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Relationship between the haplotype distribution of *Artemisia halodendron* (Asteraceae) and hydrothermal regions in Horqin Sandy Land, northern China

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ABSTRACT

The genetic diversity of *Artemisia halodendron* (Asteraceae), a constructive and dominant species in Horqin Sandy Land, was investigated to examine the genetic relationships with different hydrothermal regions in Horqin Sandy Land. We sequenced chloroplast DNA (cpDNA) fragments (*trn*L–F) of 243 plants from 10 populations across the Horqin Sandy Land. The analyses of cpDNA variation identified seven haplotypes. A low level of haplotype diversity (H_d=0.706) and nucleotide diversity (π=0.0013) was detected. Haplotypes clustered into two tentative clades. Low genetic differentiation among regions was consistently indicated by hierarchical analyses of molecular variance (AMOVA). Across the sampled populations, the haplotype distributions were differentiated with hydrothermal gradients.

Keywords: *Artemisia halodendron*; population genetics; chloroplast DNA; *trn*L-F; Horqin Sandy Land

1 Introduction

Artemisia halodendron Turcz. ex Bess. (Asteraceae, Anthemideae, Subgen. *Dracunculus*) is one of the most common semishrub species in the Horqin Sandy Land in Northeast China. It is important for vegetation rehabilitation in Horqin Sandy Land because of its high ecological value, including that (1) it is the key species of the plant communities and landscapes studies in Horqin Sandy Land (Li, 1991); (2) it plays a key role in the vegetation-restoration process due to its high drought tolerance, anti-wind erosion properties, and sand-burial resistance (Dong *et al*., 2000; Li *et al*., 2002; Zhao *et al*., 2006). Previous studies on *A. halodendron* focused on aspects of population-distribution patterns (Chao *et al*., 1999; Cao *et al*., 2008), biomass allocation (Li *et al*., 2005), breeding distribution (Li *et al*., 2005), morphological characteristics and physiological adaptations (Zhou *et al*., 1999), root longevity (Huang *et al*., 2009), genetic diversity (Huang *et al*., 2011, 2014), and establishment (Li *et al*., 2002) in Horqin Sandy Land. However, systemat-

ic comparison of genetic relationships of *A. halodendron* among populations from different hydrothermal regions has not yet been reported.

Horqin Sandy Land is located in the agropastoral transitional zone between the Inner Mongolian Plateau and the Northeast Plains (42°41′N–45°45′N, 118°35′E–123°30′E) and is one of the four largest sandy areas in northern China; it covers an area of approximately 139,300 km^2, of which up to 71,884 km^2 is desertified sandy land (Wang, 2003; Zhao *et al.*, 2003). Landscape in this area is characterized by sand dunes that alternate with gently undulating lowland areas (Li *et al.*, 2005). This area belongs to the continental semi-arid monsoon climate and is in the temperate zone, with a mean annual temperature (AMT) of 3–7 °C and mean annual rainfall (AP) of 350–500 mm (Zhao *et al.*, 2003). Over recent decades, this region has undergone severe desertification (Li *et al.*, 2000, 2004) and has displayed the northern-moving phenomenon of the interlocked agropasturing area of North China in the most recent hundred years (Zhao *et al.*, 2000, 2002).

In the present study, we used chloroplast DNA (cpDNA) *trn*L–F to examine the genetic diversity of *A. halodendron*. We specifically aimed to address the following questions: (1) What is the level of nucleotide diversity in *A. halodendron* from different hydrothermal regions? (2) How are the identified haplotypes distributed within and among populations? In particular, is there subdivision in the different hydrothermal-level populations (according to hydrothermal synthesis index)? We attempted to interpret the results to provide baseline genetic information pertinent to the restoration and management of degraded ecosystems in arid and semi-arid areas.

2 Materials and methods

2.1 Sampling

A total of 243 individuals were sampled from 10 natural *A. halodendron* populations (Tables 1 and 2). Populations 1–6 were in the low-hydrothermal-synthesis index region (average 24.97), while populations 7–10 were in the high-hydrothermal-synthesis index region (average 31.98) (Tables 1 and 2). The equation of hydrothermal synthesis index was $S = \sum_{t=1}^{12} 0.18 r_t / 1.045^t$ (r, monthly rainfall; t, monthly mean temperature) (Bailey, 1979). For each population, 17 to 30 individuals (spaced 30 m apart) were sampled (Table 3). Young and healthy leaves were randomly sampled from individuals and immediately stored with silica gel in ziplock plastic bags until DNA extractions were carried out. We chose *A. lavandulifolia* as the *out-group*.

Table 1 The average monthly rainfall in 10 years of 10 populations of *Artermisia halodendron* in Horqin Sandy Land

Population	Average monthly rainfall (mm)											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
pop1	0.1	0.3	7.5	32.3	34.8	27.1	113.1	33.9	8.0	10.9	2.0	0.0
pop2	0.1	0.3	7.5	32.3	34.8	27.1	113.1	33.9	8.0	10.9	2.0	0.0
pop3	0.1	0.3	7.5	32.3	34.8	27.1	113.1	33.9	8.0	10.9	2.0	0.0
pop4	0.1	0.3	7.5	32.3	34.8	27.1	113.1	33.9	8.0	10.9	2.0	0.0
pop5	1.6	2.3	7.4	11.5	34.0	69.8	116.7	70.4	36.6	14.4	3.8	1.4
pop6	1.6	2.3	7.4	11.5	34.0	69.8	116.7	70.4	36.6	14.4	3.8	1.4
pop7	0.7	1.1	4.0	10.9	26.9	74.3	134.7	82.0	28.7	14.5	3.4	1.3
pop8	1.5	2.1	7.1	14.2	34.1	74.0	103.9	79.3	30.5	19.0	4.9	2.8
pop9	1.5	2.1	7.1	14.2	34.1	74.0	103.9	79.3	30.5	19.0	4.9	2.8
pop10	0.2	1.6	5.8	25.4	45.2	88.6	150.2	77.4	31.0	12.5	5.6	0.6

2.2 Molecular methods

Total genomic DNA was extracted using AxyPrep Genomic DNA Mini Kits (Axygen Inc., Beijing, China) following the manufacturer's instructions. DNA quality was checked on a 1.0% agarose gel. Several pairs of cpDNA primers designed by Hamilton (1999), Taberlet *et al.* (1991), and Sang *et al.* (1997) were used in the initial screening. Two pairs of primers, *trn*L (5'-CGGAATTGGTAGACGCTACG-3') and *trn*F (5'-ATTTGAACTGGTGACACGAG-3') (Sang *et al.*, 1997), identified sequence variations in the sampled individuals and therefore were used for all remaining individuals. Polymerase chain reaction (PCR) was performed in a 25-μL reaction volume, containing 40 ng of genomic DNA, 1.0 U of Taq polymerase (Axygen Inc., Beijing, China), 3 mmol/L $MgCl_2$, 500 μmol/L each dNTP, 20 mmol/L

Tris-HCl (pH 8.3), 100 mmol/L KCl, and 0.3 μmol/L primer. The amplification condition was an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and a final 5-min extension step at 72 °C. The PCR products were determined by 1.0% agarose gel electrophoresis. The amplification products were purified using an AxyPrep PCR Purification Kit, following the manufacturer's protocol (Axygen Inc., Beijing, China). Purified DNA was sequenced by the MEIJI sequencing company in Shanghai, China, applying the PCR-primers as sequencing primers.

Table 2 The monthly mean temperature in 10 years of 10 populations for *Artermisia halodendron* in Horqin Sandy Land

Population	Monthly mean temperature (°C)											
	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.
pop1	−12.0	−6.7	0.4	10.2	17.1	22.0	23.8	22.6	16.8	8.7	−2.5	−10.4
pop2	−12.0	−6.7	0.4	10.2	17.1	22.0	23.8	22.6	16.8	8.7	−2.5	−10.4
pop3	−12.0	−6.7	0.4	10.2	17.1	22.0	23.8	22.6	16.8	8.7	−2.5	−10.4
pop4	−12.0	−6.7	0.4	10.2	17.1	22.0	23.8	22.6	16.8	8.7	−2.5	−10.4
pop5	−11.9	−8.8	−1.5	8.5	15.8	20.3	22.8	20.9	15.0	7.4	−2.3	−9.2
pop6	−11.9	−8.8	−1.5	8.5	15.8	20.3	22.8	20.9	15.0	7.4	−2.3	−9.2
pop7	−12.6	−9.0	−1.4	8.7	16.4	21.4	23.9	22.0	15.7	7.1	−3.1	−10.1
pop8	−13.5	−9.3	−1.0	8.9	16.4	21.4	24.1	22.5	16.1	7.5	−2.8	−10.8
pop9	−13.5	−9.3	−1.0	8.9	16.4	21.4	24.1	22.5	16.1	7.5	−2.8	−10.8
pop10	−13.9	−12.0	2.0	12.0	14.6	20.0	24.6	21.5	13.0	9.4	0.3	−11.8

2.3 Data analyses

DNA sequences were aligned using the CLUSTAL X program (Thompson *et al.*, 1997), with subsequent manual adjustments in MEGA4 (Tamura *et al.*, 2007). A matrix of combined sequences was constructed for the 243 individuals that we examined, and different cpDNA sequences were identified as haplotypes.

Basic population genetic parameters were estimated for three groups of populations: the low-hydrothermal-level region (populations 1–6); the high-hydrothermal-level region (populations 7–10); and finally, all populations. All parameters were calculated with $D_{NA}SP$ 5.10.01 (Librado and Rozas, 2009), including the number of segregating sites (S), the number of haplotypes (N_h), the haplotype diversity (H_d), the average number of nucleotide differences per site between two sequences in a sample, π (Nei and Li, 1979; Nei, 1987), and the average number of pairwise nucleotide differences (k).

Phylogenetic analyses of cpDNA haplotypes were performed with maximum parsimony (MP), using PAUP version 4.0 (Swofford, 2002). Heuristic search was implemented with 100 random additional sequence replicates, tree-bisection-reconnection (TBR) branch swapping, MULPARS option, and ACCTRAN optimization. To evaluate the relative robustness of the clades found in the most parsimonious tree, bootstrap analysis was conducted using 1,000 replicates with a simple taxon addition. Genetic differentiation among populations at the three different sampling levels was estimated by pairwise F_{ST} values (Wright, 1951). AMOVA was performed to analyze the source of variation among populations, using Arlequin 3.0 (Excoffier *et al.*, 2005) with 1,000 replicates of bootstrap.

3 Results

3.1 Haplotype distribution and genetic diversity

Sequence data were obtained for one loci from on average 101, 142, and 243 individuals from the low-hydrothermal-level region, the high-hydrothermal-level region, and the entire species' range, respectively. The length of the aligned *trn*L–*trn*F DNA sequences (including *trn*L and the *trn*F spacer region) ranged between 849 and 863 bp with two insertions. The analysis of cpDNA variation identified seven haplotypes (HapA-HapG) (Table 4). Haplotype C was the most abundant, occurring in three populations, followed by haplotypes A, B, D, and G, which occurred in two populations; and the remaining haplotypes were found in only a single population (Table 3 and Figure 1). We confirmed the division of the native range into two areas using hydrothermal synthesis index data (Table 3). Six populations in the low-hydrothermal-level region of the species were dominated by four different haplotypes. The other four populations in the high-hydrothermal-level region of the species were dominated by three different haplotypes (Table 3 and Figure 1).

Table 3 Origin of materials and number of samples for 10 populations of *Artemisia halodendron* from the Horqin Sandy Land

Population code	Location (abbreviation)	*S*	No. of samples	Latitude (°N)	Longitude (°E)	Altitude (m)	Haplotype	Habitats	Voucher	GenBank accession No.
pop1	Naiman (NM1)	21.8	20	42°47′12″	120°36′02″	452	D, F	Mobile dune	Huang110101-(1-20)	KX394368 KX394370
pop2	Naiman (NM2)	21.8	23	42°34′34″	120°12′33″	500	C, D	Mobile dune	Huang110102-(1-23)	KX394367 KX394368
pop3	Naiman (NM3)	21.8	17	42°58′11″	120°40′45″	357	G	Semi-mobile dune	Huang110103-(1-17)	KX394371
pop4	Naiman (NM4)	21.8	30	42°45′46″	120°35′07″	385	G	Semi-mobile dune	Huang110104-(1-30)	KX394371
pop5	Wenniute (WNT1)	31.3	22	43°10′10″	119°55′49″	434	C	Mobile dune	Huang110501-(1-22)	KX394367
pop6	Wenniute (WNT2)	31.3	30	42°54′15″	119°46′47″	479	C	Mobile dune	Huang110502-(1-30)	KX394367
pop7	Zhalute (ZLT)	29.8	30	44°25′16″	121°16′04″	340	E	Semi-mobile dune	Huang110201-(1-30)	KX394369
pop8	Tongliao (TL1)	31.0	21	43°44′38″	122°36′22″	249	A	Fixed dune	Huang110301-(1-21)	KX394365
pop9	Tongliao (TL2)	31.0	20	43°18′45″	122°14′11″	171	A, B	Fixed dune	Huang110302-(1-20)	KX394365 KX394366
pop10	Kerzuohou (KZH)	36.1	30	42°53′57″	122°17′23″	173	B	Fixed dune	Huang110401-(1-30)	KX394366

Note: *S*, the hydrothermal synthesis index.

Table 4 Variable sites of the aligned sequences of *trn*L-F in seven haplotypes of *Artemisia halodendron* in the Horqin Sandy Land

Haplotype	Variable positions				
	161	553	647	655–660	743–749
A	G	A	G	–	GAACTGG
B	G	T	G	–	GAACTGG
C	A	T	G	–	GAACTGG
D	A	T	T	–	–
E	A	T	T	ATACAT	GAACTGG
F	A	A	T	–	–
G	A	T	G	ATACAT	–
A. lavandulifolia	G	T	G	–	–

Notes: Sequences are numbered from the 5' to the 3' end in each region; – indicates lack.

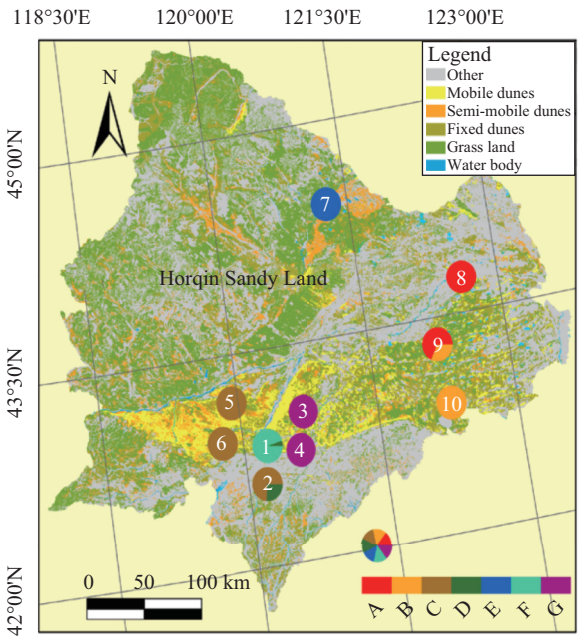

Figure 1 Geographic distribution of the seven haplotypes found on 243 individuals for *trn*L–F observed in *Artemisia halodendron* of the Horqin Sandy Land. Pie charts indicate the frequency of haplotypes within each population, and unique alleles are indicated by different colours. NDRS indicates Naiman Desertification Research Station, China Academy of Sciences. Base map data produced in 2000

We identified a total of two, three, and three segregating sites for the low-hydrothermal-level region, the high-hydrothermal-level region, and all populations, respectively. Summary statistics of sequence variation are given in Table 5. Overall haplotype diversity (H_d) and nucleotide sequence (π) diversity for *A. halodendron* were 0.706±0.001 and 0.0013±0.0001, respectively. At the regional level, haplotype diversity and nucleotide diversity between two regions varied between 0.318 (low-hydrothermal-level region) and 0.671 (high-hydrothermal-level region), and between 0.0006 (low-hydrothermal-level region) and 0.0015 (high-hydrothermal-level region), respectively. The population from the high-hydrothermal-level region had higher haplotype diversity and nucleotide diversity than the population from the low-hydrothermal-level region.

3.2 *Phylogenetic analyses and genetic structure*

Maximum parsimony analysis resulted in a single tree (length=14 and consistency index =0.5834). Two clades with low bootstrap support were identified: one consisting of the Hap C, G, F, E, and D, with four haplotypes (Hap C, G, F, and Hap D) distributed in the low-hydrothermal-level region and one haplotype (Hap E) in the high-hydrothermal-level region; and the other consisting of the rest of Hap A and Hap B, both occurring in the high-hydrothermal-level region (Figure 2).

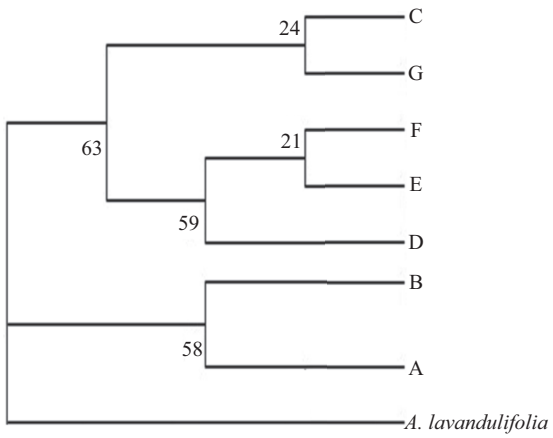

Figure 2 Phylogenetic relationships of the identified haplotypes of *Artemisia halodendron* in Horqin Sandy Land

The analysis of molecular variance (AMOVA) showed that around 17.54% of the variation was attributed to the low-/high-hydrothermal-level regions

differentiation, and between- populations variation accounted for just over one-half (55%) of the total variation, indicating very strong differentiation and little gene flow between regions and populations (Table 6). Within the low- and high-hydrothermal-level regions, between-populations differentiation was respectively 81.52% and 93.77% (Table 6). Overall, these results strongly indicate that haplotypes are geographically structured across the species' distribution range. F_{ST} values were 0.728, 0.815, and 0.938 at the species level, low-hydrothermal-level region, and high-hydrothermal-level region, respectively.

Table 5 Nucleotide variation and haplotype diversity at *trn*L-F in 10 populations of *Artemisia halodendron* from the low-hydrothermal-level region (populations 1–6), high-hydrothermal-level region (populations 7–10), and entire species' range (all populations) in the Horqin Sandy Land

Population	n	S	π	N_h	H_d	k
Low-hydrothermal region	142	2	0.0006±0.0001	3	0.318±0.046	0.541±0.183
High-hydrothermal region	101	3	0.0015±0.0001	3	0.671±0.008	1.307±0.444
All populations	243	3	0.0013±0.0001	5	0.706±0.001	1.129±0.380

Notes: n, sample size; S number of segregating sites; π, nucleotide diversity (Nei and Li, 1979; Nei, 1987); N_h, number of haplotypes; H_d, *Nei*'s haplotype diversity; k, mean pairwise nucleotide differences.

Table 6 Results of analyses of molecular variance (AMOVAs) of haplotype frequencies for populations and regional populations of *Artemisia halodendron* in Horqin Sandy Land

Populations	Source of variation	d.f.	Variance components	Percentage of variation	Fixation index	p value
Whole population	Among regions	1	0.214	17.54	F_{SC}=0.876	<0.001
	Among populations with region	8	0.769	55.32	F_{ST}=0.728	<0.001
	Within populations	233	0.117	12.96	F_{CT}=0.182	=0.04
	Total	242	1.095			
Low-hydrothermal region	Among populations	5	0.922	81.52	F_{ST}=0.815	<0.001
	Within populations	136	0.214	18.48		
High-hydrothermal region	Among populations	3	0.698	93.77	F_{ST}=0.938	<0.001
	Within populations	97	0.031	6.23		

Note: d.f., degrees of freedom.

4 Discussion and conclusion

In this study, we examined nucleotide variation at two nuclear loci of *A. halodendron* sampled from the Horqin Sandy Land. We confirmed the division of the native range into two areas, using hydrothermal synthesis index data (Table 3). The analysis of cpDNA variation identified seven haplotypes (Table 4 and Figure 2). Six populations in the low-hydrothermal-level region of the species were dominated by four different haplotypes. The other four populations in the high-hydrothermal-level region of the species were dominated by three different haplotypes (Table 3 and Figure 2).

Many studies have demonstrated that endemic species tend to possess high levels of genetic diversity (Wang *et al.*, 2010; Ge *et al.*, 2011). Compared with similar studies (Wang *et al.*, 2011), the level of haplotype diversity (H_d=0.706) and the nucleotide diversity (π=0.0013) within the whole populations of *A. halodendron* were somewhat lower than that of the endemic plants in northern of China. Such a low H_d value and π value indicated that this species was very adapted to the sandy land environment, and this character might have made it become the dominant and constructive species in Horqin Sandy Land. At the same time, the populations from the low-hydrothermal-level region exhibited lower haplotype diversity and more limited nucleotide diversity, and were a subset of that observed in the populations from the the high-hydrothermal-level region. These results lend support to the scenario described by Huang *et al.* (2011) using ISSR markers and Huang *et al.* (2013) using *psb*A-*trn*H. This fact might be due to environmental differences (Li *et al.*, 2009; Bao *et al.*, 2010; Liu *et al.*, 2010; Li and Wu, 2011). The low-hydrothermal-level region is a region of severe desertification (Zhao *et al.*, 2000; Bao *et al.*, 2010). In the process of ecological restoration, species increasing adapt to the local environment, which, in turn, reduces the genetic diversity (Wang *et al.*, 2009; Huang *et al.*, 2011).

Phylogenetic relationships analysis of the cpDNA sequences collected from *A. halodendron* in the different hydrothermal-level regions in Horqin Sandy Land did not entirely cluster according to populations or hydrothermal-level regions. This result is similar to that of some other studies (Chen *et al*., 2009; Hu *et al*., 2010; Zhou *et al*., 2010; Zhang *et al*., 2015). The MP tree indicated that the seven haplotypes formed two clades: the haplotypes in clade I all came from the high-hydrothermal-level region (63% bootstrap support); the haplotypes (except Hap E) in clade II came from the low-hydrothermal-level region (58% bootstrap support). This cluster process was closely related to the hydrothermal gradients in Horqin Sandy Land. The results of phylogenetic relationships analysis show that hydrothermal conditions change determines the increase of the haplotypes distribution difference.

The spatial analyses of genetic variation in *A. halodendron* in the different hydrothermal-level regions in Horqin Sandy Land indicate that the between-populations genetic differentiations are high within the low-, the high-hydrothermal-level region, and the total distribution range (Table 6). Our data revealed significant genetic differences among populations in different hydrothermal level regions. The increase in population genetic differentiation might be related to strong human disturbance, high rates of habitat fragmentation, and decreasing population size in Horqin Sandy Land (Zhao *et al*., 2000; Wang *et al*., 2010). It is generally accepted that genetic differentiation would have increased among the native populations due to the changed environment (Wang *et al*., 2009).

In conclusion, there are significant genetic differences between *A. halodendron* populations from different hydrothermal-level regions. This information about genetic variation has important implications for restoring and managing the degraded ecosystems in arid and semi-arid areas. It is particularly important to understand the genetic variation of extant populations over the entire range of the species' distribution. Additional research is needed to determine levels of genetic variation in *A. halodendron* throughout its entire distributional range.

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