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RESEARCH ARTICLE

Genetic variation within and among populations of *Orychophragmus violaceus* (Cruciferae) in China as detected by ISSR analysis

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Abstract *Orychophragmus violaceus*, a ground covering plant that is widely distributed in China. It has both high economical value in food, forage, health care and ornamental value in gardening. In this study, the genetic diversity of 245 individuals from nine populations in China were investigated using the inter-simple sequence repeat markers. Of the 100 primers screened, eight were highly polymorphic. Using these primers, 162 discernible DNA fragments were generated with 150 (92.59%) being polymorphic, indicating a pronounced genetic variation at the species level. Also, there were high levels of polymorphism at the population level with the percentage of polymorphic bands ranging from 85.74 to 90.06%. Analysis of molecular variance showed that the genetic variation within populations was 80.80% and the variance among populations was 16.43%. The Nei's G_{ST} (0.1643) and gene flow among populations ($Nm = 2.5760$) revealed large gene exchanges among populations. *O. violaceus* belongs to out-crossing plants. It is capable of reproducing by self-sowing, thus can influence population genetic structure. The pronounced genetic variation within populations tells us that *O. violaceus* is a proper plant for genetic research and that there is great potential of breeding this species for gardening.

Keywords Genetic variation · ISSR · *Orychophragmus violaceus* · Population structure

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Introduction

Information on genetic diversity patterns can provide insight into the evaluating and utilizing of the germplasm resources. Knowledge of how genetic variation is partitioned among populations may have important implications not only to evolutionary biology but also to conservation biology. Hence, reliable estimates of population differentiation are crucial to understanding the connectivity among populations and represent important tools in the development of conservation strategies (Balloux and Lugon-Moulin 2002; Neel and Ellstrand 2003). In addition, environmental barriers and life history may all form the genetic structure of populations (McCue et al. 1996; Donnelly and Twonson 2000).

Orychophragmus violaceus (Linn.) O.E. Schulz, belongs to the Cruciferae family, and is a biennial herb. This plant is widely distributed in the northeast, northwest, north, east and middle of China. It is found in a variety of environments, including plains,

mountains, roadsides, adjacent to buildings and so on (Zhou et al. 1987). *O. violaceus* is commonly used for forage, health care and gardening (Luo et al. 1998; Xiao and Luo 1994; Ren et al. 1998; Weng and Huang 2001), and is highly regarded for its great economic and ornamental usages. Various topics have been studied on *O. violaceus*, including distant hybridization, karyotypes, tissue culture, isozymes and genetic transformation (Li et al. 1994; Zhao et al. 1995; Liang and Tao 1997; Li 1998; Luo et al. 2000; Wu et al. 2002; Mei et al. 2003). In recent years, many cities in China (Beijing, Nanjing, Shanghai, Hangzhou et al.) have used *O. violaceus* as a ground cover plant in gardens, streets and understory in a large scale (Fig. 1; Zhang and Dai 2005). The research on ornamental characters, morphological variations were observed a few years ago, when many types of variations in this plant was found (Zhang et al. 2005; Fig. 2). Up to now, genetic diversity of it has not been reported, this made it difficult to perform effective breeding program and to protect the resources.

The inter-simple sequence repeats (ISSR) is a newly developed modification of SSR (simple sequence repeats)-based marker systems (Ziekiewica et al. 1994). It has been recognized as useful molecular markers in the analysis of genetic diversity and population genetic analysis (Lu et al. 2006; Wang et al. 2005; Kar et al. 2005; Esayas et al. 2005). The ISSR has advantages, e.g., low quantities of template DNA requirement, no need of sequence data for primer construction, random distribution throughout the genome, generation of many informative bands per reaction, and reliable and reproducible production (Ziekiewica et al. 1994; Nagaoka and Ogihara 1997; Ge 2001). Consequently, the ISSR has been widely used in marker assisted selection, genetic diversity analysis, DNA fingerprinting, and evolution and molecular ecology (Zhao et al. 2007a, b; Vijayan et al. 2006; Carvalho et al. 2005; Bolibok et al. 2005; Nybom 2004; Wang 2002).

In the present study, we applied the ISSR markers to examine the genetic diversity of *O. violaceus* in three areas, Beijing, Nanjing and Shanghai, where large populations were found. Morphological studies indicate lots of variations within each population (Zhang and Dai 2005). We were particularly interested in the level of differentiation among populations and the levels of diversity within populations. This information help us understand the genetic background and will provide a reference for the utilization of genetic resources, species protection and including future breeding program.

Materials and methods

Plant materials

Specimens were collected from nine populations of *O. violaceus* in or around Beijing, Nanjing and Shanghai. Figure 3 shows the distribution of *O. violaceus* in some literature and the three areas that were sampled. Table 1 shows the locations and the climate factors of these populations. The size of the individual populations was 30 × 30 m, and the distance between populations within the three groups was farther than 3 km. Individual plants were systematically and randomly sampled, in which a total of less than 10% of the whole population was randomly sampled. 25–35 individual plants were collected with an interplant distance of at least 5 m to increase the possibility of detecting potential individual variation (Jin and Lu 2003).

Young leaves were collected from each sample plant and dried in silica gel for subsequent DNA extraction.

Fig. 1 Landscape of *Orychophragmus violaceus* groups in Shanghai Fuxing Park (**a**) and Beijing Botanical Garden Institute of Botany, Chinese Academy of Sciences (**b**)

Fig. 2 The variations on flower color and shape of *Orychophragmus violaceus*

DNA extraction

Total DNA was extracted from young leaves following the CTAB (cetyltrimethyl ammonium bromide) method described by Doyle and Doyle (1987) with one modification. The quality of DNA was determined in 1.0% agarose gels. The purified total DNA was quantified by a BioradSmartspec3000 UV–Vis spectrophotometer. DNA samples were adjusted to concentration of 20 ng/μL with ddH_2O and subjected to PCR amplification.ISSR PCR amplification.

ISSR PCR amplification

PCR amplification was done in a Techne TC-512 thermocycler using 10 μL reaction mixture containing 20 ng DNA, 1.0 μL of 10× buffer (200 mmol/L Tris–HCl; 200 mmol/L KCl; 100 mmol/L $(NH_4)_2SO_4$; 15 mmol/L $MgCl_2$), 0.2 mM dNTP, 0.4 μM primer and 0.5 U of *Taq* DNA polymerase. The PCR schedule was 94°C for 5 min followed by 45 cycles of 94°C for 45 s, 54–60°C for 1 min, 72°C for 1.5 min and a final extension of 7 min at 72°C. ISSR-PCR products were separated by electrophoresis on 6% urea polyacrylamide gels and silver stained to visualize the bands. One hundred primers (UBC primer set no. 9, Biotechnology Laboratory, University of British Columbia, Canada) were screened initially. Two DNA templates from each population were chosen randomly for PCR amplification. Fifty ISSR primers were screened on these selected individuals by comparing the effects of magnesium concentrations and annealing temperature during amplification. Eight primers that produced clear and reproducible fragments were selected for the full survey of all 245 individuals (Table 2).

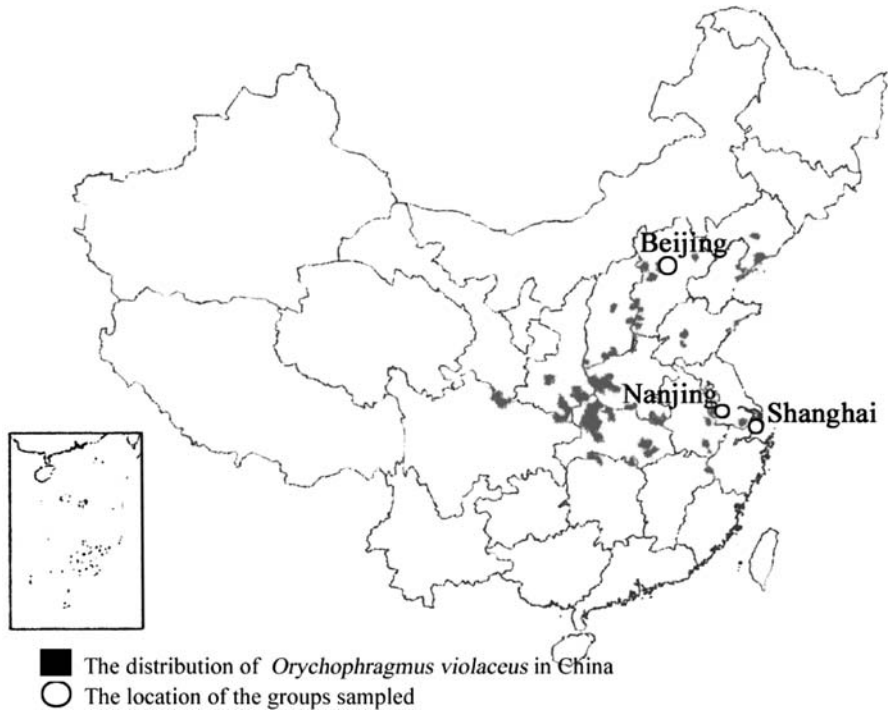

Fig. 3 Map showing the distributions of *Orychophragmus violaceus* and locations of three groups sampled from Beijing, Nanjing and Shanghai

Data analysis

Only distinct, reproducible, well-resolved fragments were scored as present (1) or absent (0) for each of the ISSR markers. The Polymorphic bands (PPB), allele number (*Ao*), effective allele number (*Ae*), Nei's (1973) expected heterozygosity (*He*), Shannon index of diversity (*I*), gene diversity among subpopulations (*Hs*) and of the total population (*Ht*), gene differentiation (*Gst*), gene flow (*Nm*) and gene distance (*D*) were analyzed using POPGENE software, version 1.31 (Yeh et al. 1999). The Analysis of Molecular Variance (AMOVA program version 1.55, Excoffier et al. 1992) was performed to describe the genetic structure and variation among groups, among populations within groups and among individuals. A dendrogram was constructed by an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA) on NTSYS-pc program (version 1.21, Rohlf 2000).

Results

Genetic diversity within populations

The ten primers generated 162 bands ranging in size from 200 to 1500 bp (Fig. 4), corresponding to an average of 20.2 bands per primer. Of these bands, 92.59% (150 in total) were PPB at the species level. Assuming Hardy–Weinberg equilibrium, the Nei's gene diversity (*He*) ranged from 0.3330 (SG) to 0.3659 (BY) for *O. violaceus*, with an average of 0.3464 at the population level. Shannon index (*I*) ranged from 0.4938 (SG) to 0.5369 (BY), with an average of 0.5150 at the population level. Among the nine populations, BY from Beijing region exhibited the greatest variability (PPB 90.06%, *He* 0.3659, *I* 0.5369). In contrast, genetic diversity in population SG was lowest with PPB 86.98%, *He* 0.3330, *I* 0.4938 (Table 3). Considering the groups of populations, those from Beijing group had, on average, higher levels of diversity than those from Shanghai group.

Genetic structure of populations

To assess the overall distribution of genetic diversity, the AMOVA program was used to analyze the distance matrix. The genetic differentiation among populations in AMOVA was highly significant ($P < 0.001$; Table 4). A large proportion of genetic variation (80.8%) existed among individuals within the populations, whereas 16.43% resided among populations. Nei's (1973) estimator of population substructure (G_{ST}) also indicated a low level of

Table 1 Sampling information of *Orychophragmus violaceus*: location, sample size, habitat, growth and climate of the collection site

Code	Location	Sample size	Habitat	Growth	Climate
BH	Houbajia nursery, Beijing	21	Cultivated plants under the trees, with abundant sunlight and big population size	Good	Beijing is located in the warm temperate continental monsoon climate zone with distinct seasons. Dry and rainless in spring, hot and rainy in summer, crisp in autumn, and dry and cold in winter
BY	Yuanmingyuan park, Beijing	29	Cultivated plants under the trees, sunshiny hillside	Vigorous	
BT	The temple of heaven, Beijing	27	Cultivated plants under the trees, broadly distributed, moist and fertile soil, shady	Flourishing and dense	
BS	The summer palace, Beijing	25	Under the trees on hillside, shady and cool condition, fertile soil, moist air and soil	Flourishing	
NL	Nanjing lovers park	24	Both sides of garden path, rows of planting, abundant sunlight, fertile soil, moist air and soil	Flourishing and higher plant	Nanjing is located in the north subtropical monsoon climate, with very distinctive seasons; with abundant sunlight and rain precipitation
NB	Nanjing botanical garden Mem. Sun Yat-Sen	25	Beneath the trees, grown with lawn, moist air, strewn distribution, cultivated	Good	
SB	Shanghai botanical garden	29	Beside the building, abundant sunlight and moist air and soil	Vigorous and higher plant	Shanghai is located in the subtropical marine monsoon climate, with distinct seasons and enough moisture
SF	Shanghai fuxing garden	35	Both sides of garden path, abundant sunlight, fertile soil	Good	
SG	Shanghai guilin garden	30	Under the trees, grown with lawn, shady, fertile soil, moist air and soil	Flourishing and orderly	

Table 2 Codes and sequences of ISSR primers used and their optimal annealing temperature (T_a)

Primer	Sequence (5′–3′)	T_a (°C)
UBC807	AGAGAGAGAGAGAGAGT	54
UBC810	GAGAGAGAGAGAGAGAT	54
UBC834	AGAGAGAGAGAGAGAGYT	55
UBC836	AGAGAGAGAGAGAGAGYA	57
UBC841	GAGAGAGAGAGAGAGAYC	59
UBC847	CACACACACACACACARC	60
UBC880	GGAGAGGAGAGGAGA	54
UBC888	BDBCACACACACACACACA	56

Y = (C, T), B = (C, G, T), D = (A, G, T)

population differentiation (G_{ST} = 0.1643). These G_{ST} translated into correspondingly high levels of gene flow (*Nm*), with 2.5760 migrants exchanged between populations (on average) each generation. There have been concerns about the direct use of G_{ST} to estimate gene flow (Whitlock and McCauley 1999). However, Tn some extent, G_{ST} remains a useful measure of the average effects of gene flow (Neigel 2002).

The genetic distance and UPGMA analysis

The UPGMA analysis indicated that there are two clusters (Fig. 5). The first cluster contains population

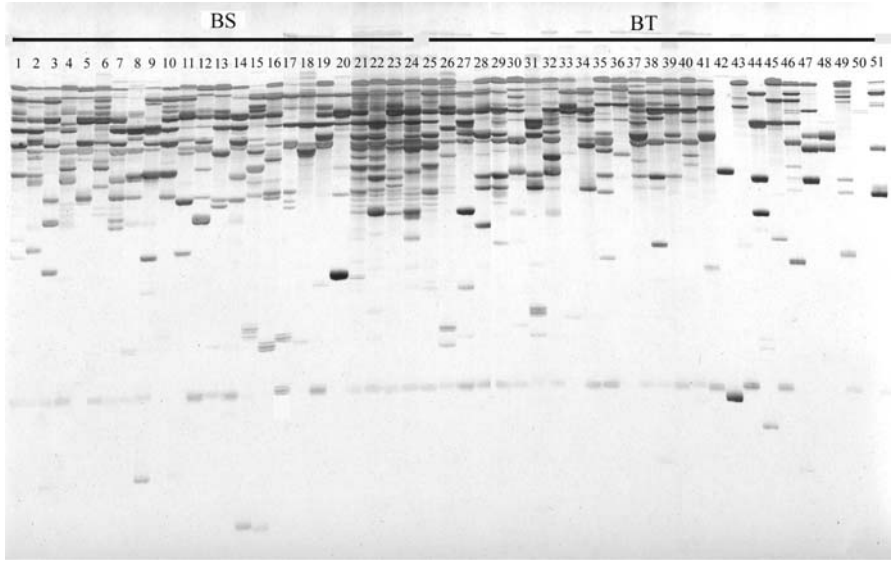

Fig. 4 Genetic profile of *Orychophragmus violaceus* populations using primer 847. Lanes 1–24 and 25–51 represent template DNA for each individual from *BS* and *BT*

Table 3 Genetic diversity of *Orychophragmus violaceus* detected by ISSR analysis

Population code	%Polymorphic bands (PPB)	Allele number (*Ao*)	Effective allele number (*Ae*)	Nei′s gene diversity (*He*)	Shannon index (*I*)
BH	86.98	1.8998	1.5651	0.3511	0.5170
BY	90.06	1.9006	1.5964	0.3659	0.5369
BT	85.74	1.8574	1.5440	0.3394	0.5007
BS	86.98	1.8698	1.5460	0.3452	0.5111
NL	86.74	1.8674	1.5453	0.3383	0.4988
NB	85.74	1.8574	1.5570	0.3475	0.5516
SB	86.36	1.8636	1.5379	0.3374	0.4988
SG	86.98	1.8698	1.5311	0.3330	0.4938
SF	87.59	1.8759	1.5863	0.3596	0.5267
Mean at population level	87.02	1.8702	1.5566	0.3464	0.5150
Mean at species level	92.59	1.9389	1.7275	0.4242	0.6103

Table 4 Analysis of molecular variance (AMOVA) for ISSR variation for *Orychophragmus violaceus* populations

Source of variation	Degrees of freedom	Variance	Mean variance	Variance component	Percentage of variance	*P*-value
Among groups	2	231.60	115.80	0.99	2.77	
Among populations	6	523.87	87.31	5.85	16.43	
Within populations	236	6792.08	28.78	28.78	80.80	<0.001
Total	244	7457.55				

P-values are the probabilities of having a more extreme variance component than the observed values alone. Probabilities were calculated by 1,000 random permutations of individuals across populations

NB from Nanjing, and populations SB and SF from Shanghai. The second cluster contains of population NL from Nanjing, population SG from Shanghai, and populations BS, BT, BY and BH from Beijing. The four populations from Beijing regions were clustered together, whereas, Nanjing and Shanghai populations had relatively closer relation.

Discussion

Genetic diversity of *Orychophragmus violaceus*

ISSRs can provide more polymorphism than isozymes and RAPD markers (Wolfe and Liston 1998; Ge and Sun 1999; Camacho and Liston 2001). By using ISSR primers, we found a high level genetic diversity for the species of *O. violaceus* with 92.59% of bands being polymorphic in all nine populations. The percentage of PPB in each population ranged from 85.74 to 90.06%. The genetic variation detected was a little higher than other species (Brassica *campestris* L., *Raphanus sativus* L. and *Brassica juncea* L.) in this family (He et al. 2002; Wu et al. 1997; Shen et al. 2004; An et al. 1999; Rabanni et al. 1998; Burton et al. 2004). In comparison, *O. violaceus* presents high genetic diversity both within and among populations.

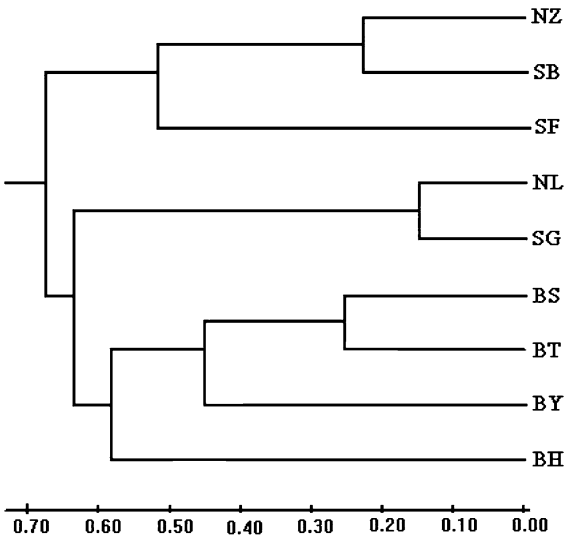

Fig. 5 UPGMA clustering of nine *Orychophragmus violaceus* populations based on genetic similarity calculated from eight ISSR markers

The level and pattern of genetic diversity detected by ISSRs in the present study highly agreed with the analysis of morphological characters, karyotypes and isozymes of *O. violaceus*. Firstly, the analysis of variation of morphological characters of *O. violaceus* revealed that there were many kinds of variations in petal color, petal shape, flower size, leaf color, leaf shape, plant shape and other features. Many peculiar variations were found, e.g., speckled petals, petal stain, toothed petal margin and speckled leaves. These characters were significantly different among populations and within populations (Zhang et al. 2005). The karyotypes of *O. violaceus* also had relatively high diversity among populations, individuals and cells (Zhang et al. 2006), and high percentage of B chromosomes and aneuploids were observed in the nucleus. Li et al. (1994) pointed that the variants of *O. violaceus* have evolved with considerable hereditary variation during the phylogenesis of the species, which can be seen in the significantly different of the locations of secondary constrictions, the chromosome arm ratio and karyotypes. In addition, analysis of POD in different environments of *O. violaceus* indicated that there were great differences among plants in isoenzyme patterns and relative enzymatic activity (Li 1998).

The high genetic variability among population in the plant may be a consequence of sexual

reproduction, mutations of somatic cells, selection, gene flow, genetic drift and changing environment (Gao and Yang 2006). The cross-pollination mechanism, sexual reproduction, high seed ratio and self-sown ability to produce offspring of *O. violaceus* could result in the accumulation of abundant genetic variation during the long evolution history of the species. The variations of *O. violaceus* was also explained in cytology, autotetraploid or allotetraploid chromosomes lead to the diversity of alleles, and the change of the number of somatic chromosomes was due to cytomixis and chromosome fractionation during the process of mitosis and meiosis. Differences of gene dosage, modification and regulation will occur in different cells of different plants (Wu et al. 1996).

Population genetic structure

ISSR markers revealed that *O. violaceus* populations had more genetic variation within populations than among populations. Little differentiation was detected among populations. There are many factors that determine the genetic structure of plant populations, including reproductive biology, gene flow, seed dissemination and nature selection. Reproductive biology is one of the most important factors (Zhao et al. 2007a, b). According to Hamrick and Godt (1989), outcrossing plant species tend to exhibit between 10 and 20% genetic variation among populations while selfing species exhibit, on average, 50% variation between populations. Studies on the biology of flowering and pollination indicate *O. violaceus* is an outcrosser (Zhang et al. 2007). It is also supported by the genetic differentiation ($G_{ST} = 0.1643$) among populations of *O. violaceus*, which was very close to the average genetic differentiation ($G_{ST} = 0.144$) in outbred populations (Bussell 1999).

The variation among individuals within populations was the main source of variation of *O. violaceus*. The reasons for this population genetic structure are as follows: (1) *O. violaceus* is widely distributed in the northeast, northwest, north, east and middle of China and is found in various kinds of environments. The strong adaptability and wide distribution results in little differentiation between populations. (2) *O. violaceus* exchanged its genes mainly by seeds and pollen. The spread of seeds and pollen is the main way of gene flow in natural populations of plants (Li and Chen 2004). Seeds and pollen are both small and dispersed by wind. Due to the high frequency of strong winds, the effects of long distance dispersal of seeds and pollen by wind are similar. (3) Gene flow is the most important factor in making population genetic structure homogeneous. The greater the amount of gene flow among populations, the less gene differentiation occurs (Slatkin 1985). The large gene flow ($Nm = 2.5760 > 1$) of *O. violaceus* could counteract most of the gene differentiation which is caused by genetic drift within populations.

We also found little genetic differentiation among three groups, which suggests that the populations from different sides are poorly differentiated. Only 2.77% of the total variation was found among three geographic regions (Beijing, Nanjing, and Shanghai). This small vitiation might be due to the continuous distribution in China. Though there is no direct transport of seeds and pollen among three regions, gene flow could influence the evolution process of different regions. Another possible reason is that the populations we selected were cultured populations not natural population. Seeds were collected around the cities and it is difficult to know their real source.

Implications for development

The information about population genetic diversity represents population adaption to environments which conditions on the level of its adaptive evolution. It is also of critical importance to the conservation and management of plants, including the assessment of the conservation value and status of special populations (Hogbin and Peakall 1999; Bawa and Ashton 1991; Hamrick and Godt 1996). The high genetic diversity maintained within populations of *O. violaceus* is encouraging. In addition, the plant possesses great ornamental value and developmental potential. It is necessary to protect existing natural populations and its habitat in order to preserve as much genetic variety as possible. Further research on the reproductive mechanism and law of inheritance and variation of some characters (petal color and shape, leaf shape) of *O. violaceus* will help us to understand its natural hybrids and promote its better utilization in the future.

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