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RESEARCH ARTICLE

Genetic diversity and differentiation in Chinese sour cherry *Prunus pseudocerasus* Lindl., and its implications for conservation

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Abstract In this study, the genetic diversity and differentiation of 10 natural *Prunus pseudocerasus* Lindl. populations were investigated using inter-simple sequence repeat (ISSR) markers. Totally, 18 selected primers generated 150 loci, with an average of 8.33 bands per primer. The results showed that the percentage of polymorphic bands (*PPB*) was pretty low at the population level (*PPB* = 1.13–32%), but relatively high at the species level (*PPB* = 84%). Besides, a high level of genetic differentiation among populations was detected based on the gene differentiation coefficient ($G_{ST} = 0.7118$) and the hierarchical analysis of molecular variance (AMOVA) ($\Phi_{ST} = 64.53\%$, $P < 0.001$), in line with the low inter-population gene flow ($N_m = 0.2025$). Moreover, Mantel test revealed a significant correlation between genetic and geographic distances among the populations ($r = 0.5272$, $P < 0.005$). The high level of intraspecific genetic diversity was probably related with its life history traits, while its small population size and the resultant high levels of genetic drift and inbreeding might explain the low genetic diversity within populations. The relatively high inter-population genetic differentiation was largely attributed to its small population size, habitat fragmentation, the mode of pollen and seed dispersal, and geographic isolation. Based on the present study, conservation strategies were proposed to preserve this valuable natural germplasm resource.

Keywords Cherry · Genetic differentiation · Genetic diversity · Inter-simple sequence repeats (ISSRs) · *Prunus pseudocerasus* Lindl.

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Introduction

As a fundamental component of biodiversity, genetic diversity is essential to the long-term survival of any species, especially in environments which are subject to climate changes or the introduction of new pests, pathogens or competitors (Rajora and Mosseler 2001). Reduced genetic diversity usually has deleterious effects on species fitness, e.g. an increased risk of inbreeding effects, and may threaten the survival of species or populations (Malone et al. 2003; Reed and Frankham 2003). Thus, an accurate estimate of genetic diversity of the targeted species

or populations is essential to the effective conservation and utilization of genetic resources.

Prunus pseudocerasus Lindl., commonly known as Chinese sour cherry, is an insect-pollinated, perennial species with a long history of cultivation, and belongs to the family Rosaceae, genus *Prunus*, subgenus *Cerasus*. It originates from Southwest China and is widely distributed in the temperate zone of Northern Hemisphere, occurring along the south slope of mountains or ditch sides (Yu and Li 1986). The fruit of *P. pseudocerasus* contains rich nutritional ingredients and trace elements, such as proteins, carotene, Vitamin C, saccharides, iron and phosphorus (Yu and Li 1986). It is also a widely used material for the cultivation of new cherry cultivars (Ochatt et al. 1987; Gutièrrez-Pesce et al. 1998; Mandegaran et al. 1999; Pesce and Rugini 2004). To adapt to ecologically diverse habitats, it has accumulated considerable genetic variations associated with various characters of great value for the fruit farming. In addition, its flower, leaf, root, bark and core are of high medicinal value. Because of its economic values, it has been playing an important role in fruit industry. However, as indicated by our field survey in 2002 and 2003, largely due to anthropogenic activities (e.g. road construction, deforestation and grazing), its natural germplasm resource has been seriously devastated and needs urgent preservation and restoration. To date, all previous studies of *P. pseudocerasus* have mainly been focused on its taxonomy (Chang et al. 2007), propagation (Feucht and Dausend 1976; Chen et al. 2003), morphology (Chu et al. 1995; Xin et al. 1996), phylogeography (Cao et al. 2007), phytochemistry (Gao et al. 2003; Takahashi et al. 2006; Sun 2007) and physiology (Atkinson and Taylor 1996; Zhao et al. 2005). No efforts have been reported on its genetic diversity and structure.

Genetic markers have contributed to the study of plant evolutionary ecology by providing methods for detecting genetic differences among individuals (Cruzan 1998). During the past decades, their popularity has enabled the significant progresses in understanding the population genetics of plant species. Among these marker systems, inter-simple sequence repeats (ISSRs) are a microsatellite-derived genetic finger printing method based on the amplification of the specific regions between two SSR (simple sequence repeat) sequences (Zietkiewicz et al. 1994). Practically, due to the longer SSR-based primers, ISSRs enable higher-stringency DNA amplifications and can yield stable and reproducible bands (Wolfe et al. 1998), and has already established wide applications in studies of genetic diversity, phylogenetics, genetic mapping and evolutionary biology in a wide range of plant species (e.g. Qiu et al. 2004; Zhang et al. 2006; Chennaoui-Kourda et al. 2007; Li and Jin 2007).

In the present study, we investigated ISSR markers in 10 natural populations of *P. pseudocerasus*. The aims were to: (1) characterize its genetic diversity at both population and species levels; (2) reveal the partitioning of genetic variation within and among populations; and (3) eventually provide some references for its practical conservation strategies and resource exploitation.

Materials and methods

Plant materials and DNA extraction

Eighty individuals, which corresponded to 10 populations, were sampled across five provinces in China, including Shaanxi, Sichuan, Chongqing, Guizhou and Yunnan (Table 1; Fig.1). Young leaves were collected, dried in a plastic bag with silica gel, transported to the laboratory and stored in −80°C freezer until use. Corresponding to each population, parameters such as longitude, latitude and altitude were determined and recorded for further analysis.

Total genomic DNA was extracted from dried leaves using a modified CTAB method (Wang and Fang 1998). Then, the DNA extract was suspended in 200 µl 0.1× TE buffer (10 mM pH 8.0 Tris–HCl; 1 mM pH 8.0 EDTA), and kept at −20°C for long-term storage or at 4°C for immediate use.

ISSR-PCR amplification

One hundred primers (UBC Primer Set #9, Biotechnology Laboratory, University of British Columbia, Canada) were initially screened, and 18 primers, which yielded bright and reproducible bands, were obtained and used for the further analysis of all eighty DNA samples (Table 2).

PCR was performed in a 10-µl reaction volume containing 50 ng of genomic DNA, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U Taq polymerase and 1 µM

Table 1 Locations and sample sizes of *Prunus pseudocerasus* populations in this study

Populations	Sampling locations	Longitude (°E)	Latitude (°N)	Altitude (m)	Sample size
YJ	Yinjiang, Guizhou Province	108°36′	27°55′	917	8
YN	Heilongtan, Yunnan Province	102°54′	25°02′	1,923	8
MZ	Mengzi, Yunnan Province	103°47′	23°24′	1,902	7
PB	Pingbian, Yunnan Province	103°52′	23°13′	2,019	3
WL	Wolong, Sichuan Province	103°13′	31°04′	1,950	3
BX	Baoxing, Sichuan Province	102°45′	30°30′	1,950	5
CK	Chengkou, Chongqing	108°43′	32°03′	1,248	14
FP	Foping, Shaanxi Province	107°48′	33°34′	1,346	17
XX	Xixiang, Shaanxi Province	108°15′	33°45′	1,616	6
AK	Ankang, Shaanxi Province	108°53′	32°09′	820	9

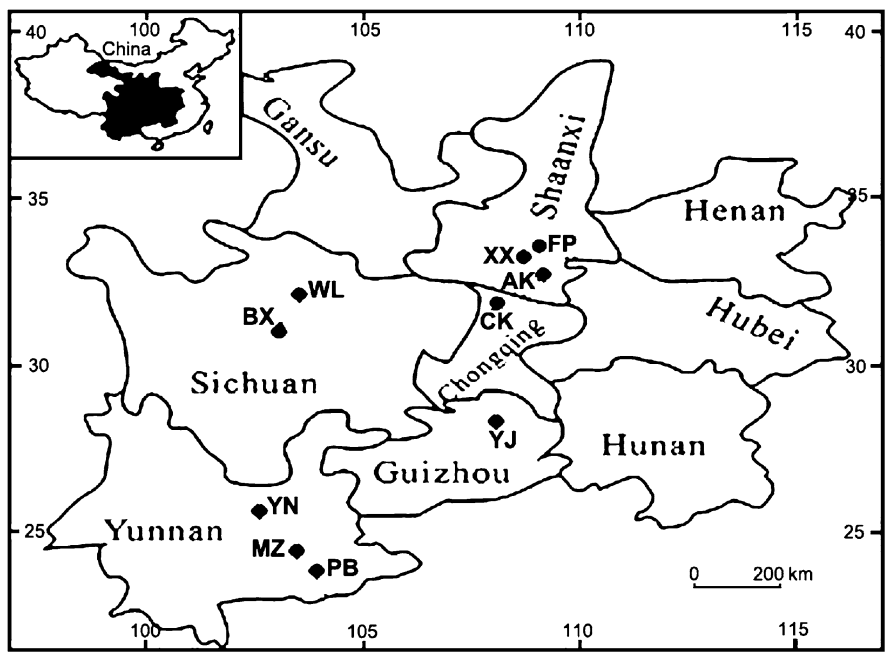

Fig. 1 Sampling locations of the 10 *P. pseudocerasus* populations in China

primers. The amplifications were performed in the thermal cycler PTC-200 (MJ Research) with the following program: initial denaturation at 94°C for 2 min; followed by 35 cycles of 94°C for 30 s, appropriate annealing temperature (see Table 2 for details) for 45 s, 72°C for 90 s; and last synthesis at 72°C for 7 min.

Amplified products were electrophoresed in 2% agarose gel at 100 V for 1.5 h along with the molecular weight marker GeneRuler DL2000 (Shanghai Sangon Biological Engineering Technology & Service Co., Ltd, China), stained with ethidium bromide, and photographed with Bio-RAD Gel Documentation System.

Statistical analysis

Since ISSR markers were dominantly inherited, we assumed that each band represented the phenotype at a single biallelic locus (Williams et al. 1990).

Table 2 ISSR primers used in this study

Primer code	Sequence (5′→3′)	N_L	Size range (bp)	T_A (°C)
UBC813	$(CT)_8T$	7	448–1,545	52
UBC816	$(CA)_8T$	7	312–1,753	52
UBC822	$(TC)_8A$	6	459–1,062	56
UBC824	$(TC)_8G$	8	322–1,823	52
UBC827	$(AC)_8G$	9	234–1,753	52
UBC850	$(GT)_8YC$	5	720–1,663	52
UBC853	$(TC)_8RT$	11	252–1,800	52
UBC854	$(TC)_8RG$	11	287–1,401	50
UBC857	$(AC)_8YG$	7	281–1,458	55
UBC866	$(CTC)_6$	4	592–1,414	57
UBC868	$(GAA)_6$	8	373–1,868	57
UBC873	$(GACA)_4$	11	337–1,986	56
UBC878	$(GGAT)_4$	8	647–1,882	56
UBC880	$(GGAGA)_3$	13	231–1,378	55
UBC881	$(GGGTG)_3$	8	426–1,426	50
UBC892	TAGATCTGATATCTGAATTCCC	6	553–1,500	52
UBC893	NNNNNNNNNNNNNNN	8	390–1,815	52
UBC899	CATGGTGTTGGTCATTGTTCCA	13	308–1,752	55

N = (A, G, C, T); R = (A, G); Y = (C, T); N_L, number of loci scored; T_A, annealing temperature (°C)

Amplified fragments were scored for presence (1) or absence (0) of homologous bands. Assuming Hardy-Weinberg equilibrium, the resulting binary data matrix of the ISSR phenotypes was analyzed using POPGENE version 1.31 (Yeh et al. 1997), to estimate various genetic diversity parameters: the percentage of polymorphic bands (*PPB*), the effective number of alleles per locus (n_e), observed number of alleles per locus (n_a), Nei's gene diversity (h), Shannon's information index (I), gene differentiation coefficient (G_{ST}) and the level of gene flow (N_m). Nei's (1978) unbiased genetic identity (I) and genetic distance (D) between populations were also computed using this program.

The hierarchical analysis of molecular variance (AMOVA) was used to calculate variance components within and among populations (Excoffier et al. 1992). DCFA 1.1 program (Zhang and Ge 2002) was employed to generate Squared Euclidean distance matrix, which was used as the input for the AMOVA analysis. To reveal the genetic relationship among populations, a dendrogram was constructed using an unweighted pair-group method with arithmetic mean (UPGMA) of MEGA 3.1 (Kumar et al. 2004). In addition, Mantel test (Mantel 1967) was performed using Mantel 2.0 (Liedloff 1999), to determine the correlation between inter-population genetic and geographic distance matrices.

Results

Genetic diversity

The 18 selected primers generated 150 bands ranging in size from 231 to 1,986 bp, corresponding to an average of 8.33 bands per primer (Table 2). At the population level, the percentage of polymorphic bands (*PPB*) varied between 1.33% and 32.00%, with an average of 18.67%, while the value rose sharply to 84.00% at the species level. Table 3 showed the details about Nei's gene diversity index (h) and Shannon's information index (I). As indicated by these three parameters, the highest level of variability occurred in Population YJ ($PPB = 32.00\%$; $h = 0.1092$; $I = 0.1643$), whereas the lowest level in Population WL ($PPB = 1.33\%$; $h = 0.0065$; $I = 0.0091$).

Genetic structure

POPGENE analysis revealed that the gene differentiation coefficient among populations (G_{ST}) was

Table 3 Genetic diversity of *P. pseudocerasus* detected by ISSR analysis

Populations	n_a	n_e	h	I	*PPB* (%)
YJ	1.3200	1.1848	0.1092	0.1643	32.00
YN	1.2667	1.1730	0.0986	0.1459	26.67
MZ	1.1467	1.0867	0.0502	0.0755	14.67
PB	1.0800	1.0552	0.0315	0.0463	8.00
WL	1.0133	1.0127	0.0065	0.0091	1.33
BX	1.1189	1.0871	0.0471	0.0684	11.33
CK	1.2867	1.1592	0.0933	0.1408	28.67
FP	1.2533	1.1369	0.0819	0.1244	25.33
XX	1.2667	1.1715	0.0986	0.1462	26.67
AK	1.1200	1.0742	0.0438	0.0653	12.00
Mean value	1.1872	1.1141	0.0661	0.0986	18.67
At species level	1.8400	1.3648	0.2242	0.3479	84.00

n_a, Observed number of alleles; n_e, Effective number of alleles; h, Nei's gene diversity; I, Shannon's information index; *PPB*, Percentage of polymorphic bands (%)

0.7118, while AMOVA analysis showed that 64.53% of total variance occurred among populations (Table 4), both of which indicated a high level of inter-population genetic differentiation. This was further confirmed by the low level of gene flow ($N_m = 0.2025$) among populations.

Nei's (1978) unbiased genetic distances among populations were calculated based on 150 markers scored. Values ranged from 0.0796 (PB vs. WL) to 0.3472 (YN vs. MZ) (Table 5). Based on the obtained distance matrix, cluster analysis (UPGMA) was performed to further reveal the genetic relationships among populations. The 10 populations were clustered into two groups (Fig. 2). Group I was further subdivided into three subgroups: one including Population FP, XX and AK, one including Population YJ and CK, and the remainder including Population PB, YN and MZ. Group II comprised two populations (WL & BX), both of which were sampled from Sichuan Province. This topology was basically consistent with the geographic distribution of these populations, indicating a possible correlation.

In addition, Mantel test revealed a significant correlation between geographic and genetic distances among populations ($r = 0.5272$, $P < 0.005$).

Discussion

Effects of sample size on population genetics parameters

Since individuals of *P. pseudocerasus* usually occurred sporadically in small populations or over relatively wide areas, sample sizes of some populations in this study were pretty small. For example, only three individuals were observed in Population PB and WL, respectively. This inevitably raised doubts about the validity of the conclusions for these small populations.

Nei and Roychoudhury (1974) concluded that far more information can be obtained in an electrophoretic study by increasing the number of loci rather than the number of individuals. This was later empirically confirmed by Gorman and Renzi (1979), who demonstrated that both heterozygosity and genetic distance estimates are more significantly affected by the number of loci sampled than by the number of individuals sampled. Studies involving estimates of genetic distance are relatively sample size independent, and even a sample of two individuals usually yields a heterozygosity estimate within 2.5% of the % heterozygosity calculated for a much larger sample if a sufficient number of loci are examined (Gorman and Renzi 1979). Thus, despite the small sample sizes of some populations, the population genetics parameters calculated in this study can still hold true.

Genetic diversity of *P. pseudocerasus*

Our ISSR assay of the ten *P. pseudocerasus* populations revealed a relatively high *PPB* value (84.00%) at the species level, which was higher than those observed in other species in the family Rosaceae, e.g.

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

Source of variance	df	SSD	MSD	Variance component	Ratio of variance (%)	P
Among populations	9	1071.97	101.670	12.254	64.53	<0.001
Within populations	70	503.68	6.735	6.735	35.47	<0.001

Table 5 Nei's (1978) unbiased genetic identity (above diagonal) and genetic distance (below diagonal) among *P. pseudocerasus* populations

Populations	YJ	YN	MZ	PB	WL	BX	CK	FP	XX	AK
YJ	****	0.8446	0.8469	0.8103	0.8000	0.8492	0.8801	0.8080	0.8397	0.7781
YN	0.1689	****	0.9234	0.8492	0.7313	0.7779	0.8318	0.7911	0.8004	0.8108
MZ	0.1661	0.0796	****	0.8813	0.7242	0.7692	0.7922	0.7667	0.8283	0.7890
PB	0.2104	0.1635	0.1264	****	0.7067	0.7082	0.7699	0.7435	0.8124	0.7476
WL	0.2232	0.3130	0.3227	0.3472	****	0.8626	0.7971	0.7894	0.7717	0.7244
BX	0.1635	0.2511	0.2625	0.3451	0.1478	****	0.8121	0.7608	0.7507	0.7394
CK	0.1278	0.1842	0.2329	0.2615	0.2268	0.2082	****	0.8657	0.8355	0.8517
FP	0.2132	0.2344	0.2656	0.2964	0.2365	0.2734	0.1442	****	0.8708	0.8300
XX	0.1748	0.2227	0.1883	0.2078	0.2591	0.2867	0.1797	0.1383	****	0.8441
AK	0.2509	0.2097	0.2370	0.2909	0.3225	0.3019	0.1606	0.1864	0.1695	****

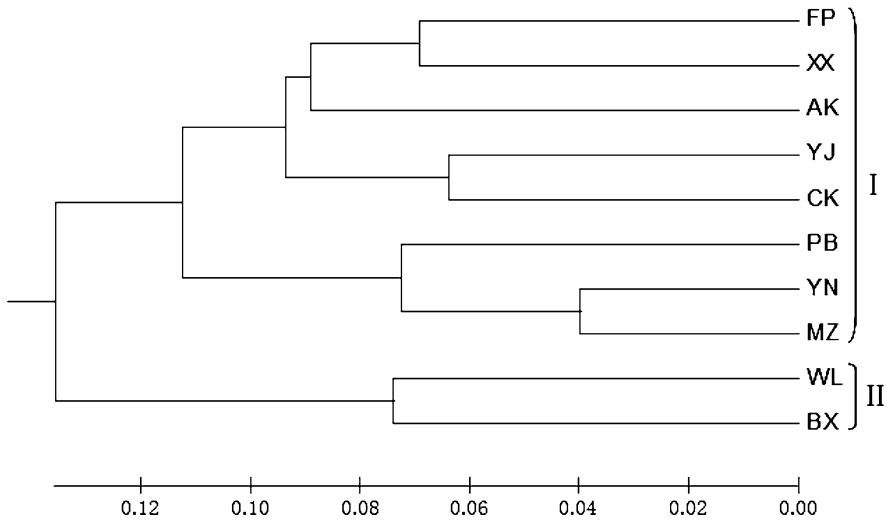

Fig. 2 UPGMA dendrogram for the 10 *P. pseudocerasus* populations based on Nei's (1978) genetic distance

79.78% in *Prunus persica* (L.) Batsch (Wang et al. 2001) and 76.2% in *Prunus mume* Sieb. et Zucc. (Zhang and Bao 2007). In contrast, the genetic diversity within populations was pretty low (*PPB*: 1.33 ~ 32.00%; Average: 18.67%).

This high level of intraspecific genetic diversity was probably linked with its life history traits. It is now well documented that the level of genetic diversity may be attributed to several life history traits, such as geographic range, seed dispersal mechanism, mating system, life form and taxonomic status (Hamrick and Godt 1989). Generally, woody species with large geographic ranges, outcrossing breeding systems, and wind or animal-ingested seed dispersal have more genetic diversity within species than woody species with other combinations of traits (Hamrick et al. 1992), basically in line with the traits of *P. pseudocerasus*. As a long-lived perennial woody plant, *P. pseudocerasus* is widely distributed in the temperate zone of Northern Hemisphere (Yu and Li 1986). To adapt to ecologically diverse habitats, it has probably accumulated considerable genetic variation within species. Besides, it adopts a animal-ingested seed dispersal system (Cai 2006). Despite the poor knowledge about its breeding system, all these traits together have probably resulted in its present high genetic diversity at the species level.

On the other hand, this study also revealed a pretty low level of genetic diversity within population, which could be largely explained by its small population size, the effects of genetic drift and probably of inbreeding. Small populations tend to have increased levels of genetic drift, which can be

enforced by high incidences of inbreeding, and the resultant loss of genetic diversity (Thingsgaard 2001; Mateu-Andrés and Segarra-Moragues 2003). According to our field survey, *P. pseudocerasus* usually occurred in fragmented habitats, and most of its populations had a very small size. In consequence, genetic drift predominated, and resulted in increased homozygosity and loss of genetic variability. Despite the unavailability of definite records, *P. pseudocerasus* has probably adopted a facultative breeding system (i.e. the coexistence of self-pollination and cross-pollination) since self-compatibility was previously reported (Li et al. 2007).

Genetic structure

Population genetic structure reflects the interactions among species' long-term evolutionary history (e.g. habitat fragmentation, population specialization), mutation, recombination, genetic drift, breeding system, gene flow and natural selection (Slatkin 1987; Schaal et al. 1998). In this study, a high level of genetic differentiation ($G_{ST} = 0.7118$; $\Phi_{ST} = 64.53\%$) and pretty low gene flow ($N_m = 0.2025$) were revealed among *P. pseudocerasus* populations. Typically, $N_m < 1$ indicates strong differential selection, in which case population differentiation may be maintained (Slatkin 1985).

The present high level of inter-population genetic differentiation could be largely attributed to several factors. Firstly, it was probably closely connected with its small population size and habitat fragmentation. The theory of population genetics indicates that genetic differentiation caused by genetic drift and inbreeding depression should increase in progressively smaller and isolated populations (Templeton et al. 1990), as supported by previous reports (Buza et al. 2000; Shea and Furnier 2002; Tomimatsu and Ohara 2003). Despite its wide geographic distribution, the habitats of *P. pseudocerasus* were fragmented into small patches largely due to geographic barriers and anthropogenic activities, and the sizes of the isolated populations, e.g. Population PB and WL, were usually pretty small. As a result of habitat fragmentation, these populations may have accumulated considerable 'localized' genetic variations, which help adapt themselves to their specific local environments and distinguish themselves from others. Secondly, the mode of pollen and seed dispersal, which determines the gene flow among populations (Li and Chen 2004), may partly account for this high level of genetic differentiation. Flowers of *P. pseudocerasus* are mainly pollinated by insects (e.g. bees), which usually have a limited range of flight (Cai 2006), while natural populations of *P. pseudocerasus* usually occur in discrete mountainous regions with varying physical conditions (e.g. topography, altitude, humidity and temperature). Different physical conditions can lead to fruit ripening and flowering asynchrony, the latter of which in turn results in the substantial decrease or even lack of gene flow via pollen dispersal. Also, for the animal-ingested seed dispersal system, its efficiency largely depends on the migration habits and capacity of animals. Especially for those obligate fruit-eaters, which feed mainly or solely on the fruit of *P. pseudocerasus* during the ripening period, when fruit ripening asynchrony occurs, they may confine their activities within the areas where ripe fruits are available. As a result, the seed exchange among *P. pseudocerasus* populations will be discounted. Thirdly, Mantel test revealed a significant correlation between geographic and genetic distances ($r = 0.5272$, $P < 0.005$), indicating the role of geographic isolation as an important factor in the evolution of *P. pseudocerasus*' genetic structure. In addition, other factors, such as the low seed germination rate and breeding system (probably the coexistence of self-pollination and cross-pollination), may also partly contribute to the present population genetic structure in *P. pseudocerasus*.

Conservation considerations

The level and partitioning of genetic diversity are likely to affect the evolutionary potential of species and/or populations (Futuyma 1986). And, the main objective in any plant genetic resources conservation program should be to maintain the highest possible level of genetic variability present across the gene pool of a given species or crop both in its natural range and in a germplasm collection. As revealed in this study, the most immediate threat to *P. pseudocerasus* appears to be the genetic drift and inbreeding depression, and anthropogenic activities should take major responsibility for the reduction in population size.

An effective conservation strategy should integrate different conservation actions, 'which together leads to an optimum sustainable use of genetic diversity

existing in a target gene pool, in the present and future' (IPGRI 1993). To preserve this valuable natural germplasm resource, the following measures are proposed. Firstly, efforts should be made to protect its natural habitats. According to our field survey, the habitats of *P. pseudocerasus* have been seriously deteriorated and fragmented largely due to anthropogenic activities, such as road construction, deforestation and grazing. In consequence, these populations usually have a pretty small size, and thus tend to have increased levels of genetic drift and inbreeding depression. However, the conflict between conservation and local interests is a difficult issue. And also for the economic reasons, we suggest the emphasis for *in situ* conservation be placed on populations with high genetic diversity, such as Population YJ, CK, YN, XX and FP. Especially, Population FP, with the biggest size and relatively high genetic diversity, is located in a national nature reserve, and thus deserves more attention. Secondly, *ex situ* conservation should be a good complement to the *in situ* measures. Translocations are becoming increasingly popular since they are effective in both removing detrimental variation associated with inbreeding depression as well as restoring neutral genetic variation to historical levels (Bouzat et al. 2008). Seeds can be collected and exchanged among populations, so that they can increase their sizes and genetic diversity by natural regeneration. Thirdly, an appropriate number of seeds can be sampled from all these populations, and either be stored in a gene bank (e.g. seed bank, germplasm bank) or be used for the establishment of artificial populations in appropriate sites or botanical gardens. Besides, new techniques or methods should be developed to promote its applications in fruit industry, which will in turn benefit its conservation.

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