pop3), RuiAF (Pop4), and RuiAT (Pop5) (Figure 1). The samples were cleaned and then stored at -80°C.

Genomic DNA was extracted using the CTAB method, with the slight modification of purification being conducted twice (Allen et al., 2006). The DNA samples were dissolved in TE-RNase buffer. The DNA concentration and quality were tested using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and 1.0% agarose gel electrophoresis visualized by ultraviolet (UV) light, respectively. All of the DNA samples were adjusted to meet the following criteria: 1) The ratio of A260/280 was between 1.6 and 1.8; 2) The concentration of the DNA sample was diluted to 40 ng/μL.

### PCR amplification

Ten RAPD primers and 10 ISSR primers were used for the PCR amplifications, which were screened from 50 RAPD primers and 50 ISSR primers, respectively (Table 1). PCR amplifications were performed in a 25-μL mixture of 1X PCR buffer, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 1.0 μM primer, 40 ng DNA, and 1.5 U Taq DNA polymerase (TaKaRa). The PCRs for both the RAPD and ISSR analyses were conducted using a T-Personal thermocycler (Biome-

tra, Göttingen, Germany). The RAPD analysis was conducted under the following conditions: 5 min at 94°C, followed by denaturing for 35 cycles of 30 s at 94°C, annealing for 60 s at 52°C, and extension for 90 s at 72°C, followed by 10 min at 72°C for the final extension. For the ISSR analysis, the conditions were as follows: 5 min at 94°C, followed by denaturing for 40 cycles of 30 s at 94°C, annealing for 90 s at 36°C, extension for 90 s at 72°C, followed by 10 min at 72°C for the final extension. The PCR products were separated using 1.2% agarose gel electrophoresis buffered with 0.5X TBE (44.5 mM Tris-HCl, 44.5 mM boric acid, and 1 mM EDTA). Gels were stained with ethidium bromide (0.5 µg/mL) for 15 min and photographed under UV light using a GelDoc-It® Imaging system (UVP, Upland, CA, USA). All of the PCR amplifications were repeated at least twice in order to obtain clear, stable, and reproducible bands.

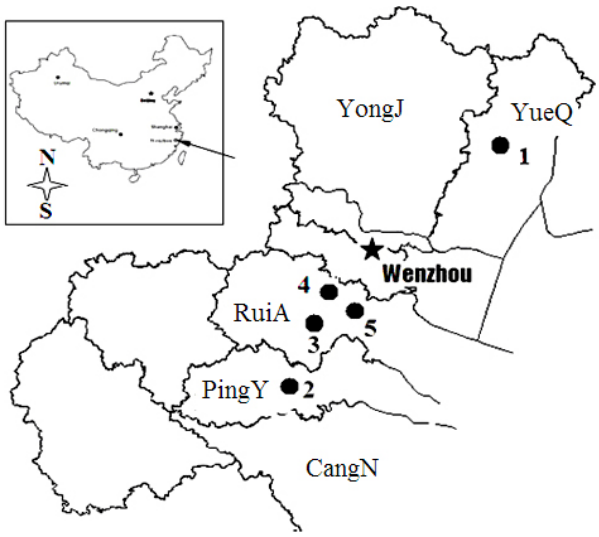


**Figure 1**. Geographical locations of *Curcuma wenyujin* populations in this study (Wenzhou, China). Populations 1 to 5 represent the populations YueQ, PingY, RuiAM, RuiAF, and RuiAT, respectively.


**Figure 2.** *Curcuma wenyujin* cultivars. **A.** Flowering period of *C. wenyujin* individuals; **B.** eight-month development phase of *C. wenyujin* cultivars from PingY, Wenzhou, China.

**Table 1.** Primers and sequences used in the study.

| RAPD analysis | | ISSR analysis | |
|---|---|---|---|
| Primer | Sequence | Primer | Sequence |
| TubeS-247 | 5'-CCTGCTCATC-3' | ISSR-822 | 5'-TCT CTC TCT CTC TCT CA-3' |
| TubeS-248 | 5'-GGCGAAGGTT-3 | ISSR-830 | 5'-TGT GTG TGT GTG TGT GG-3' |
| TubeS-258 | 5'-GAGGTCCACA-3' | ISSR-834. | 5'-AGA GAG AGA GAG AGA GYT-3' |
| TubeS-269 | 5'-GTGACCGAGT-3 | ISSR-835 | 5'-AGA GAG AGA GAG AGA GYC-3' |
| TubeS-270 | 5'-TCGCATCCCT-3 | ISSR-840 | 5'-GAG AGA GAG AGA GAG AYT-3' |
| TubeS-273 | 5'-CACAGCGACA-3 | ISSR-841 | 5'-GAG AGA GAG AGA GAG AYC-3' |
| TubeS-275 | 5'-ACACCGGAAC-3 | ISSR-843. | 5'-CTC TCT CTC TCT CTC TRA-3' |
| TubeS-276 | 5'-CAGCCTACCA-3' | ISSR-845 | 5'-CTC TCT CTC TCT CTC TRG-3' |
| TubeS-278 | 5'-TTCAGGGCAC-3' | ISSR-849 | 5'-GTG TGT GTG TGT GTG TYA-3' |
| TubeS-280 | 5'-TGTGGCAGCA-3' | ISSR-851 | 5'-GTG TGT GTG TGT GTG TYG-3' |

RAPD, random amplified polymorphic DNA; ISSR, inter-simple sequence repeat.

## Data processing and cluster analysis

Bands were scored for the presence (1) or absence (0) of each genotype for each primer. The available binary matrix was analyzed using the PopGene software (version 1.32) (Yeh et al., 1999). The following parameters were used to evaluate genetic diversity (and variation) at the species and population levels: the percentage of polymorphic loci (*PPL*), Shannon's information index (*I*), the expected heterozygosity ($H_E$) (Nei, 1973), and the coefficient of genetic differentiation ($G_{ST}$), which is equal to ($H_T$ - $H_E$) / $H_T$ ($H_T$ is the species level $H_E$) (Yeh et al., 1999).

Nei's (1972) genetic relationships between the five populations were assessed using PopGene. A phylogenetic dendrogram was constructed based on the available matrices of genetic distances using the unweighted pair group method with arithmetic average (UPGMA) with 1000 bootstrap permutations, using the TFPGA software (version 1.3) (Miller, 1997). Correlations between the geographical distances and average genetic distances between the five populations were also determined.

## RESULTS

## Polymorphisms detected by RAPD and ISSR analysis

The DNA amplified by RAPD ranged from 200 to 2300 bp in length, and by ISSR from 300 to 1800 bp in length. At the species level, 10 RAPD primers for 100 samples produced 56 polymorphic loci out of the total 57 DNA loci, indicating that the total *PPL* was 98.25%. Ten ISSR primers for 100 samples produced 42 DNA loci, and the total *PPL* was 100%. At the population level, using RAPD the *PPL* ranged from 54.39% (Pop2) to 73.68% (Pop1), with an average of 66.32%, and using ISSR, it ranged from 61.90% (Pop2) to 78.57% (Pop1), with an average of 67.14% (Table 2). The results indicated that the frequency of species-level polymorphisms was higher than that of population-level polymorphisms.

**Table 2.** Genetic variation in five populations of *Curcuma wenyujin* revealed by random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) analyses.

| Marker | Population | Sample size | *H* | *I* | *PPL* | $G_{ST}$ |
|---|---|---|---|---|---|---|
| RAPD | Pop1 | 20 | 0.2791 | 0.4105 | 73.68% | 24.24% |
| | Pop2 | 20 | 0.2057 | 0.3047 | 54.39% | 44.16% |
| | Pop3 | 20 | 0.2710 | 0.3948 | 68.42% | 26.44% |
| | Pop4 | 20 | 0.2766 | 0.4036 | 68.42% | 24.92% |
| | Pop5 | 20 | 0.2599 | 0.3795 | 66.67% | 29.45% |
| | Average | 20 | 0.2585 | 0.3786 | 66.32% | 29.83% |
| | Total | 100 | 0.3684 | 0.5462 | 98.25% | |
| ISSR | Pop1 | 20 | 0.3361 | 0.4857 | 78.57% | 18.06% |
| | Pop2 | 20 | 0.2512 | 0.3729 | 61.90% | 38.76% |
| | Pop3 | 20 | 0.2530 | 0.3730 | 66.67% | 38.32% |
| | Pop4 | 20 | 0.2797 | 0.4016 | 64.29% | 31.81% |
| | Pop5 | 20 | 0.2711 | 0.3911 | 64.29% | 33.91% |
| | Average | 20 | 0.2792 | 0.4049 | 67.14% | 31.93% |
| | Total | 100 | 0.4102 | 0.5936 | 100.00% | |

Pop1-5 represent samples YueQS, PingYM, RuiAM, RuiAF and RuiAT, respectively; *H*, Nei's gene diversity; *I*, Shannon's information index; *PPL*, percentage of polymorphic loci; $G_{ST}$, coefficient of genetic differentiation.

## Genetic diversity and variation

The $H_E$ and *I* results are presented in Table 2. At the species level, the results showed that the $H_E$ and *I* were 0.3684 and 0.5462, respectively, by RAPD, and 0.4102 and 0.5936, respectively, by ISSR. At the population level, the RAPD analysis revealed that the lowest $H_E$ was in Pop2 (0.2057) and the highest was in Pop1 (0.2791), with an average value of 0.2585; the lowest *I* was in Pop2 (0.3047) and the highest was in Pop1 (0.4105), with an average value of 0.3786. Similarly, the ISSR analysis revealed that the $H_E$ ranged from 0.2512 (Pop2) to 0.3361 (Pop1), with an average of 0.2792, and *I* ranged from 0.3729 (Pop2) to 0.4857 (Pop1), with an average of 0.4049. Both the RAPD and ISSR markers indicated that the lowest *PPL*, $H_E$, and *I* values were in Pop2, and the highest were in Pop1 (Table 2), which suggested that Pop2 had the lowest diversity and Pop1 had the highest. The data also implied that genetic diversity at the species level was much higher than that at the population level, indicating that the genetic diversity of *C. wenyujin* was mainly within populations.

The $G_{ST}$ results are presented in Table 2. Regardless of the type of analysis, the highest *Gst* values were in Pop2 (44.16% by RAPD and 38.76% by ISSR), while the lowest $G_{ST}$ values were in Pop1 (24.24% by RAPD and 18.16% by ISSR), which suggested that genetic differentiation between populations was relatively low and most of the genetic variation occurred within populations (Table 2).

## Phylogenetic analysis

Table 3 presents Nei's genetic distances between the five populations, as revealed by RAPD and ISSR analyses. RAPD markers revealed that Nei's genetic distance ranged from 0.1424 to 0.2867, and with ISSR markers, the distance ranged from 0.1904 to 0.3500, suggesting a high genetic similarity between the populations. Both DNA markers revealed that the shortest genetic distance was between Pop1 and Pop2 (0.1424 by RAPD and 0.1904 by ISSR).

RAPD analysis indicated that the greatest genetic distance (0.2867) was between

 

Pop4 and Pop5, while the ISSR analysis found that the greatest distance (0.3500) was between Pop2 and Pop3. This difference may be explained by the following reasons: 1) RAPD and ISSR markers provided different kinds of information; 2) The sample sizes of each population were not large enough to provide the complete genetic information of the populations.

Figure 3 shows phylogenetic dendrograms of the five populations. In Figure 3A, Pop1 and Pop2 were closely clustered together and Pop5 was in the same group, and Pop4 and Pop3 were clustered together in a separate group. The populations in Figure 3B shared a similar tree structure with their counterparts in Figure 3A, i.e., Pop1, Pop2, and Pop5 form one group while Pop4 and Pop3 form another. Although the largest genetic distances were between different populations in the different analyses (between Pop4 and Pop5 by RAPD and between Pop2 and Pop3 by ISSR), both markers revealed a similar tree structure: one group with three populations (Pop1, Pop2, and Pop5) and the other with two populations (Pop3 and Pop4), suggesting that the two markers were suitable for analyzing the genetic diversity of *C. wenyujin* populations and that the data revealed the genetic relationships between the populations.

**Table 3.** Nei's genetic distances between five populations of *Curcuma wenyujin*, based on random amplified polymorphic DNA (RAPD) (below diagonal) and inter simple sequence repeat (ISSR) data (above diagonal).

| Population | Pop1 | Pop2 | Pop3 | Pop4 | Pop5 |
|---|---|---|---|---|---|
| Pop1 | - | 0.1904 | 0.2133 | 0.2873 | 0.2320 |
| Pop2 | 0.1424 | - | 0.3500 | 0.2160 | 0.2373 |
| Pop3 | 0.1486 | 0.1912 | - | 0.2448 | 0.2947 |
| Pop4 | 0.2666 | 0.2392 | 0.1579 | - | 0.3122 |
| Pop5 | 0.1874 | 0.2304 | 0.2069 | 0.2867 | - |

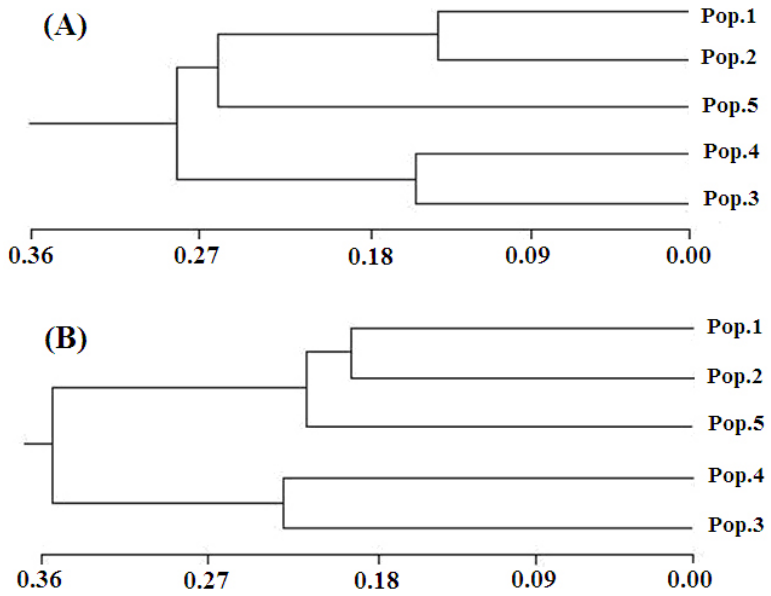


**Figure 3.** Unweighted pair group method with arithmetic average (UPGMA) dendrograms of five populations of *Curcuma wenyujin* based on Nei's unbiased genetic distance, with 1000 bootstrap permutations using random amplified polymorphic DNA (**A**) and inter simple sequence repeat data (**B**).

## Correlations between genetic distances and geographical distances

Table 4 presents the correlations between the average Nei's genetic distances (combined RAPD and ISSR data) and the geographical distances between the populations. The genetic distances ranged from 0.1664 (between Pop1 and Pop2) to 0.2995 (between Pop4 and Pop5), with an average of 0.2318, which suggested that there was low genetic diversity between the populations. The three geographically close populations of Pop3, Pop4, and Pop5 exhibited large genetic distances between each other, whereas the two geographically distant populations (Pop1 and Pop2) exhibited the shortest genetic distance. There were no significant correlations between the populations.

**Table 4.** Average Nei's genetic distances (below diagonal) and geographical distances (km) (above diagonal) between five populations of *Curcuma wenyujin*.

| Population | Pop1 | Pop2 | Pop3 | Pop4 | Pop5 |
|---|---|---|---|---|---|
| Pop1 (YueQ) | - | 80.4000 | 69.1000 | 63.1000 | 60.50 |
| Pop2 (PingY) | 0.1664 | - | 25.0000 | 21.6000 | 22.60 |
| Pop3 (RuiAM) | 0.1810 | 0.2706 | - | 6.8000 | 7.70 |
| Pop4 (RuiAF) | 0.2770 | 0.2276 | 0.2014 | - | 5.20 |
| Pop5 (RuiAT) | 0.2097 | 0.2339 | 0.2508 | 0.2995 | - |

## DISCUSSION

Currently, two (or more) DNA markers are commonly employed for genetic diversity analysis, molecular identification, etc. (Das et al., 2011; Mohanty et al., 2011; Singh et al., 2012). The present study, using two kinds of DNA marker (RAPD and ISSR), revealed that the average Nei's genetic distance ranged from 0.1664 to 0.2995, with an average of 0.2318 (Table 4), which meant that the genetic similarities ranged from 0.7005 to 0.8336, with an average of 0.7682. In previous studies, a genetic similarity of over 88% was found between six populations of *C. wenyujin* by ISSR analysis (RuiAT, RuiAM, RuiAB, YongJW, PingYM, and YueQS) (Tao et al., 2007, 2009). Wang et al. (2008) reported much higher genetic similarities (ranged from 0.9700 to 0.9991) among five populations of *C. wenyujin* (GX4, FJ5, ZJ6, ZJ7, and ZJ8). Singh et al. (2012) also found relatively high genetic similarities between *C. longa* plants (another Zingiberaceae species) from 10 different agro-climatic regions, whose Nei's genetic diversity (*H*) ranged from 0.181 to 0.257.

The levels of *H* found in our study were higher than those found in previous studies. Possible reasons for this were as follows: 1) The samples were not taken from the same geographical regions; 2) There were 20 samples from each population in this study, more than that in other studies; 3) Two markers were more reliable than a single marker; 4) The genetic diversities of the populations may have recently been artificially increased. The present study found no significant correlations between genetic distances and geographical distances, which was consistent with the results of earlier studies (Tao et al., 2007, 2009; Wang et al., 2008). This may be due to frequent germplasm exchanges, and the geographical proximity of the populations in Wenzhou. There were large genetic differences between populations of *C. wenyujin* and populations of *Curcuma phaeocaulis* and *Curcuma kwangsiensis* (Wang et al., 2008).

Reproduction is the main driver of plant evolution, genetic diversification, and speciation (Holsinger, 2000). For *C. wenyujin,* it is a cross-pollinated plant, and ineffective hy-

bridization usually happens, which is similar to that of *C. longa* (Ravindran, 2007). Therefore, asexual reproduction dominates the life cycle of *C. wenyujin*. In practice, farmers often select robust rhizomes to generate new generations of individuals with genetically identical genomes, and new generations of individuals are more likely to accumulate deleterious mutations that reduce genotype diversity (Holsinger, 2000; Robertson et al., 2010; Ludwig et al., 2013). In addition, farmers often remove flowers during their early developmental period for a greater yield. This practice not only decreases the energy and nutrient consumption of floral organs, but also makes pollination impossible and further decreases the genetic diversity of populations.

What factors could increase the genetic diversity and variation of *C. wenyujin* populations without effective hybridization? Previous studies have found that environmental factors, as well as artificial germplasm exchanges, cause increases in genetic diversity, not heredity (Tao et al., 2007, 2009).

In light of the results of the present and previous studies, we supposed that the following factors would influence genetic diversity: 1) Genetic variation, including DNA mutations and chromosomal variation (particularly polyploidization), caused by environmental factors such as radiation and mutagens. Although these phenomena occur at an extremely low frequency in nature, and most variation (or mutation) is harmful, a small number of beneficial mutations probably accumulate even fix simultaneously (Keightley et al., 1998; de Meeus et al., 2007). Polyploidization could be the main driver of diversification (Soltis et al., 2009; Robertson et al., 2010). 2) Artificial treatment could significantly increase variation (or the rate of evolution) by radiation or other methods, consequently increasing population diversity. Germplasm exchange is a popular practice, and to some extent it does increase population diversity, as different geographical populations of plants may genetically differ (Zhu et al., 2000; Tao et al., 2007). Individual plants with genes for particular traits could be artificially eliminated, since only robust rhizomes should be selected as precursors. Additionally, annual harvesting may result in a low survival rate and a decrease in genetic diversity (Tao et al., 2009). 3) Human interference and environmental pressures (e.g., industrial pollution, chemical insecticides, global warming, and agricultural pests) could lead to the disappearance of wild individuals and decrease population genetic diversity (Chen et al., 2007; Tao et al., 2007, 2009).

## Conservation implications

Population genetic diversity affects population productivity, growth, and stability (Hughes et al., 2008), and is important in the development, utilization, and protection of plant resources (Shah et al., 2008; Ding et al., 2013). Previous studies have found that industrial pollution, habitat destruction and fragmentation, climate change, human overexploitation, annual harvesting, as well as wild resource disappearance result in low genetic diversity and population decreases (Tao et al., 2009; Ding et al., 2013). Unfortunately, these phenomena have occurred in populations of *C. wenyujin*, and will eventually result in the species having a low genetic diversity in China (Tao et al., 2007, 2009).

Low population genetic diversity results in a decreased ability to adapt to the environment, and decreases in product quality and yield (Smithson and Lenne, 1996; Holsinger, 2000; Zhu et al., 2000; Robertson et al., 2010; Ludwig et al., 2013). Based on the results of this and previous studies, we recommended the following: 1) The establishment of a germplasm bank. Large areas of genetically diverse populations should be established, or explants should be

cryopreserved to protect germplasm resources (Mohanty et al., 2011); 2) The protection of wild individuals. The *in situ* protection and rapid propagation of wild individuals are economical and effective measures (de Meeus et al., 2007). In addition, local government departments should take measures to prohibit the exploitation of wild individuals; 3) Last but not least, the breeding and propagation methods of cultivars with desirable characteristics should be revised (de Meeus et al., 2007). Wenzhou is an economy-oriented region where farmers practice annual harvesting to maximize their profits; however, crop rotation should be conducted rather than continuous cropping.

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