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Clonal Diversity and Genetic Differentiation in Rhizomatous Herb, *Iris japonica* (Iridaceae) Populations on Jinyun Mountain, Southwest China

(Kepelbagaian Klon dan Pembezaan Genetik dalam Populasi Tumbuhan Berizom, *Iris japonica* (Iridaceae) di Gunung Jinyun, Barat Daya China)

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ABSTRACT

Many plants can reproduce both clonally and sexually, and the relative importance between the two modes of reproduction varied among habitats. Clonal diversity and genetic differentiation of the rhizomatous herb, Iris japonica *populations, was analyzed by 12 randomly amplified polymorphic DNA markers to determine the extent that reproductive mode varied locally between two habitats, open area of forest edge (OAFE, 2 populations OAFE 1 and OAFE 2) and bamboo forest (BF, 2 populations BF 1 and BF 2) on Jinyun Mountain, southwest China. Total clonal diversity and genetic diversity of* I. japonica *populations were high. The clonal diversity and genetic diversity of* I. japonica *populations in BF habitats were lower than those in OAFE habitats. Neighbor-joining tree by Jaccard's genetic distance showed two genetically distinct groups: OAFE group and BF group. AMOVA indicated that about half of the total variation existed within the four populations, and that genetic differentiation among habitats (BF populations and OAFE populations) was also significant. OAFE habitats were helpful in maintaining clonal diversity and genetic diversity of* I. japonica *populations than BF habitats. Habitat differences might have distinguished effects on the relative significance of clonal propagation and sexual reproduction, and further have a fatal influence on clonal diversity, genetic diversity and genetic differentiation of* I. japonica *populations in Jinyun Mountain, southwest China.*

Keywords: Clonal diversity; genetic diversity; Iris japonica*; population differentiation; RAPD*

ABSTRAK

Banyak tumbuhan boleh membiak secara klon dan seksual, dan kepentingan relatif di antara kedua-dua cara pembiakan berbeza-beza mengikut habitat. Kepelbagaian klon dan pembezaan genetik bagi populasi tumbuhan berizom, Iris japonica*, dianalisis dengan 12 penanda polimorfik DNA teramplikasi rawak untuk menentukan perbezaan setempat cara pembiakan antara dua habitat: kawasan terbuka pinggir hutan (OAFE; 2 populasi – OAFE 1 dan OAFE 2) dan hutan buluh (BF; 2 populasi – BF 1 dan BF 2) di Gunung Jinyun, barat daya China. Kepelbagaian total klon dan genetik bagi populasi* I. japonica *adalah tinggi. Kepelbagian klon dan genetik bagi populasi BF adalah lebih rendah berbanding populasi OAFE. Pohon Neighbor-joining berdasarkan jarak genetik Jaccard menunjukkan kewujudan dua kumpulan genetik yang berbeza: kumpulan OAFE dan kumpulan BF. AMOVA menunjukkan separuh daripada variasi terkandung dalam empat populasi, dan pembezaan genetik antara habitat (populasi BF dan populasi OAFE) adalah signifikan. Habitat OAFE lebih berguna dalam pengekalan kepelbagaian klon dan genetik populasi* I. japonica *jika dibandingkan dengan habitat BF. Perbezaan habitat mungkin mempunyai kesan membezakan yang relatif signifikan pada propagasi klon dan pembiakan seksual, dan ini seterusnya memberikan kesan kemandirian pada kepelbagian klon, kepelbagaian genetik dan pembezaan genetik* I. japonica *di Gunung Jinyun, barat daya China.*

Kata kunci: Iris japonica*; kepelbagaian genetik; kepelbagaian klon; pembezaan populasi; RAPD*

INTRODUCTION

Many plants could reproduce both clonally and sexually (Mandujano et al. 1998; Prati & Schmid 2000). How such plants partitioned their reproductive effort into clonal propagation and sexual reproduction was a fundamental question in plant ecology and evolution (Wilk et al. 2009). Sexual reproduction has been thought to create a new combination of genomes, and it lead to acceleration of adaptation and speciation in response to selection (Kanno & Seiwa 2004). Clonal propagation, on the other hand, could have noticeable adaptations to severe habitats with limited resources (e.g., light, temperature and nutrients) by physiological integration, and reserve the same genotype of a local population (Mandujano et al. 1998; Wang et al. 2009). Clonal propagation was thought to maintain lower genetic diversity than sexual reproduction for populations (Pollux et al. 2007; Wilk et al. 2009). The relative importance of two reproduction modes was usually modified by variation of abiotic and biotic conditions in different habitats (Mandujano et al. 1998; Young et

al. 2002). Morphological, physiological as well as life-history traits (including sexual reproduction and clonal propagation) were found to respond to environmental variation (competition and light) in many clonal plants (Kanno & Seiwa 2004; Prati & Schmid 2000). Thus, trade-offs between clonal propagation and sexual reproduction might influence gene variations within population and among populations, the size and spatial pattern of genets, mating patterns, population size, and the local adaptation and geographical evolution (Tarasjev 2005; Young et al. 2002). Finally, it might lead the variation of clonal diversity and genetic diversity in clonal plants (Jacquemyn et al. 2006; Pollux et al. 2007; Wilk et al. 2009). Therefore, habitat difference, even on a small spatial scale, was the key to maintaining genetic differentiation. Analysis on the variation of genetic diversity and differentiation of clonal plant in different habitats can lead us understand the adaptation of two reproduction modes.

Iris japonica Thunb. (Iridaceae), is a perennial herb reproducing by both seeds and vegetative rhizomes. It occurs preferentially in the understory of sparse forest understory, forest gap, forest edge and the moist grassland, forming mono-dominant groups. Its geographical distribution is Asia, including south China and Japan. Plants are flowering from March to April and fruiting from May to June (Wang et al. 2008).

DNA markers, such as randomly amplified polymorphic DNA (RAPD) (Yamagishi et al. 2010), microsatellite (Wilk et al. 2009), and noncoding spacer sequences of the plastid genome (Honjo et al. 2004), have been employed to analyze genetic diversity in plant populations. RAPD markers, without sequencing nucleotides and developing primers specific, could estimate variation in a simpler way and in a shorter period (Yamagishi et al. 2010). In this study, RAPD markers were employed to estimate the clonal diversity and genetic differentiation of four *I. japonica* populations from two habitats, open area of forest edge (OAFE, 2 populations OAFE 1 and OAFE 2) and bamboo forest (BF, 2 populations BF 1 and BF 2) on Jinyun Mountain, southwest China. In detail, we discussed (1) the level of clonal diversity within population and among populations, (2) the extent of genetic diversity and differentiation within population and among populations, and (3) whether clonal diversity and genetic diversity of *I. japonica* depend on localized differences in habitat or not.

Materials and Methods

Study Area

The study area was located in Jinyun Mountain Nature Reserve of Chongqing city, Southwest China. It belonged to subtropics climate with moist monsoon. *I. japonica* populations were mainly distributed in sparse forest and at the edge of evergreen broad-leaved forest. The first habitat type that open area of forest edge (OAFE) included two study sites (OAFE 1 and OAFE 2) (29°50'15"N, 106°23'22"E) was located within Reserve at the edge (with a distance of 10 m) of a *Cinnamomum camphora* evergreen broad-leaved forest. The second type that bamboo forest (BF) also had two sites (BF 1 and BF 2) (29°49'38"N, 106°23'4"E), was located 5 km away, at these sites, overstory wood species *Phyllostachys pubescens*, a giant bamboo with sparse canopy. Two study sites of each habitat type had a distance of 800 m.

DNA Extraction and RAPD Amplification

In four sites (BF 1, BF 2, OAFE 1 and OAFE 2) above, leaf samples of 80 (each population for 20 samples) randomly selected genets (distance > 1 m between genets) were collected and stored at −80°C until required for genetic analysis. The CTAB method of Yang (2006) was applied to isolate high-molecular-weight DNA from leaf tissue.

DNA was amplified in PTC-200 DNA Engine Cycler (Bio-Rad Inc., California, USA). We first screened DNA of four randomly selected leaf samples for twenty primers from the previous DNA analysis on *Iris*, including *Iris japonica* (Zhang & Huang 2008). Primers that did not produce well-amplified, clearly distinguishable bands or reproducible fingerprints were excluded (Sangon Biotech (Shanghai) Co. Ltd). Out of the twenty primers examined, twelve primers were selected yielding polymorphic bands (Table 1). These twelve primers were chosen for the subsequent analysis of the 80 samples.

All RAPD–PCRs were done in 25 μL with 1 μL of diluted DNA (approximately 50 ng), 1.0 μL primer (4.0 μ mol L^{-1}), 2.5 μL of 10 × reaction buffer including 15 mM $MgCl_2$, 1.0 μL of 2.5 mM combined dNTPs (Sangon Biotech (Shanghai) Co. Ltd) and 1.5 unit *Taq* polymerase (Sangon Biotech (Shanghai) Co. Ltd). All amplifications were carried out in a PTC-200 DNA Engine Cycler under the following conditions: PCR was began with an 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C. PCR was finished with an extension of 7 min at 72°C. After the final cycle, samples were stored at 4°C.

The amplified products were separated on 1.0% agarose gels in 1 M TAE buffer (Tris-Acetate-EDTA buffer) containing Goldview (2 μL) (SBS Genetech Co. Ltd, Beijing, China), using Bio DL2000 molecular size standard (Bioer Technology Co. Ltd, Hangzhou, China). For data scoring, the banding patterns were recorded using the gel documentation system Gel Doc 1000 (Bio-Rad Inc., California, USA). Individuals that did not give clear signals were omitted from the analysis.

Data Analysis

The data concerning band presence (1)/absence (0) in the RAPD analysis were used to determine genetic identity. Random amplified polymorphic DNA data were converted into a matrix with presence (1)/absence (0) data for all scorable bands obtained by the twelve primers. The following measures of genotypic (clonal) variation were calculated for each OAFE and BF habitats. Samples with

TABLE 1. Primer characteristics, total number and number of polymorphic bands by RAPD-PCR analysis

Primer name	Nucleotide sequence (5'-3')	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Primer name	Nucleotide sequence (5'-3')	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)
S27	GAAACGGGTG	10	7	70.00	S1155	GAAGGCTCCC	9	7	77.78
S133	GGCTGCAGAA	7	6	85.71	S1159	GTTCTCGGAC	5	4	80.00
S306	ACGCCAGAGG	11	10	90.91	S1177	ACGGCACTCC	9	7	77.78
S358	TGGTCGCAGA	9	8	88.89	S1202	CACCTGCTGA	8	6	75.00
S1141	AAGACGACGG	6	3	50.00	S1213	GGGTCGGCTT	7	6	85.71
S1144	AACAGGGCAG	8	5	62.50	S1263	ACGAAACGGG	13	11	84.62

an identical banding pattern were assumed belonging to the same RAPD phenotype (= same genotype). However, scoring errors, polymerase chain reaction artifacts and somatic mutations might cause small differences in the data of individuals from the same genotype, which lead to an erroneous assignment to the same or different genotype. Therefore, clonal diversity was estimated using the program GenoTypeGenoDive with a threshold value of 5% (Rusterholz et al. 2009). Parameters of clonal diversity were as follows: (1) The proportion of distinguishable genotypes (*PD*) (Ellstrand & Roose 1987) was measured as G/N, where G is the number of genets (distinct genotypes) and N is the total number of individuals (ramets) sampled. (2) Simpson's diversity index (D) modified for finite sample size (Pielou 1969) was calculated as $D = 1 - \sum[N_i(N_i - 1)/N(N - 1)]$, where N_i is the number of plants of genotype i and N is the number of plants sampled. D ranges from 0 to 1, with 1 being the maximal diversity.

As measurement of genetic diversity, *PPL* (the percentage of polymorphic loci), *Nei*' gene diversity index (h), *Shannon–Wiener* index (I), total gene diversity (h_t), gene diversity within population (h_s), total genetic diversity (I_t), genetic diversity within population (I_s), coefficient of gene differentiation (G_{ST}) and gene flow among population (N_m) corrected for sample sizes were calculated with the program POPGENE 32 (Yeh & Boyle 1997). Neighbor-joining tree of 80 *Iris japonica* individuals was estimated by 12 polymorphic RAPD markers and *Jaccard*'s genetic distance. The distribution of genetic variability among and within populations and habitats was investigated by an analysis of molecular variance (AMOVA) by GenAlEx 6.2 (Peakall & Smouse 2006).

RESULTS AND DISCUSSION

CLONAL DIVERSITY OF *IRIS JAPONICA* POPULATIONS

The distinguishable genotypes, *PD* and *D* of the *I. japonica* populations in BF habitats (BF 1, BF 2 and total BF population) were lower, and mean size of genotype was higher than those in the corresponding OAFE habitats (OAFE 1, OAFE 2 and total OAFE population). Our value for the mean *PD* ranged from 0.400 to 0.750 among populations (Table 2). Studies on clonal plants have reported such a large range in genotype diversity among populations (Ellstrand & Roose 1987). The special genotypes might occur in different habitats for a long time. Thus, habitat differences could lead the differences in genotypes diversity in many clonal plants (Ellstrand & Roose 1987; Young et al. 2002), including *Iris pumila* (Tucic et al. 1988) and *Iris hexagona* (Ruan et al. 2005). OAFE habitats were helpful to maintaining clonal diversity (genotypes) than BF habitats. Because clonal propagation could reserve the same genotype of a local population, these might indicate considerable variation in two reproduction modes (Mandujano et al. 1998).

GENETIC DIVERSITY AND GENETIC DIFFERENTIATION OF *I. JAPONICA* POPULATIONS

The PCR amplification pattern of BF1 population generated with primer S1263 is shown in Figure 1 (other PCR amplifications are available from the corresponding author). Eighty (78.43%) of 102 RAPD fragments showed polymorphism among 80 *I. japonica* samples. Furthermore, in BF habitats, *I. japonica* had an obviously reduced *Nei*' and *Shannon–Wiener* index of genetic diversity and a lower percentage of polymorphic loci compared with OAFE habitats (Table 2). Neighbor-joining tree of 80 individuals by Jaccard's genetic distance showed two groups: OAFE group (samp 1-40) and BF group (samp 41-80), indicating that OAFE and BF populations were genetically distinct from each other. BF populations had larger clones (e.g. one clone including samp 43, 44, 45, 65, 71 and 76, and another clone including samp 41, 42, 77, 78 and 79) than OAFE populations, which indicated the predominant clonal propagation in BF populations (Figure 2).

The relative importance between clonal propagation and sexual reproduction was usually modified by variation of abiotic and biotic conditions in different habitats (Mandujano et al. 1998). Study on six *Iris* (*I. hexagona*) populations showed that the genetic diversity was higher in salty habitats than in freshwater wetland habitats, which resulted from salty habitats was in favor of sexual reproduction and wetland habitats was for clonal

TABLE 2. Clonal and genetic diversity of *Iris japonica* population ns in OAFE and BF sites

Population (*n*)	Clonal diversity				Genetic diversity		
	Genotypes	*PD*	*D*	Mean size of genotype	*PPL* (%)	*Nei*	*Shannon-Wiener*
OAFE 1 (20)	12.0	0.600	0.890	1.67	54.90	0.215	0.316
OAFE 2 (20)	15.0	0.750	0.920	1.33	63.73	0.256	0.374
Total OAFE (40)	23.0	0.575	0.864	1.74	73.53	0.257	0.385
BF 1 (20)	10.0	0.500	0.875	2.00	42.16	0.163	0.238
BF 2 (20)	8.0	0.400	0.825	2.50	44.12	0.161	0.240
Total BF (40)	13.0	0.325	0.676	3.08	55.88	0.180	0.273

n was the ramet sample size, genotypes were the number of multilocus genotypes found, *PD* was the proportion of distinguishable multilocus genotypes and *D* is the Simpson index. *PPL* (the percentage of polymorphic loci), Nei' gene diversity index and Shannon-Wiener index were using in genetic diversity.

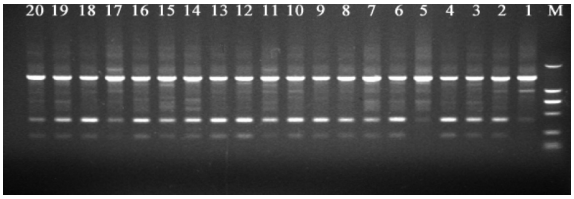

FIGURE 1. The PCR amplification pattern of BF 1 population generated with primer S1263 1-20: Individual 1-20; M: DL2000 standard molecular weight marker (means 100, 250, 500, 750, 1000, 2000bp from the bottom to top)

propagation (Ruan et al. 2005). In our previous studies, open area of forest edge habitat was helpful to sexual reproduction of *I. japonica* populations due to adequate light, poor physical properties of soil (low water content and high unit weight) and strong intra- and inter-specific competition (Wang et al. 2008; 2010). Such as *Syneilesis palmate*, stressful conditions (intra-species competition and disturbance) might increase sexual reproduction (Nishitani et al. 1999). On the contrary, bamboo forest habitat was in favor of clonal propagation (such as root and rhizome system availability) for sufficient water

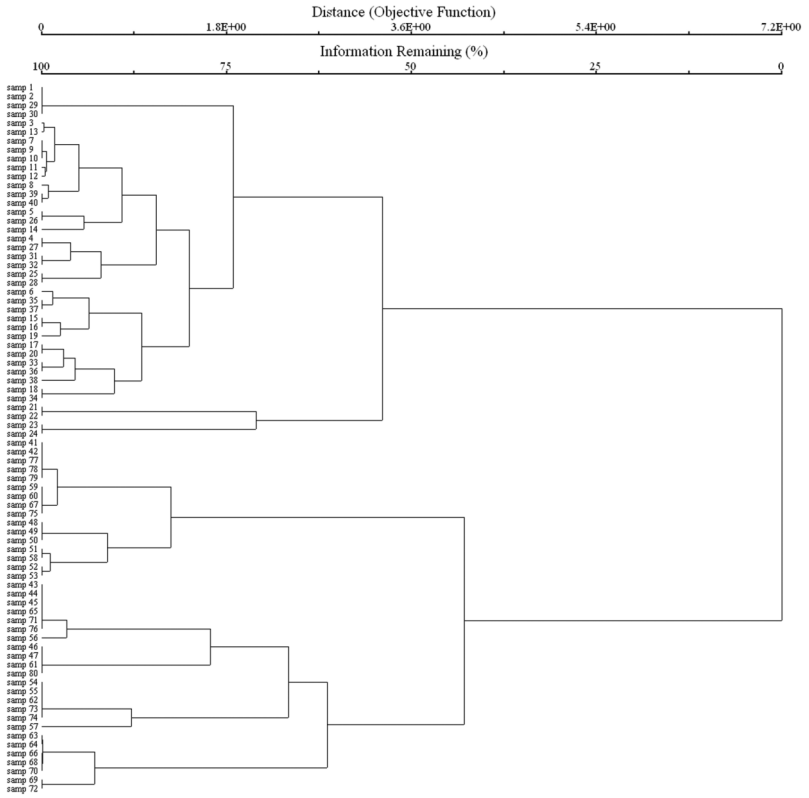

FIGURE 2. Neighbor-joining tree of 80 *Iris japonica* individuals estimated by 12 polymorphic RAPD markers and Jaccard's genetic distance. Samp 1-80 indicate OAFE 1 (sample 1-20), OAFE 2 (sample 21-40), BF 1 (sample 41-60) and BF 2 (sample 61-80), respectively

content in soil and growth space (Wang et al. 2008; Wang & Fang 2010). Such as *Hydrangea paniculata* and *Opuntia rastrera*, clonal propagation increased in forest understory for the less nutrient-demanding (e.g., light, nutrients) than seed production (Mandujano et al. 1998; Kanno & Seiwa 2004). Therefore, variation in two reproduction modes might lead widespread genotypes and low genetic diversity in BF habitat, and various genotypes and high genetic diversity in OAFE habitat for long-time adaptation in *I. japonica* populations.

Genetic variation within and among populations based on *Nei*' index indicated that about 28-29% (G_{ST} = 0.281) of variation existed among populations. It had high gene flow among populations (1.279) (Table 3). According to the AMOVA, 49.63% of the total variation existed within populations ($P < 0.01$), 41.77% among habitats (meant long-distance populations, between BF populations and OAFE populations) ($P < 0.01$), and 8.60% among populations (meant among sub-populations, between BF 1 and BF 2 populations, or OAFE 1 and OAFE 2 populations) ($P < 0.01$) (Table 4). This result indicated that about half of the total variation existed within the four populations, and that genetic differentiation among habitats (BF populations and OAFE populations) was also significant. *I. hexagona* populations showed 62% of the genetic variation existed within populations, and 38% of variation existed among populations (Ruan et al. 2005). The extent of genetic variation among populations in various plant species (by RAPD markers) was 0-27% in cross-pollinating species and 37-43% in self-pollinating species (hermaphrodites, as *I. japonica*) (Yamagishi et al. 2010), which was consistent with our results.

I. japonica can reproduce sexually and asexually. The clonal growth usually developed by fine rhizome. Fine rhizomes could be largely produced at the same time, which formed a large clonal population in one or two growing season in BF habitat. On the other hand, *I. japonica* invested more resources in sexual reproduction in OAFE habitat. Allocation of total biomass to flowers of *I. japonica* was more than 25% in OAFE habitat (Wang et al. 2010). Although the populations had low fruit set, germination ratio and settling of seedlings, they could maintain high genetic variation due to the regeneration success by chance. It was hermaphrodites and self-pollinating. Without animal and human disturbance, the fruits and seeds could not be long-distance spreading due to the high weight. Therefore, populations had high genetic variation between BF and OAFE habitats/populations for the differences in reproductive significance, and had high variation within populations for high sexual reproduction in OAFE habitats.

Conclusion

OAFE habitats were helpful in maintaining clonal diversity and genetic diversity of *I. japonica* populations than BF habitats. Habitat types have a fatal influence on clonal diversity, genetic diversity and genetic differentiation of *I. japonica* populations in Jinyun Mountain, southwest China. Habitat differences might have distinct effects on the relative significance of clonal propagation and sexual reproduction. Due to the long-term effects, clonal diversity and genetic diversity of populations will vary in OAFE and BF habitats. We suggest analysis on characteristics of two reproductive modes of *I. japonica* populations in field and simulated experiments are significant to further understand the effects of habitats and populations genetics.

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TABLE 3. Analysis of genetic structure in subdivided populations

Population (*n*)	*Nei*' gene diversity				*Shannon-Wiener* diversity	
	h_s	h_t	G_{ST}	N_m	I_s	I_t
Total (80)	0.199	0.276	0.281	1.279	0.292	0.412

h_s: gene diversity within populations; h_t: total gene diversity; G_{ST}: Coefficient of gene differentiation; I_s: genetic diversity within populations; I_t: total genetic diversity; N_m: Gene flow among populations.

TABLE 4. Analysis of molecular variance (AMOVA) among habitat types and populations, and within populations

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*P*-value
Among habitat types	1	321.8	7.103	41.77	<0.01
Among populations	2	75.35	1.462	8.60	<0.01
Within populations	76	641.3	8.438	49.63	<0.01

'Among habitat types' meant long-distance populations, between BF populations and OAFE populations; 'Among populations' meant among sub-populations, between BF1 and BF2 populations, or OAFE1 and OAFE 2 populations.

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