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Genetic Variation and Clonal Diversity of *Psammochloa villosa* (Poaceae) Detected by ISSR Markers

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Genetic variation and clonal diversity of seven *Psammochloa villosa* (Poaceae) populations from northwest China were investigated using inter simple sequence repeat (ISSR) markers. Of the 84 primers screened, 12 produced highly reproducible ISSR bands. Using these primers, 173 discernible DNA fragments were generated with 122 (70·5 %) being polymorphic, indicating considerable genetic variation at the species level. In contrast, there were relatively low levels of polymorphism at the population level with the percentage of polymorphic bands (PPB) ranging from 6·1 to 26·8. Analysis of molecular variance (AMOVA) showed that a large proportion of genetic variation (87·46 %) resided among populations, while only 12·54 % resided among individuals within populations. Clonal diversity was also high with 98 genets being detected from among 157 individuals using 12 ISSR primers. The evenness of distribution of genotypes in *P. villosa* populations varied greatly, with all of the genotypes being local ones. No significant differences in genetic or clonal diversity were found between populations in mobile or fixed dunes. The main factor responsible for the high level of differentiation among populations and the low level of diversity within populations is probably the clonal nature of this species, although selfing may also affect the population genetic structure to some extent. The efficiency of ISSRs in identifying genetic individuals was much higher than that of allozymes. An approximately asymptotic correlation was found between the number of genets detected and the number of polymorphic loci used, suggesting that use of a high number of polymorphic bands is critical in genet identification.

Key words: *Psammochloa villosa*, ISSRs, genetic variation, clonal diversity.

INTRODUCTION

Most perennial plants possess the capacity for clonal growth and, as a group, plants clone themselves in an intriguing variety of ways (Eckert, 1999). Over the last decades, the significance of studies on clonal plants has been widely appreciated and great progress has been made in researching the morphology, physiology, ecology and evolution of clonal plants (Asker and Jerling, 1992; Eckert, 1999). To date, much of the interest in clonal plants has focused on how their pattern of development influences the way they grow, how they capture resources, and how they respond to environmental variation in space and time (Eckert, 1999). Relatively few studies have been conducted into the population genetics and evolution of clonal plants due mainly to the difficulty in identifying genets (Widen *et al*., 1994). Although allozyme analysis has long been used to identify clones and to study population genetics of clonal plants, it usually underestimates genetic polymorphism and has a limited ability to distinguish genetic individuals (Esselman *et al*., 1999; Mueller and Wolfenbarger, 1999; Wang *et al*., 1999).

In recent years, a number of PCR-based DNA markers, such as RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and ISSR (inter simple sequence repeats), have been widely used to investigate clonal diversity and population genetic structure because they overcome the limitations of allozyme markers (Tani *et al*., 1998; Esselman *et al*., 1999; Rossetto *et al*., 1999). Of these, the most popular marker is RAPD, which has been successfully used in a wide variety of fields (Wolfe and Liston, 1998). As a less widely used PCR-based marker, ISSR has a few advantages over other markers. ISSR primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no prior knowledge of target sequences is required for ISSRs (Godwin *et al*., 1997). Also, the sequences that ISSRs target are abundant throughout the eukaryotic genome and evolve rapidly; consequently ISSRs may reveal a much higher number of polymorphic fragments per primer than RAPDs (Fang and Roose, 1997; Esselman *et al*., 1999). In addition, studies have indicated that ISSRs produce more reliable and reproducible bands compared with RAPDs because of the higher annealing temperature and longer sequence of ISSR primers (Tsumura *et al*., 1996; Nagaoka and Ogihara, 1997; Qian *et al*., 2001). Therefore, ISSRs have proved to be useful in population genetic studies, especially in detecting clonal diversity and fingerprinting closely related individuals (Zietkiewicz *et al*., 1994; Wolfe and Liston, 1998; Esselman *et al*., 1999).

Psammochloa villosa (Trin.) Bor (Poaceae) is a rhizomatous grass and one of the pioneers of sandland vegetation succession usually found in dune habitats (Dong and Alaten, 1999). It is distributed mainly in northwest China

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where the landscape comprises large sand-covered areas, and desertification is becoming problematic (Lu and Kuo, 1987). As a typical sand-fixation plant, *P. villosa* exhibits rapid clonal growth by rhizome production, a character that has played an important role in its life cycle (Lu and Kuo, 1987; Dong and Alaten, 1999). The clonal integration, ramet population features and other ecological characteristics of *P. villosa* have been extensively studied in recent years (Dong *et al*., 1999; Dong and Alaten, 1999). Nevertheless, almost nothing was known about its population genetics until Wang *et al*. (1999) conducted a preliminary study into genetic diversity using allozyme analysis and found relatively low genetic variation and clonal diversity of this species in Ordos sandland.

In the present study, we investigated the genetic polymorphism and clonal diversity of seven *P. villosa* populations utilizing ISSRs. The specific goals of the study were (1) to demonstrate the resolving power of ISSRs for the study of clonal plants; (2) to investigate the genetic diversity within and among populations of this important sand-adapted species, and (3) to measure the clonal diversity and the correlation between clonal growth and the sand-covered environment. Such information may be relevant to restoration ecology of sand dune habitats in this region, and contribute to a better understanding of the evolutionary significance of clonal growth in general.

MATERIALS AND METHODS

Population sampling

In September 1999, seven natural populations of *P. villosa* were sampled from Ordos Sandland Ecological Stations (OSES), the Chinese Academy of Sciences. Two populations (P3, P6) came from the Shihuimiao Station, and the other five (P1, P2, P4, P5, P7) from the Shilongmiao Station. Of these, three populations were sampled from fixed dunes, three from mobile dunes, and only one population from a semi-fixed dune (Fig. 1). Twenty-one to 25 plants were collected at random from each population at intervals of at least 4 m. The sizes and habitats of the sampled populations and their approximate locations are shown in Fig. 1.

Total DNA extraction

Leaves were harvested and stored with silica gel in zip-lock plastic bags until DNA isolation. Total DNA was extracted using a modification of the protocol of Doyle and Doyle (1987). Dried leaf material was ground to a fine powder in a mortar and then transferred to a 2 ml Eppendorf tube filled with 950 µl of preheated 2 × CTAB extraction buffer containing 0·3 % mercaptoethanol. After being incubated at 65 °C for 60 min, the homogenate was mixed with 950 µl chloroform: isoamylalcohol (24 : 1, v/ v). Samples were shaken gently for 10 min and centrifuged at 10 000 r min^{-1} for 10 min at room temperature. The supernatant was reserved and mixed with 2/3 vol ice-cold isopropanol. DNA was then recovered as a pellet by centrifugation at 11 000 r min^{-1} for 6 min at 4 °C, washed with 300 µl of ethanol, dried, and dissolved in 200 µl of 1 × TE buffer. DNA quality and quantity were determined in 0·8 % agarose gels.

ISSR PCR amplification

Eighty-four primers from Shengong Inc. (Shanghai, China) were tested for PCR. DNA amplification was performed in a Rapidcycler 1818 (Idaho Tech., Idaho Falls, Idaho, USA), and commenced with 2 min at 94 °C, followed by 38 cycles of 2 s at 94 °C, 2 s at 50 °C, 1 min at 72 °C, and ended with 7 min at 72 °C. Reactions were carried out in a volume of 10 µl containing 50 mM Tris-HCl (pH 8·3), 500 µg ml^{-1} BSA, 10 % Ficoll, 1 mM tartrazine, 2 mM $MgCl_2$, 200 µM dNTP, 1 µM primer, 5 ng of DNA template, 0·5 U Taq polymerase and 4 % DMSO. Amplification products were resolved electrophoretically on 2·0 % agarose gels run at 100 V in 1 × TBE, visualized by staining with ethidium bromide, and photographed under ultraviolet light. Molecular weights were estimated using a 100 bp DNA ladder.

Data analysis

ISSR bands were scored as present (1) or absent (0) for each DNA sample, and the Jaccard coefficient was employed to calculate pairwise band similarities for all 157 individuals using the NTSYS program (Rohlf, 1994). Genetic diversity was measured by the percentage of polymorphic bands (PPB), which was calculated by dividing the number of polymorphic bands at population and species levels by the total number of bands surveyed. The Shannon

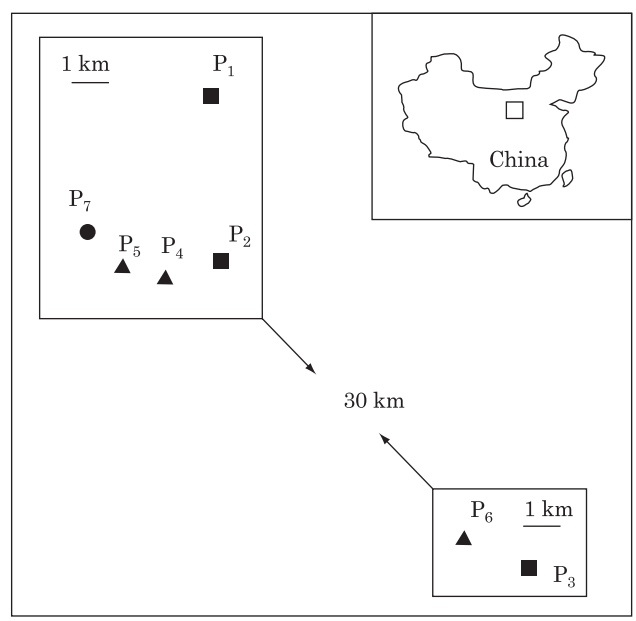

FIG. 1. Localities and habitats of seven populations of *Psammochloa villosa*. Squares represent fixed dunes, triangles represent mobile dunes, and the circle represents the semi-fixed dune. P1 (21), P2 (25), P4 (21), P5 (21), P7 (23), Popultions sampled from Shilongmiao; P3 (25), P6 (21), populations sampled from Shihuimiao. Sample sizes in parentheses.

index of diversity (I) was also calculated using the POPGENE program developed by F. Yeh, R-C Yang and T. Boyle (downloadable from http : //www.ualberta.ca/ ~ fyeh/).

A distance matrix was constructed by the RAPDistance program (Armstrong *et al*., 1994), and the nonparametric Analysis of Molecular Variance (AMOVA) program version 1·5 was used to describe the genetic structure and variability among populations, as described by Excoffier and coworkers (1992). A dendrogram was also constructed by an unweighted paired group method of cluster analysis using arithmetic averages (UPGMA).

Clonal diversity was evaluated by the following indexes (Fager, 1972; Ellstrand and Roose, 1987): (1) number of genotypes, G; (2) the mean clone size, $N_c = N/G$, where N represents the sample size; (3) proportion distinguishable $PD = G/N$; (4) a modified version of the Simpson diversity index, $D = 1 - [N_i(N_i - 1)/N(N - 1)]$, where N_i is the number of samples of the ith genotype; and (5) Fager index, $E = (D - D_{min})/(D_{max} - D_{min})$, where $D_{min} = (G - 1)(2N - G)/N(N - 1)$ and $D_{max} = (G - 1)N/G(N - 1)$.

RESULTS

ISSR polymorphism

Eighty-four ISSR primers were screened on two randomly selected individuals. By comparing the effects of magnesium concentrations and annealing temperature during amplification, 12 primers that produced clear and reproducible fragments were selected for further analysis (Table 1). These 12 selected primers generated 173 bands ranging in size from 220 to 1400 bp, corresponding to an average of 14·4 bands per primer. Of these bands, 70·5 % (122 in total) were polymorphic among 157 individuals, i.e. the percentage of polymorphic bands (PPB) for this species was 70·5 %. Every primer produced polymorphic bands when all of the seven populations were considered. Genetic diversity varied greatly among populations with PPB values ranging from 6·1 (P4) to 26·8 (P3). The Shannon index (I) showed the same trends (Table 2). We observed no significant differences in polymorphism between populations in mobile dunes and those in fixed dunes ($0{\cdot}4 < P < 0{\cdot}5$). However, the two populations from Shihuimiao station had higher PPB values than the five populations from Shilongmiao station (Table 2).

Genetic structure of populations

To assess the overall distribution of genetic diversity, the AMOVA program was used to analyse the distance matrix given by the RAPDistance program. AMOVA showed highly significant ($P < 0{\cdot}001$) genetic differentiation among populations. A large proportion of genetic variation (87·46 %) resided among populations, whereas only 12·54 % resided among individuals within populations. When populations were grouped based on their origin, genetic variation within stations (74·12 %) was much higher than that among stations (25·88 %), suggesting that the genetic differentiation occurred mainly among populations rather than between the two stations. A cluster analysis (UPGMA) indicated that the two populations from the Shihuimiao station clustered together before forming a cluster with any other population, while the populations in the Shilongmiao station did not cluster together as a clade (Fig. 2). Both the AMOVA analysis and the cluster dendrogram demonstrated that there was no distinct genetic differentiation between the two stations (Shilongmiao and Shihuimiao).

TABLE 1. *Primers used for ISSR amplification*

Primer	Sequence of primer
SSR1	$(ACTG)_4$
SSR2	$(GACA)_4$
SSR4	$BDB(CA)_6$
SSR5	$VHV(GT)_7$
SSR7	$GC(AC)_5$
SSR15	$CCC(GT)_6$
SSR16	$GSG(GT)_6$
SSR17	$CSC(GA)_6$
SSR21	$GCG(AC)_6A$
SSR22	$SSWN(GACA)_3$
IS-5	$(AC)_8TG$
IS-36	$(AG)_8TC$

B = C/G/T D = A/G/T H = A/C/T V = A/C/G S = G/C W = A/T.

TABLE 2. *Percentage of polymorphic bands (PPB) and Shannon index (*I*) for seven* P. villosa *populations*

Population	No. of bands	No. of polymorphic bands	PPB	*I*
P1	124	11	8·9	0·0218 (0·0965)
P2	129	12	9·3	0·0219 (0·0965)
P3	127	34	26·8	0·1160 (0·2288)
P4	114	7	6·1	0·0163 (0·0950)
P5	127	21	16·5	0·0479 (0·1505)
P6	120	23	19·2	0·0574 (0·1692)
P7	137	26	19·0	0·0485 (0·1496)
Species	173	122	70·5	—

Clonal diversity of P. villosa

The clonal diversity and evenness of distribution of genotypes in seven populations of *P. villosa* are summarized in Table 3. Twelve selected ISSR primers identified 98 genotypes in 157 individuals, and all the populations examined were comprised of more than one genet. The largest number of genets was found in P3 (20), and the lowest in P4 (9). Across seven populations, mean clone sizes ranged from 1·24 (P6) to 2·33 (P4). Simpson diversity index (D) was originally developed as a measure of species diversity, and has been employed to measure clonal diversity within populations (Parker, 1979; Ellstrand and Roose, 1987). The value ranges from zero in a population composed of a single clone to unity in a population where every individual sampled is a distinct genotype (Eckert and Barrett, 1993). In addition, Fager's E value describes the

TABLE 3. *Clonal diversity and distribution uniformity in seven populations of* Psammochloa villosa

	N	*G*	$N_c(=N/G)$	$PD(=1/N_c)$	*D*	*E*
P1	21	12	1·75	0·571	0·943	0·889
P2	25	14	1·79	0·560	0·940	0·854
P3	25	20	1·25	0·800	0·963	0·328
P4	21	9	2·33	0·429	0·838	0·687
P5	21	16	1·31	0·762	0·967	0·688
P6	21	17	1·24	0·810	0·976	0·659
P7	23	10	2·30	0·435	0·866	0·751
Mean		14·1	1·71	0·6243	0·944	0·688
			(0·4713)	(0·1665)	(0·5379)	(0·1835)
Species	157	98	1·60	0·624	0·990	0·956

N, Sample size; G, number of genotypes; N/G, average size of genotype; D, Simpson index; E, Fager index. Standard errors in parentheses.

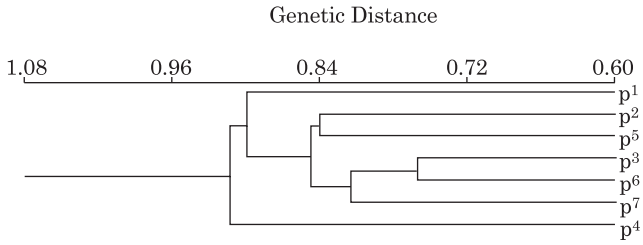

FIG. 2. Genetic distances among seven *P. villosa* populations (Populations P1–P7 correspond to those in Fig. 1).

evenness of distribution of genotypes within a population, and varies between zero (when all individuals in a population possess the same genotype) and one (when a population has completely uniform genotype frequencies). In *P. villosa*, the mean values of *D* and *E* were 0·944 and 0·956, respectively (Table 3).

Clonal diversity of the two populations from Shihuimiao station (P3 and P6) was higher than that of the five populations from Shilongmiao station, which parallels the higher genetic diversity measured in Shilongmiao station. No significant differentiation of genetic and clonal diversity was found between populations in mobile and fixed dunes ($P > 0{\cdot}5$). A dendrogram based on genetic distances between individuals showed that all individuals from the same population formed a group (data not shown), indicating that none of the 98 genotypes were found at more than one location. Therefore, they were all local genotypes and no widespread genotype occurred.

DISCUSSION

ISSR polymorphism and genet identification

Clonal plants present special problems when analysing populations because a single genetic individual (genet) may comprise numerous morphological units (ramets) that appear distinct (Parks and Werth, 1993). Therefore, it is difficult to detect population dynamics precisely because the number of genets in a population is unknown. Estimates of individuals based on population size are usually subject to error because regardless of ramet numbers, populations of clonal plants consisting of few genets tend to be subject to similar genetic processes that affect any small population, such as genetic drift and inbreeding (Sipes and Wolf, 1997). Thus for clonal species, genet identification is usually a necessary precursor to population genetic studies.

To date, data accumulated from studies of genotype diversity and spatial distribution of genets in clonal plants have been obtained by allozyme methods. However, given the fact that measurement of genotypic diversity depends on the number and variability of genetic markers (Eckert and Barrett, 1993), the limited number of enzymes available and small number of polymorphic loci detected make allozyme techniques inadequate for accurate identification of genotypes and population structures (Widen *et al*., 1994; Wang *et al*., 1999). Compared with allozymes, ISSR markers are much more effective in genotype identification because the amplification of ISSRs is based on SSRs, which are extremely variable in eukaryotes. In a previous allozyme study of four *P. villosa* populations in Ordos, Wang *et al.* (1999) detected 12 genotypes in 158 individuals. In our case, a total of 157 individuals was identified from seven populations sampled from similar areas in Ordos. Based on using ISSR markers, we found 98 genotypes, suggesting that ISSRs are much more informative and powerful tools by which to identify genets and to assess genetic variability in *P. villosa*. Tani *et al*. (1998) and Esselman *et al*. (1999) also demonstrated the greater discriminative power of ISSR markers over allozymes for genet identification in their studies of *Pinus pumila* and *Calamagrostis porteri*, respectively. Furthermore, much evidence has shown that ISSRs can produce even more information than RAPD markers and, therefore, show great potential in studies of clonal diversity and genet identification (Nagaoka and Ogihara, 1997; Esselman *et al*., 1999; Qian *et al*., 2001).

It should be mentioned that the efficiency and accuracy of genet identification might be affected by the number of polymorphic bands available in a given species. In the present study, 98 genotypes were found in seven populations based on 122 polymorphic bands. However, this number of genotypes should be considered the minimum estimate because additional genotypes might be recovered if more polymorphic bands were used.

Population genetic structure and clonal diversity

A growing body of data indicates that populations of clonal plants can maintain considerable amounts of genetic

diversity (Eckert and Barrett, 1993; Widen *et al*., 1994). Our ISSR survey of seven natural populations of *P. villosa* revealed a high level of genetic variation at the species level with 70·5 % of bands being polymorphic—a much higher level of variation than detected by allozymes (Wang *et al*., 1999). However, relatively low genetic diversity existed within populations where PPB values ranged from 6·1 to 26·8, implying that a larger proportion of variation resided among populations. AMOVA analysis showed that most of the genetic diversity (87·46 %) resulted from differentiation among populations, which is in general agreement with a previous allozyme analysis in which 62·2 % of the total variation resided among four populations (Wang *et al*., 1999).

Similarly, there is considerable clonal diversity in *P. villosa* populations. The wide range of *E* values suggested that the evenness of distribution of genotypes in *P. villosa* populations varied greatly, with all of the genotypes being local ones. This result is similar to that obtained by allozyme analysis (Wang *et al*., 1999), and is consistent with the population genetic structure in this species.

The population genetic structure of a species is affected by a number of evolutionary factors including mating system, gene flow and seed dispersal, mode of reproduction as well as natural selection (Hamrick and Godt, 1990). As stated above, no genetic differentiation was found between either station or between the two habitats (mobile *vs*. fixed dunes), suggesting that selection-favouring adaptations to local environments have not played a critical role in determining the current genetic profile of this species, at least in terms of allozyme and ISSR markers. *Psammochloa villosa* possesses a certain capacity for sexual reproduction by high-rate selfings (Wang *et al*., 1999). It is not unexpected, therefore, that a much larger proportion of genetic diversity resides among populations of *P. villosa*; selfing species often maintain as much diversity as outcrossing species (Wolff and Morgan-Richards, 1999). More importantly, recent studies have shown that *P. villosa* populations produce very few seedlings by sexual reproduction in Ordos sandland (Dong and Alaten, 1999). They maintain themselves primarily by the growth of rhizomes, particularly in Ordos sandland where water shortage, strong evapotranspiration, infertility and habitat patchiness are common (Dong *et al*., 1999). In this context, the low level of genetic variation within populations and high genetic differentiation among populations in *P. villosa* may be attributed mainly to the clonal nature of this species because expansion to new sites by vegetative means is highly unlikely (Esselman *et al*., 1999, Wang *et al*., 1999).

Estimates of the extent and pattern of clonality will depend on the number and spatial distribution of ramets sampled and the number and variability of the genetic markers assayed (Eckert and Barrett, 1993). Given high variation in clonal diversity of different populations, and differences in sizes of the clones in *P. villosa* (Tables 2 and 3), questions arise about whether or not dominant clones occur, the spatial pattern of different clones, and whether the extent and pattern of clonality of *P. villosa* correlates with habitat, etc. These studies are currently underway. Such information is crucial to a thorough understanding of the evolutionary and ecological significance of clonality in *P. villosa*, and of plants of general.

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