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ORIGINAL RESEARCH

Genetic Diversity and Geographical Differentiation of *Desmodium triflorum* (L.) DC. in South China Revealed by AFLP Markers

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Abstract High levels of genetic variation enable species to adapt to changing environments and provide plant breeders with the raw materials necessary for artificial selection. In the present study, six AFLP primer pairs were used to assess the genetic diversity of *Desmodium triflorum* (L.) DC. from 12 populations in South China. A high percentage of polymorphic loci (P=76.16%) and high total gene diversity (H_T=0.310) were found, indicating that the genetic diversity of *D. triflorum* is high at the species level. Genetic diversity was also relatively high at the population level (P=55.85%, H_e=0.230). The coefficient of gene differentiation among populations (G_{ST}) was 0.255, indicating that while most genetic diversity resided within populations, there was also considerable differentiation among populations. AMOVA also indicated 24.29% of the total variation to be partitioned among populations (Φ_{ST}=0.243). UPGMA clustering analysis based on genetic distances showed that the 12 populations could be separated into three subgroups: an eastern, a western, and a central-southern subgroup. However, a Mantel test revealed no significant correlation (r=0.286, p=0.983) between the geographical distances and genetic distances separating these populations; mountain barriers to gene flow and human disturbance may have confounded these correlations. The present study has provided some fundamental genetic data that will be of use in the exploitation of *D. triflorum*.

Keywords AFLP · *Desmodium triflorum* (L.) DC. · Genetic diversity · Population structure

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Introduction

Despite abundant resources of native, wild, warm-season turf grass (Liu and He 1996), few species have been cultivated in China. In recent years, lawn workers in China have recognized problems associated with the large-scale introduction of exotic turf grasses. Notably, it is expensive to maintain and manage turf grasses that have been introduced from geographic regions with environmental conditions that differ from those at sites where they have been newly established. Thus, there is an urgent need to exploit native wild turf grass species (Bai et al. 2002; Liu et al. 2003; Xuan et al. 2005; Xi et al. 2004).

Desmodium triflorum (L.) DC. (Fabaceae) is a perennial herb with a pantropical distribution ranging from Asia and Oceania to America (Yang 1995). There are about 27 *Desmodium* species in China, the most common, *D. triflorum*, being distributed in Zhejiang, Fujian, Jiangxi, Guangdong, Hainan, Guangxi, Yunnan, and Taiwan. The natural habitats of this species are usually natural lawns, roadsides, and riversides (Yang 1995). Although in recent years *D. triflorum* has appeared widely in cultivated lawns in South China where it was initially considered to be one of the main weeds of warm-season turf grass (Lin et al. 2004; Huang et al. 2007), it also has several characteristics typical of an ideal turf grass. Firstly, individual plants of *D. triflorum* are about 1.5 to 2.5 cm tall (Ma et al. 2003) with

stolons that can grow up to 1 m long (Yang 1995). Secondly, *D. triflorum* can reproduce both sexually by seeds and asexually by stolon extension, the latter mode facilitating its rapid propagation and expansion in lawns. Thirdly, *D. triflorum* is extremely drought-tolerant, being able to tolerate drought for 2 months in the greenhouse (Ma and Yang 2002). Furthermore, in Baise, Guangxi province, *D. triflorum* has also been observed to have good tolerance of cold (Zeng et al. 2006). These characteristics make it an ideal new candidate for consideration as a warm-season turf grass.

Genetic diversity, or the heritable variation within and between populations, forms the genetic variation on which the natural or artificial selection of a species is based. Thus, since *D. triflorum* has the potential to be cultivated as a novel warm-season turf grass, basic genetic information is required to underpin any scientific breeding programs.

Previous research on *D. triflorum* focused on its morphology and physiology (Ma and Yang 2002; Ma et al. 2003; Zeng et al. 2006). The genetic relationships of two *Desmodium* species and two closely related species (*Dendrolobium triangulare*, *Desmodium gangeticum*, *Desmodium heterocarpon*, and *Tadehagi triquetrum*) have been resolved (Heider et al. 2009), but the genetic structures of the extant populations of *D. triflorum* have never been studied despite a general understanding of a population's genetic structure being crucial for its scientific exploitation (Yang et al. 2007). AFLP analysis has great potential as a powerful technique for fingerprinting DNA samples of any origin or complexity (Vos et al. 1995). It is an efficient and reliable marker system for studying genetic diversity and genetic relationships in plants (e.g., Yang et al. 2007; Bayazit et al. 2007). In the present study, AFLP was used to evaluate genetic diversity among 201 individuals of *D. triflorum* and to determine the genetic structure of geographic populations, thereby providing basic genetic information to facilitate its future exploitation.

Materials and Methods

Plant Materials

A total of 201 individuals of *D. triflorum* from 12 populations were collected in Guangdong, Jiangxi, Guangxi, and Hainan provinces in South China (Table 1 and Fig. 1). Plants were sampled with intervals of at least 10 m between individuals to avoid collecting more than one sample of the same individual from any extended growth of its stolons. Leaf materials were stored with silica gel in Ziplock plastic bags until required for DNA isolation.

DNA Extraction and AFLP Assay

Genomic DNA was extracted from leaf tissues using a modified cetyltrimethylammonium bromide (CTAB)-based method (Reichardt and Rogers 1993). AFLP assays were carried out according to Vos et al. (1995) with minor modifications. Briefly, total genomic DNA was digested with *Eco*RI and *Mse*I for 3 h at 37°C. Ligations to adapters were carried out at 20°C for 3 h to generate template DNA for amplification. After ligation, a pre-amplification (30 s at 94°C, 30 s at 56°C, and 1 min at 72°C) was performed using template DNA and a pair of primers based on the sequences of the *Eco*RI and *Mse*I adapters, including an additional selective nucleotide at the 3′-ends of both the *Mse*I primer (*Mse*I+C) and the *Eco*RI primer (*Eco*RI+A). The pre-amplified PCR products were then diluted 50-fold and used as the template for selective amplifications, with a total of six primer combinations: E-AAG/M-CAA, E-ACA/

Table 1 Sampling information of 12 populations of *D. triflorum*

Population (abbreviation)	Sample size	Altitude (m)	Longitude	Latitude	Annual temperature (°C)	Annual precipitation (mm)
Ganzhou, Jiangxi (JX)	15	168	114°57.50′ E	25°49.24′ N	18.9	1,605
Shaoguan,Guangdong (SG)	16	56	113°32.42′ E	24°49.48′ N	19.6	1,522
Meizhou, Guangdong (MZ)	15	393	116°06.83′ E	24°34.48′ N	21.2	1,473
Jieyang, Guangdong (JY)	15	54	116°21.06′ E	23°34.07′ N	21.4	1,723
Guanghzou Guangdong (GZ)	18	23	113°17.60′ E	23°05.94′ N	21.7	1,755
Huizhou, Guangdong (HZ)	16	25	114°22.84′ E	23°03.15′ N	21.2	1,800
Nanning, Guangxi (NN)	20	106	108°22.22′ E	22°48.36′ N	21.7	1,300
Luoding, Guangdong (LD)	19	125	111°34.47′ E	22°47.80′ N	21.5	1,202
Zhuhai, Guangdong (ZH)	19	57	113°35.03′ E	22°21.60′ N	21.8	2,231
Maoming, Guangdong (MM)	15	26	110°53.56′ E	21°41.94′ N	23.2	1,806
Zhanjiang, Guangdong (ZJ)	15	30	110°21.85′ E	21°11.47′ N	23.2	1,567
Qionghai,Hainan (QH)	18	65	110°27.79′ E	19°13.84′ N	24.0	2,072

Fig. 1 Sampling locations of the 12 populations of *D. triflorum*

M-CAA, E-AAC/ M-CAA, E-ACT/ M-CTC, E-AAC/ M-CAT, and E-AGC/M-CTC. The first phase of the selective amplification was performed with a 5-min pre-denaturing at 96°C, followed by 12 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C, with the annealing temperature decreasing stepwise from 65.0°C to 56.6°C. The latter phase was performed with 25 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. The *Eco*RI primers were labeled with fluorescent 6-carboxy fluorescein (6-FAM) on the 5′ nucleotide. The amplified products were separated using an ABI Prism 377 DNA sequencer and 6% denaturing sequencing polyacrylamide gel in 1×TBE running buffer.

Data Analysis

Data were collected and analyzed using GeneScan software (version 2.1, PE Company), and peak values were obtained using Genotyper (version 1.2, PE Company). The bands within the readable range from 50 to 500 bp were scored as binary characters and then transformed into 1 for presence and 0 for absence of a band of a particular size by Peak Matcher 6.0 (DeHaan et al. 2002). Intra- and inter-population genetic statistics were generated using the program POPGENE 1.32 (Yeh et al. 1997) and included: the mean expected heterozygosity (H_e), the effective number of alleles (N_e), Shannon's diversity index (I), the percentage of polymorphic loci (P), total genetic diversity (H_T) and gene diversity within populations (H_S), the coefficient of gene differentiation ($G_{ST} = (H_T - H_S)/H_T$), and the levels of gene flow (N_m), which were calculated using the formula: $N_m = 0.25(1 - G_{ST})/G_{ST}$. Analysis of molecular variance (AMOVA) was conducted to calculate variance components and their significance levels for variation among populations and within populations using AMOVA1.55 (Excoffier et al. 1992). Unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis was used to generate a dendrogram of the relationships among the 12 sampled populations based on Nei (1978) and unbiased minimum distance using tools for population genetic analysis (TFPGA; Miller 1997). Bootstrapping analysis with 1,000 replicates was performed to assess the credibility of the dendrogram. Nei's (1972) unbiased genetic identity (I) was also calculated among populations. The latitudes and longitudes of the sites where the 12 populations were sampled were used to calculate geographical distances between them with the Mapinfo 8.0 Program. Finally, a Mantel test (Mantel 1967; Sokal 1979) was carried out to calculate the correlation between geographic and genetic distances using NTSYS pc v2.02j (Rohlf 1998).

Results

Genetic Diversity of *D. triflorum*

During the fieldwork, we also looked for, but did not find, *D. triflorum* in other regions such as Hengyang (Hunan province) ang Longyan (Fujian province). Although we could not cover the entire range of *D. triflorum* in China,

Table 2 Genetic diversity of 12 *D. triflorum* populations in South China

Population	H_e	N_e	I	P (%)
JX	0.197	1.351	0.285	46.85
SG	0.265	1.479	0.383	63.56
MZ	0.201	1.358	0.29	47.40
JY	0.223	1.407	0.319	50.68
GZ	0.260	1.469	0.376	62.74
HZ	0.200	1.356	0.290	49.04
NN	0.174	1.302	0.257	46.30
LD	0.262	1.467	0.381	63.84
ZH	0.215	1.381	0.315	55.07
MM	0.241	1.429	0.351	58.90
ZJ	0.253	1.454	0.367	61.10
QH	0.269	1.482	0.389	64.66
Average	0.230	1.411	0.334	55.85
Total	0.310	1.561	0.449	76.16

H_e expected heterozygosity, N_e effective number of alleles, I Shannon's diversity index, P percentage of polymorphic bands

we did include its main locations, which should be sufficient to generate preliminary data on the genetic diversity and population differentiation of the species in South China. In this study, a total of 365 unambiguous AFLP markers were detected from 12 populations of *D. triflorum* in South China using six primer combinations, of which 278 (76.16%) were polymorphic. At the species level, the mean expected heterozygosity (H_e), the effective number of alleles (**N_e**), and Shannon's index (I) were 0.310, 1.561, and 0.449, respectively (Table 2). At the population level, the mean values of H_e, N_e, I, and P were 0.230, 1.411, 0.334, and 55.85%, respectively. The values of P and H_e varied from 46.30% and 0.174 in the Nanning population to 64.66% and 0.269 in the Qionghai population (Table 2).

Population Genetic Structure of *D. triflorum*

Based on Nei's genetic diversity analysis, the total gene diversity (H_T) of *D. triflorum* was 0.310, of which 0.231 was partitioned within populations (H_S), much higher than that between populations (0.079), indicating that the genetic diversity of *D. triflorum* resided mainly within populations. The coefficient of genetic differentiation among populations (G_{ST}) was 0.255 and was consistent with the results of the AMOVA (Φ_{ST}=0.243, p<0.001), which showed that most variance (75.7%) resided within populations, with the remainder (24.3%) partitioned among populations. Both analyses suggested that there is considerable genetic differentiation between these populations of *D. triflorum*. At the species level, the gene flow (N_m) was 0.730.

Genetic Distances and Relationships Among Populations of *D. triflorum*

The genetic identity (I) coefficients between populations were obtained following the method of Nei (1972) and are presented in Table 3. These values of genetic identity were fairly high, ranging from 0.845 to 0.958, with a mean of 0.888. The highest genetic identity value was between populations MM and ZJ and the lowest between populations ZH and LD. The Mantel test indicated that there was no significant correlations (r=0.286, p=0.983) between genetic distances and geographic distances.

A UPGMA tree was constructed based on unbiased minimum genetic distances between the 12 populations of *D. triflorum*, and some populations were clustered with high bootstrap values (Fig. 2). The 12 populations could be

Table 3 Nei's (1972) genetic identity among 12 populations of *D. triflorum*

Population	JX	SG	MZ	JY	GZ	HZ	NN	LD	ZH	MM	ZJ	QH
JX												
SG	0.889											
MZ	0.909	0.882										
JY	0.885	0.872	0.914									
GZ	0.874	0.888	0.880	0.893								
HZ	0.874	0.893	0.915	0.895	0.877							
NN	0.880	0.874	0.890	0.888	0.889	0.869						
LD	0.868	0.881	0.871	0.885	0.952	0.876	0.892					
ZH	0.877	0.933	0.869	0.846	0.860	0.877	0.848	0.845				
MM	0.870	0.891	0.875	0.896	0.915	0.915	0.857	0.914	0.875			
ZJ	0.865	0.889	0.875	0.900	0.916	0.907	0.853	0.911	0.871	0.958		
QH	0.886	0.91	0.869	0.866	0.916	0.887	0.872	0.915	0.917	0.916	0.910	

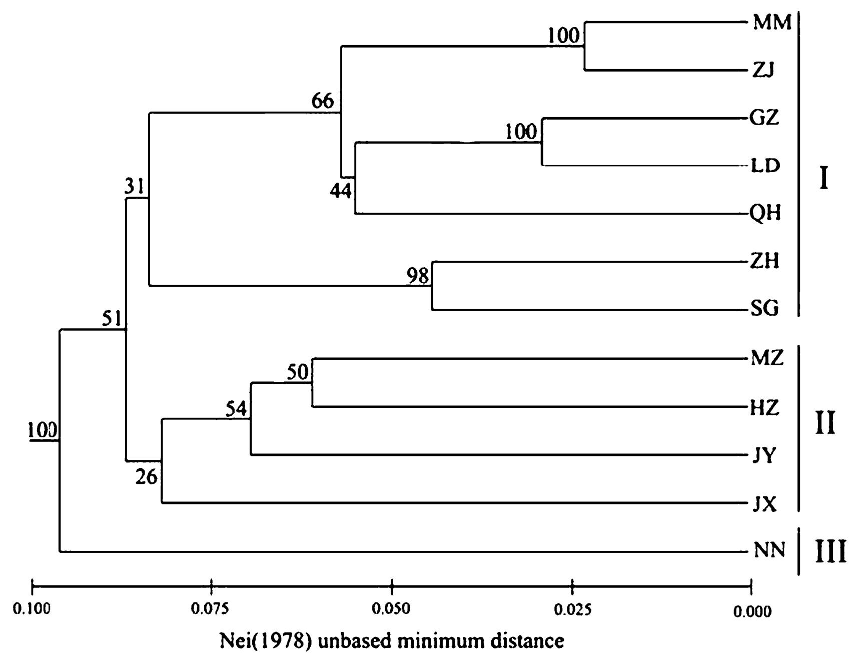

Fig. 2 UPGMA dendrogram of the 12 populations of *D. triflorum* based on Nei (1978) unbiased minimum distance

divided into three subgroups: The western population NN alone formed one subgroup; the eastern populations JY, MZ, HZ, and JX formed the second subgroup; and the central-southern populations were included in the third subgroup.

Discussion

Genetic Diversity of *D. triflorum*

Compared with the congeneric species *D. gangeticum*, *D. heterocarpon*, and *D. triquetrum* (polymorphism, P=34.5, 37.5, and 33.3%, respectively; Heider et al. 2009), *D. triflorum* (H_e=0.310, N_e=1.561, I=0.449, P=76.16%) appears to be more polymorphic due to its relatively high level of genetic variation. Even higher levels of polymorphism have been reported for other legume species, e.g., *Cratylia argentea* (91.8%; Andersson et al. 2007) and *Flemingia macrophylla* (95%; Heider et al. 2007). The maintenance of genetic diversity within a plant species has been shown to be influenced by its biological characteristics (Hamrick and Godt 1990) such as life history traits and mating system and its geographical distribution. In particular, the wide geographical distribution of *D. triflorum* should theoretically enable this species to maintain high levels of genetic diversity since widely distributed plant species tend to maintain more variation than more narrowly distributed species. Accordingly, since it is widely distributed in tropical and subtropical regions of the world, *D. triflorum* has become adapted to many different environments, thereby maintaining high levels of genetic variation. Its long lifespan also positively affects the genetic diversity of *D. triflorum* by necessitating an adaptability to changing environmental conditions (Vrijenhoek 1985; Ledig 1986). Thus, its relatively high level of genetic variation at the species level makes *D. triflorum* an ideal candidate for exploitation.

Population Genetic Differentiation of *D. triflorum*

Indications of significant differences in the genetic structures between *D. triflorum* populations were obtained by two different analytical approaches: The coefficient of gene differentiation (G_{ST}) was 0.255, under the assumption of Hardy–Weinberg equilibrium, and AMOVA showed significant genetic differentiation between populations (Φ_{ST}= 0.243, p<0.001). Similar results have been found in other studies on species of Fabaceae. For example, among groups of Chinese common bean, G_{ST} values between 0.08 and 0.41 (average 0.1883) have been obtained (Zhang et al. 2008) and among groups of cowpea G_{ST} values between 0.120 and 0.400 (average 0.3175; Coulibaly et al. 2002). However, our results show N_m between populations of *D. triflorum* to be 0.730, which is lower than the reported values for the common bean (N_m = 2.45; Blair et al. 2007) and Argentinean *Acacia* species (N_m=0.999; Casiva et al. 2002).

Clustering analysis divided the 12 populations into three subgroups: a western, an eastern, and a central-southern subgroup. The results correspond well with their natural distribution, but the genetic differentiation among the 12 populations appears to have little correlation with the geographic distances between them. The lack of any

significant correlation between genetic distance and geographic distances of *D. triflorum* indicates that it does not conform to the standard isolation by distance model, possibly because mountains form effective barriers to gene flow between its populations (Echt et al. 1998; Chase et al. 1996; Forcioli 1998) since *D. triflorum* only occurs at low altitudes and does not grow well in shady environments. The presence of many mountains and areas of dense forests in the study area in South China (e.g., there are 164 mountains with altitudes exceeding 1,000 m in Guangdong province) may therefore prevent gene flow, even between nearby populations of *D. triflorum* which has a scattered distribution in South China.

Exploitation

Genetic variation within a species is the basis for both natural and artificial selection. Therefore, accurate estimates of genetic structure are very useful for the management and exploitation of plant genetic resources (Hamrick et al. 1991). The high level of genetic variation at the species level, observed in the present study on *D. triflorum*, should greatly facilitate any future program of artificial selection. Similarly, the high level of genetic diversity observed within populations was also encouraging since it should provide a broad genetic basis to maintain the species' potential to respond to new selection pressures imposed by environmental changes (Ge and Sun 2001). Our results show that the highest genetic diversity of *D. triflorum* is found mainly within the populations of Guangdong and Hainan. These places, therefore, should be prioritized as locations from which to select germplasm for breeding programs. On the other hand, the considerable genetic divergence detected among different regional populations of *D. triflorum* suggests that regional populations probably carry unique alleles from which hybrid vigor might arise if these different populations were crossed. It should also be noted, however, that two marginal populations, JX and NN, have much lower genetic diversity because they have often been rendered locally extinct by human activity. Nevertheless, these populations may be adapted to more extreme environments (for example, population JX may be more adapted to low temperatures of about −5°C). Therefore, these populations may still be of value since if incorporated into breeding programs, they may confer on new cultivars an elevated tolerance to extreme conditions.

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