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Genetic diversity of *Ceratoides arborescens*, a species endemic to China, detected by inter-simple sequence repeat (ISSR)

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ABSTRACT. In order to investigate genetic diversity and population structure of *Ceratoides arborescens*, six populations were selected from different steppe types in Inner Mongolia grasslands of China. Inter-simple sequence repeat (ISSR) markers were used to assess the genetic diversity within and among natural populations of *C. arborescens*. Thirteen ISSR primers generated 154 discernible DNA bands, of which 151 (98.05%) were polymorphic. High genetic diversity was detected at the species level [percentage of polymorphic loci (PPB) = 98.05%; H = 0.2984; I = 0.4557], whereas, relatively low genetic diversity existed within populations (PPB = 80.62%; H = 0.2675; I = 0.4031). Analysis of molecular variance showed that variation existed mainly within populations (73.25%) rather than among populations (26.75%), which was in line with the high level of gene flow (N_m = 4.3332). The Mantel test found no significant correlation between genetic distance and geographic distance (r = 0.7522, $P < 0.05$). Six populations were clustered into two main groups, a desert steppe group and a typical steppe group.

Key words: *Ceratoides arborescens*; Inter-simple sequence repeat (ISSR); Genetic diversity; Conservation implications

INTRODUCTION

The analysis of genetic diversity is a key element for the study of biodiversity, ecosystem functioning, and the consequences of anthropogenic impact on natural systems (Li et al., 2012; Wang et al., 2012; Yang et al., 2014). Genetic diversity plays an important role in the survival and adaptability of a species, and it is largely attributed to several life history traits, such as geographic range, seed dispersal mechanism, mating system, life form, and taxonomic status (Zhang et al., 2006; Tian et al., 2012; Zhao et al., 2012; Liu et al., 2013; Ghaffari et al., 2014). Compared to widespread species, many endemic and rare species usually show low levels of genetic diversity because of their habitat fragmentation (Yang et al., 2012; Sow et al., 2014). The loss of genetic variability usually has deleterious effects on the species response to natural selection and may threaten the survival of the species or populations (Jugran et al., 2011; Teixeira et al., 2014). Thus, an accurate estimate of genetic diversity of a species is important for the conservation and sustainable exploitation of the species.

Ceratoides arborescens (Chenopodiaceae), a plant species endemic to China, is widely distributed in northwest China, especially in the typical steppes and desert steppes of Inner Mongolia. In addition, it often occurs on farmlands alongside the annual and perennial crops, at altitudes ranging from 800 to 1800 m and the rainfall from 200 to 500 mm per annum (Yi et al., 2004; Tong et al., 2010; Dong et al., 2012). As a multipurpose species of great economic and environmental value, it has been used for grassland improvement because of its high nutritional value and high stress tolerance (Yi et al., 2004; Han and Yi, 2008; Tong et al., 2010). However, due to habitat degradation and overgrazing, the species exhibits a fragmented distribution pattern. Conservation and rational use of this species are thus of primary importance. Selection and assessment of genotypes could facilitate breeding processes by separating populations with genetic similarity.

Previous studies have focused on morphology and anatomy (Bai et al., 1998; Liu et al., 2007; Zhou et al., 2007), reproductive biology (Han and Yi, 2008; Lu et al., 2009), and population ecology (Xie et al., 2007). No study has investigated the species' genetic diversity and population structure, although such information is essential for the formulation of effective conservation and sustainable exploitation strategies. Inter simple sequence repeat (ISSR) method has been applied to assess genetic diversity especially in plants with no or only few available specific primers, such as *Dactylis glomerata* (Madesis et al., 2014) and *Brachiaria ruziziensis* (both from Poaceae) (Azevedo et al., 2011). The objective of this study is to evaluate genetic diversity and population structure within and among the populations of *C. arborescens* in Inner Mongolia grasslands of China, in order to improve the management of genetic resources.

MATERIAL AND METHODS

Study sites and sampling

A total of 120 individuals of *C. arborescens* were collected from six natural populations in the middle and eastern regions of Inner Mongolia, in the area of the typical geographical distribution of the species (Figure 1, Table 1). Fresh tender leaves were collected randomly from 20 mature individuals in each population. The distances between sampled individuals varied between 30 and 50 m, depending on the population size. The sampled leaves were kept

at 4°C in sealed bags and stored at -70°C at the Inner Mongolia Agriculture University until DNA extraction.

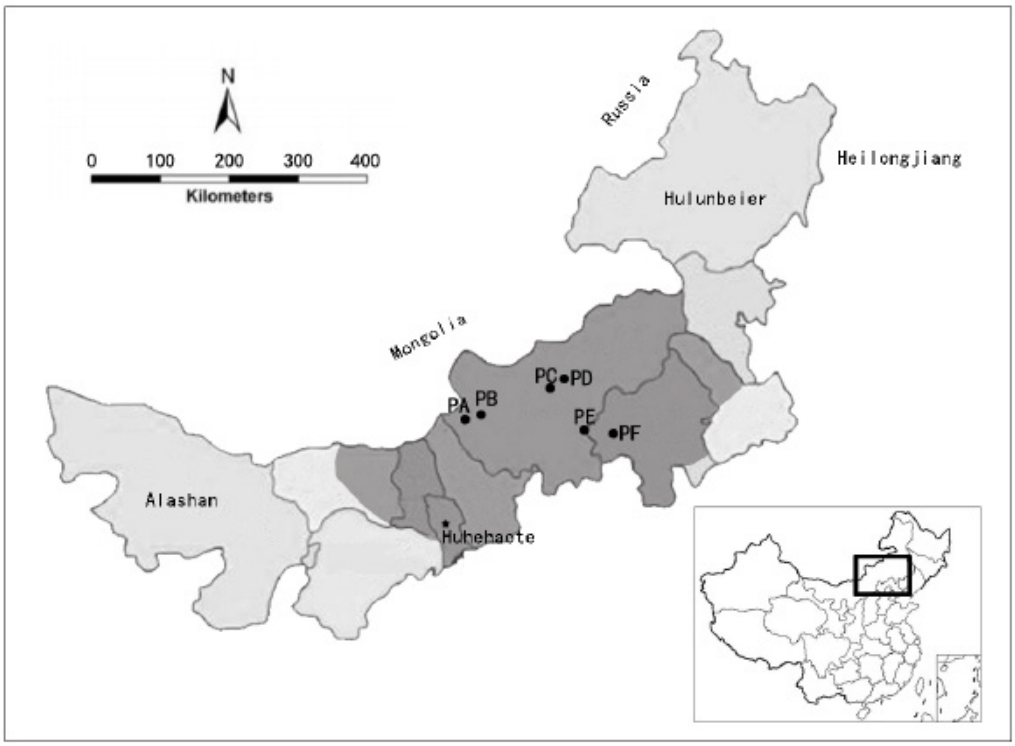

Figure 1. Map of the six sample populations of *Ceratoides arborescens* in Inner Mongolia grassland of China.

Table 1. Populations of *Ceratoides arborescens* examined in the ISSR analysis.

Population code	Place of collection	Elevation (m)	Geographic coordinates	Vegetation type	Sample size (Number of clumps)
PA	West Baiyinxile, Siziwang, Wulanchabu	1397	42º10'37"N, 112º10'38"E	Desert steppe	>300
PB	East Baiyinxile, Siziwang, Wulanchabu	1446	42º08'35"N, 112º11'38"E	Desert steppe	>300
PC	Southern suburbs, Xilinhaote, Xilinguole	1085	43º48'53"N, 116º06'01"E	Typical steppe	267
PD	Southwestern suburbs, Xilinhaote, Xilinguole	1190	43º47'21"N, 116º07'52"E	Typical steppe	179
PE	Kangjiaying, Jingpeng, Keshiketeng	1250	43º19'06"N, 117º32'58"E	Typical steppe	135
PF	Sizhangfang, Wudan, Wengniute	704	43º04'31"N, 118º53'14"E	Typical steppe	69

DNA extraction and ISSR-PCR amplification

DNA was extracted using a modified CTAB method (Zhang et al., 2006) and dissolved in 1X TE buffer for subsequent use. DNA quality and quantification were estimated by spectrophotometer analysis (Eppendorf BioPhotometer, Eppendorf, Hamburg, Germany). ISSR-PCR amplifications were performed in a GeneAmp PCR System 9700

DNA Thermal Cycler (PerkinElmer, Waltham, MA, USA) with cycling profile: 3 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 48°C, and 1.5 min at 72°C, and ending with 10 min at 72°C. One hundred primers (Biotechnology Laboratory, Inner Mongolia Agriculture University) were screened initially to identify the well-amplified polymorphic bands among the populations. Of the 100 primers tested, 13 produced bright, clear, and reproducible bands. These primers were selected for further study of the 120 *C. arborescens* individuals.

PCR was carried out in a total volume of 20 μL, which included 50 ng template DNA, 2 μL10X PCR buffer (Mg^{2+} Plus), 2 μL 25 mM $MgCl_2$, 1.0 μL 2.5 mM dNTPs Mixture, 1.0 U *Taq* polymerase (TaKaRa Bio, Dalian, China), and 0.4 μM of each primer (Shanghai Sangon Bio, Shanghai, China). Amplification products, along with a GeneRuler100 ladder (Fermentas, Burlington, Canada), were separated via electrophoresis on 1.5% (w/v) agarose gels with 0.5 X TBE buffer at 120 V for 3-4 h and stained with 0.1 mg/μL ethidium bromide. They were then photographed with an Epson digital still AF camera (Epson, Suwa, Japan). Negative controls, which lacked template DNA, were also included in each PCR set to test for possible contamination.

Genetic diversity analysis

The experiment was repeated three times and only clear and reproducible bands were recorded. They were scored as 1 for presence or 0 for absence for each sample, and a matrix of different ISSR phenotypes was assembled for statistical analysis. These genetic indexes including the observed number of alleles, effective number of alleles, percentage of polymorphic loci (PPB), Shannon index (*I*) of diversity, and Nei's population differentiation (G_{ST}) were calculated using the POPGENE version 1.32 software (Peakall and Smouse, 2006). A dendrogram based on Nei's genetic distance was constructed by cluster analysis using an unweighted pair-group method with arithmetic averages (UPGMA) implemented in MEGA program version 3.1 (Kumar et al., 2004). Molecular analysis of variance (AMOVA) was performed to analyze genetic distance among populations using ARLEQUIN (Excoffier et al., 2005). In addition, the Mantel test was performed using Mantel 2.0 to determine the correlation between inter-population genetic and geographic distance matrices (Mantel, 1967).

RESULTS

Genetic diversity

The thirteen selected primers generated 154 bands, corresponding to an average of 11.9 bands per primer (Table 2). Of the 154 bands, 151 were polymorphic (98.05%) at the species level. The genetic diversity parameters of *C. arborescens* populations are shown in Table 3. Nei's gene diversity was estimated to be 0.2675 at the population level and 0.2984 at the species level. *I* was 0.4031 (at the population level) and 0.4557 (at the species level). Among our six populations, the highest level of variability was found in population of west Baiyinxile (PA) (PPB = 87.66%; $H = 0.2765$; $I = 0.4206$) and the lowest level in population of Sizhangfang (PF) (PPB = 74.68%; $H = 0.2545$; $I = 0.3810$).

Table 2. Sequences and numbers of bands for 13 primers.

Primer code	Sequence (5'-3')	Total bands	Polymorphic bands	PPB (%)
UBC807	$(AG)_8T$	14	13	92.9
UBC808	$(AG)_8C$	15	14	93.3
UBC810	$(GA)_8T$	14	14	100.0
UBC812	$(GA)_8A$	14	14	100.0
UBC817	$(CA)_8A$	8	8	100.0
UBC818	$(CA)_8G$	11	11	100.0
UBC820	$(GT)_8C$	9	8	88.9
UBC825	$(AC)_8T$	13	13	100.0
UBC827	$(AC)_8G$	10	10	100.0
UBC840	$(GA)_8YT$	15	15	100.0
UBC856	$(AC)_8YA$	11	11	100.0
UBC857	$(AC)_8YG$	11	11	100.0
UBC866	$(CTC)_6$	9	9	100.0
Total		154	151	98.05
Mean		11.9	11.6	

Y = (C, T).

Table 3. Genetic diversity of *Ceratoides arborescens* detected by ISSR analysis.

Populations	N_A	N_E	H	I	PPB (%)
PA	1.8766	1.4622	0.2765	0.4206	87.66
PB	1.8701	1.4638	0.2753	0.4176	87.01
PC	1.7792	1.4511	0.2646	0.3977	77.92
PD	1.7922	1.4664	0.2708	0.4060	79.22
PE	1.7727	1.4455	0.2633	0.3954	77.20
PF	1.7468	1.4402	0.2545	0.3810	74.68
Mean value	1.8063	1.4549	0.2675	0.4031	80.62
Species level	1.9805	1.5002	0.2984	0.4557	98.05

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 loci.

Genetic differentiation among populations

The genetic differentiation among the populations assessed by gene differentiation coefficient (G_{ST}) was 0.1154, which indicated that 11.54% of the total genetic variation was among the populations. A similar level of genetic differentiation among the populations was obtained from AMOVA, 13.21% among the populations and 86.79% within populations. The genetic differentiation values of *C. arborescens* populations were highly significant ($P < 0.001$) and F_{ST} was 0.132 (Table 4). The level of gene flow (N_m) was 4.3332, which indicated high level of gene exchange among the populations.

Table 4. Analysis of molecular variance (AMOVA) within/among *Ceratoides arborescens* populations.

Source of variation	d.f.	Sum of squares	Mean squares	Variance components	Percentage of variation (%)	Fixtion index (F_{ST})	P value
Among populations	5	447.217	89.443	3.366	13.21	0.132	<0.001
Within populations	114	2521.000	22.114	22.114	86.79		<0.001
Sum	119	2968.217	111.557	25.480	100.00		

Genetic relationship

The UPGMA tree (Figure 2), inferred from the Nei's genetic distance for *C. arborescens*, resolved the six populations into two clades: a desert steppe group and a typical steppe group. The former included populations PA and of East Baiyinxile (PB), whereas populations of southern suburbs (PC), southwestern suburbs (PD), Kangjiaying (PE), and PF made the latter group. This topology was consistent with the geographic distribution of these populations, indicating a possible correlation. Nei's unbiased genetic distances among the populations were calculated based on 151 markers scored. Values ranged from 0.0283 (PA *vs* PB) to 0.0674 (PA *vs* PE) (Table 5). The Mantel test identified a significant correlation between geographic and genetic distances among the populations ($r = 0.7522$, $P < 0.05$).

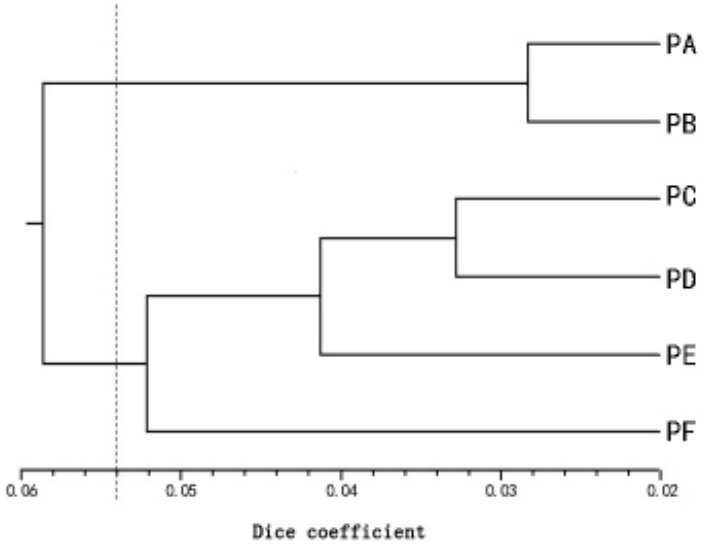

Figure 2. UPGMA dendrogram based on Nei's genetic distances among six populations of *Ceratoides arborescens*.

Table 5. Nei's unbiased genetic identity (above diagonal) and genetic distance (below diagonal) among *Ceratoides arborescens* populations.

Population	PA	PB	PC	PD	PE	PF
PA	-	0.9721	0.9562	0.9420	0.9348	0.9376
PB	0.0283	-	0.9501	0.9423	0.9361	0.9365
PC	0.0447	0.0512	-	0.9678	0.9611	0.9459
PD	0.0598	0.0594	0.0328	-	0.9580	0.9526
PE	0.0674	0.0660	0.0397	0.0429	-	0.9491
PF	0.0644	0.0656	0.0556	0.0486	0.0522	-

DISCUSSION

Genetic diversity

Previous studies have shown that endemic species and species with narrow distribu-

tion usually show lower levels of genetic diversity than widespread species (Tanahara and Maki, 2010; Chen et al., 2013; Duan et al., 2013; Nguyen et al., 2013; Aoki et al., 2014). However, *C. arborescens* was found to possess an unexpectedly high rate of genetic diversity (Table 3), indicating the species ability to adapt to varying environmental conditions. As a long-lived perennial plant, *C. arborescens* is widely distributed in the mid-eastern Inner Mongolia of China. To adapt to ecologically diverse habitats, it probably adopts outcrossing breeding systems, the wind-pollinated and wind-dispersed system. All these traits together have probably resulted in its presently high level of genetic diversity at the species level.

Moreover, many researchers have considered that habitat fragmentation, location, and population size have high impact on genetic diversity within populations (Feng et al., 2006; Arunkumar et al., 2012; Liu et al., 2013; Ghaffari et al., 2014). In the present study, the desert steppe group (PA, PB) exhibits a higher degree of genetic diversity than the typical steppe group (PC, PD, PE, and PF), This result was consistent with population size, suggesting that fragmented habitats, migration (decreased or absent) between populations, and genetic drift have probably contributed to the loss of diversity in the populations, especially in PF.

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 (Adoukonou-Sagbadja et al., 2007). In this study, genetic differentiation was consistent with the pattern of strong variation within populations and weak variation among populations (Table 4). The genetic differentiation was low (G_{ST} = 0.1154) and gene flow (N_m = 4.333) was high among *C. arborescens* populations. Typically, $N_m > 1$ indicates weak differential selection (Rossi et al., 2009; Oja and Talve, 2012; Munthali et al., 2013). Therefore, the present low level of genetic differentiation among populations could be largely attributed to several factors. First, the mode of pollen and seed dispersal, which determines the N_m among populations (Li et al., 2009a; Bellucci et al., 2011; Nestmann et al., 2011; Wang and Li, 2011), can facilitate N_m among populations, minimizing the population differentiation. Many studies have demonstrated that wind-pollinated and wind-dispersed species exhibit low levels of genetic differentiation (Li et al., 2009b; Adamski et al., 2012; Thomas et al., 2012), approaching the levels observed in *C. arborescens*. Second, genetic diversity may be influenced by geographic distances, especially longitudinal differences, as revealed by the Mantel test. These are expected to result in relatively low levels of genetic differentiation among *C. arborescens* populations due to frequent large-scale N_m.

Implications for future conservation

This study has implications for the use and conservation of this species through its potential role for multiple uses in the changing grassland environment of Inner Mongolia. Information obtained from this study provides valuable baseline data on the population genetics of *C. arborescens*, which suggest that the higher percentage of total variation is still harbored within population and indicates that in the Inner Mongolia grassland environment, *C. arborescens* is not genetically depauperate. Recent human intervention through overgrazing may be the main factor responsible for the fragmented status of this species. Therefore, habitat sites protection of *C. arborescens* should be concentrated on restoring the natural ecological bal-

ance through reducing livestock grazing.

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