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GENETIC DIVERSITY IN *BREONADIA SALICINA* BASED ON INTRA-SPECIES SEQUENCE VARIATION OF CHLOROPLAST DNA SPACER SEQUENCE

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Abstract

Assessment and knowledge of the genetic diversity and variation within and between populations of rare and endangered plants is very important for effective conservation. Intergenic spacer sequences variation of *psbA-trnH* locus of chloroplast genome was assessed within *Breonadia salicina* (Rubiaceae), a critically endangered and endemic plant species to South western part of Kingdom of Saudi Arabia. The obtained sequence data from 19 individuals in three populations revealed nine haplotypes. The aligned sequences obtained from the overall Saudi accessions extended to 355 bp, revealing nine haplotypes. A high level of haplotype diversity (Hd = 0.842) and low level of nucleotide diversity (Pi = 0.0058) were detected. Consistently, both hierarchical analysis of molecular variance (AMOVA) and constructed neighbor-joining tree indicated null genetic differentiation among populations. This level of differentiation between populations or between regions in *psbA-trnH* sequences may be due to effects of the abundance of ancestral haplotype sharing and the presence of private haplotypes fixed for each population. Furthermore, the results revealed almost the same level of genetic diversity in comparison with Yemeni accessions, in which Saudi accessions were sharing three haplotypes from the four haplotypes found in Yemeni accessions.

Introduction

Saudi Arabia has different scenarios of ecosystem degradation. Over-exploitation including over-grazing, expanding urban activities, selective removal of species, habitat destruction and mutilation which all cause genetic erosion. Fragmentation of habitats, endangerment, rarity, paucity of regeneration, reproductive inefficiencies, thin populations or narrow genetic base of germplasms etc., are physical syndromes of ebbing for survival, which appear in higher rate in the Kingdom displays (Thomas, 2011) and lead eventually to extinction of many endangered species including species under current study. Therefore, the conservation of threatened and endangered medicinal species is indispensable for the future of humankind (Rahman *et al.,* 2004).

In the present study, *Breonadia salicina* (Vahl) Hepper & Wood (family: Rubiaceae) was selected as one of the most critically endangered plant species in Saudi Arabia (Al-farhan *et al.,* 2005; Al-Turki, 2002) with dwindling population size that might lead to its extinction and thus it needs urgent conservation efforts. *Breonadia* Ridsdale is a monotypic genus, distributed in Tropical Africa and Southern Arabia. *B. salicina* is a medium sized to tall tree about 40 m in height and 2 m in diameter. It usually grows along high escarpments from 500 to 2000 m, near the banks or in the water of permanent streams and rivers (Mahlo, 2009; Al-farhan *et al.,* 2005).

Molecular analysis of genetic variation among individuals of a population can offer a means of monitoring the genetic variability of a declining population and assess genetic consequences of fragmentation on remnant populations (Cruzan, 2001; Wallace, 2002). Chloroplast DNA (cpDNA) is commonly used in studies assessing variation between populations, species, genera and sometimes even higher taxonomic levels.

Chloroplast DNA markers proved to be one of the most powerful tools for cytoplasmic polymorphism studies and plant molecular systematics over the last decade through the analysis of DNA sequencing, nucleotide substitutions, restriction site mutations and structural rearrangements (Harding *et al.,* 2000; Molvray *et al.,* 1999). The usefulness of cpDNA markers have been widely used in investigations of genetic structure, phylogeography, and the reconstruction of the evolutionary history of endemic and endangered species (Artyukova *et al.,* 2009; Ayele *et al.,* 2009; Ikeda *et al.,* 2008; Prentice *et al.,* 2003; Wang *et al.,* 2009; Khan *et al.,* 2013). Universal primers for amplification of several chloroplast introns, intergenic spacers and genes have been published, allowing to address a broad range of systematic and evolutionary questions at all taxonomic levels (Cronn *et al.,* 2002; Tate & Simpson, 2003).

Intergenic spacer *psbA-trnH* of cpDNA exhibits considerable ease of amplification, genetic variability and divergence, short sequence length, conserved flanking sites for developing universal primers and ease of alignment (Kress *et al.,* 2005; Kress & Erickson, 2007). The aim of this study was to investigate the patterns of genetic diversity within and among natural populations of *B. salicina* in Saudi Arabia using cpDNA intergenic spacer *psbA-trnH*.

Materials and Methods

Collection of plant materials: Leaf samples from fully-grown trees of *B. salicina* were collected through all natural populations in Kingdom of Saudi Arabia and only one individual plant found in Al-Baha. Moreover, another population was collected from the Republic of Yemen for better comparison of genetic diversity (Table 1). After collection, the samples were labeled and preserved in silica gel, then dipped in liquid nitrogen and stored at -80˚C upon arrival to the laboratory. Identification was done based on morphological characters of flowers, leaves and fruits.

Table 1. Samples collected from different populations of *B. salicina* and their locations.

Populations	Location	Province (region)	Number of accessions	Coordinates
Population 1	Wadi Lejib	Jizan (KSA)	6	17° 36' N 42° 56' E
Population 2	Rabuaa (Wadi Afkah)	Asir (KSA)	7	17° 33' N 43° 18' E
Population 3	Rabuaa (Main Entrance)	Asir (KSA)	5	17° 34' N 43° 19' E
Al-Baha Individual	Jabal Shada Al-Aala	Al-Baha (KSA)	1	19° 51' N 41° 18' E
Yemeni population	Mapyan	Hajjah (Yemen)	8	15° 43' N 43° 34' E

Extraction and purification of genomic DNA: A modified CTAB (cetyl trimethyl ammonium bromide) procedure based on the protocol of Khan *et al*., (2007) was followed for obtaining good quality and high yield of genomic DNA. DNA concentration and purity were determined through Thermo Scientific Nanodrop 8000 spectrophotometer, while the DNA quality was checked using 1% agarose gel in 1X TBE buffer stained with ethidium bromide; then electrophoresis and visualization of the genomic DNA was done using the Ingenius Syngene Bio-imaging gel documentation system.

Amplification of chloroplast intergenic spacer *psbA-trnH* locus: The intergenic spacer between *psbA* and *trnH* genes of the cpDNA was amplified with a pair of universal primers (Sang *et al.,* 1997; Tate & Simpson, 2003). According to the protocol followed by Al-Qurainy *et al*., (2011), polymerase chain reaction (PCR) was performed in a 25µl volume using PCR beads (GE healthcare, UK). The standardized components mixture in each single reaction were 20ng/µl DNA and 10pmol/µl from both forward (5-GTTATGCATGAACGTAATGCTC-3) and reverse (5-CGCGCATGGTGGATTCACAAATC-3) primers, while there was a modification in annealing temperature of thermo-cycling conditions. The amplification was performed using the Thermocycler, Applied Biosystems AB Veriti 96 well. The amplified products were resolved on a 1.2% agarose gel containing ethidium bromide (0.5µg/ml). PCR products were visualized under UV light and photographed using Ingenius Syngene Bio-imaging gel documentation system.

Sequencing of amplified *psbA-trnH*: The sequencing was done at Maceogen Inc., Korea. Cycle sequencing was conducted using the same primers as used in amplification and BigDye vers.3 reagents and an ABI PRISM 3100 DNA Analyzer (Perkin-Elmer, Applied Biosystems). The PCR program consisted of first initial denaturing set at 94°C for 5 min, followed by 30 cycles of 96°C for 10 sec, annealing at 50°C for 5 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min. The sequenced product was precipitated with 17 µl of deionized sterile water, 3 µl of 3 M sodium acetate solution, and 70 µl of 95% ethanol. The sequencing was performed using the ABI 3100 automated DNA sequencer (Perkin-Elmer, Applied Biosystems).

Data analysis: DNA sequences were aligned with the ClustalX Version 2 software (Larkin *et al.,* 2007) and then manually refined. DnaSP, version 5.1 (Librado & Rozas, 2009) was used to calculate statistical values, such as haplotype diversity (Hd), average number of pairwise nucleotide differences (k), the nucleotide composition, nucleotide diversity (Pi) and number of polymorphic sites (S). The phylogenetic tree was constructed using the neighbor-joining (NJ) method with MEGA version 5 (Tamura *et al.,* 2011). Analysis of molecular variance (AMOVA) was performed using GenAlEx 6.5 (Peakall & Smouse, 2006, 2012).

Results

The length of cpDNA *psbA-trnH* intergenic spacer varied from 337 bp to 342 bp. All sequences were BLAST as a query sequence in GenBank database (http://www.ncbi.nlm.nih.gov/). BLAST analysis showed that our amplified and sequenced *psbA-trnH* showed 97% sequence identity with that of *psbA-trnH* intergenic spacer of *Uncaria sinensis* (GenBank accession no. GQ435236.1). *Uncaria sinensis* belongs to the same family, subfamily and tribe as *B. salicina* (Rubiaceae, Cinchonoideae and Naucleeae), respectively.

Haplotype distribution and genetic diversity: The length of aligned sequences was 355 bp long, with 8 nucleotide substitutions and 24 indels. In total, 9 haplotypes (Table 2) were identified among Saudi accessions. Haplotype H1 was the most abundant which occurred in 7 accessions, followed by haplotypes H2 and H6 occurred in 3 accessions whereas, the remained haplotypes were found in only a single sample (Table 4).

Table 2. Summary of different haplotype sequences in *B. salicina.*

Haplotype #	Nucleotide positions							
	291	292	296	300	304	311	313	314
Hap_1	G	G	A	A	A	G	A	A
Hap_2	G	G	T	A	A	G	A	A
Hap_3	A	A	A	A	A	G	A	A
Hap_4	G	A	A	A	A	G	A	A
Hap_5	A	A	T	A	A	G	A	A
Hap_6	A	A	A	A	A	G	A	G
Hap_7	G	G	A	A	T	T	A	A
Hap_8	G	G	A	A	A	G	G	A
Hap_9	G	G	A	C	A	G	A	A
Hap_10	G	G	T	C	A	G	A	A

Table 3. Descriptive statistics results of *psbA-trnH* sequences in different *B. salicina* populations.

Sampling population	n	H	S	Hd	K	P*i*
Pop. 1	6	5	3	0.933 ± 0.122	1.73	0.00519 ± 0.00085
Pop. 2	7	5	6	0.857 ± 0.137	1.71	0.00518 ± 0.00171
Pop. 3	5	3	5	0.700 ± 0.218	2.0	0.00604 ± 0.00234
Saudi accessions*	19	9	8	0.842 ± 0.067	1.92	0.00579 ± 0.00090
Yemeni accessions	8	4	5	0.750 ± 0.139	1.82	0.00550 ± 0.00153
Total*	27	10	8	0.843 ± 0.048	1.93	0.00582 ± 0.00064

n, sample size; **H,** number of haplotypes; **S,** number of polymorphic sites; **Hd,** haplotype diversity; **k,** Average number of nucleotide differences; **Pi,** nucleotide diversity; *Al-Baha individual included

Table 4. Geographical distribution of haplotypes in different *B. salicina* populations.

Population	Haplotype										No. of individuals
	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	
1	1	2	1	1	1	-	-	-	-	-	6
2	3	1	-	-	-	1	-	1	1	-	7
3	3	-	-	-	-	1	1	-	-	-	5
Al- Baha individual	-	-	-	-	-	1	-	-	-	-	1
Saudi Arabia	7	3	1	1	1	3	1	1	1	-	19
Yemen	2	-	4	-	-	1	-	-	-	1	8
No. of individuals	9	3	5	1	1	4	1	1	1	1	27

Overall haplotype diversity (Hd) and nucleotide sequence (Pi) diversity for Saudi accessions were 0.842 ± 0.067 and 0.00579 ± 0.0009, respectively. At the population level, haplotype diversity and nucleotide diversity among Saudi populations (Al-Baha individual was excluded to avoid statistical error) varied between 0.933 (population_1) and 0.7 (population_3), and between 0.00604 (population_3) and 0.00518 (population_2), respectively (Table 3). Hence, the highest level of haplotype diversity and nucleotide diversity occurred in population_1 and population_3, respectively.

In the case of Yemeni accessions, a total of 4 haplotypes were identified from which three haplotypes (H1, H3 and H6) were similar to their counterparts in Saudi haplotypes and only one was different H10 (Table 4). The overall haplotype diversity (Hd) and nucleotide sequence (Pi) diversity in Yemeni accessions were about 0.75 ± 0.139 and 0.0055 ± 0.00153, respectively (Table 3).

Phylogeny and patterns of population structure: The phylogenetic analysis conducted using the Neighbor-Joining (NJ method) which revealed two strongly supported groups (a and b) (Fig. 1).

However, this phylogenetic analysis revealed that haplotypes from different geographic regions were almost clustered into the same clade, whereas haplotypes from the same population are not grouped within the same clade. This may be because of lack of distinct geographic distribution structure of haplotype and disperse into different clades.

Table 5. Analysis of molecular variance (AMOVA) for populations of *B. salicina* based on haplotype sequences of *psbA-trnH* intergenic regions.

		Source of variance	df	Sum of square	Variance component	% Total of variance	Significance
1.	Groups based on geographical origin	Variance among regions	1	5.306	0.264	6%	p<0.001
		Variance among populations	1	3.240	0.000	0%	
		Variance within populations	15	67.010	4.467	94%	P<0.001
		Total	17	75.556	4.731	100%	
2.	Without grouping	Variance among populations	2	8.546	0.000	0%	
		Variance within populations	15	67.010	4.467	100%	p<0.001
		Total	17	75.556	4.467	100%	

df = Degree of freedom

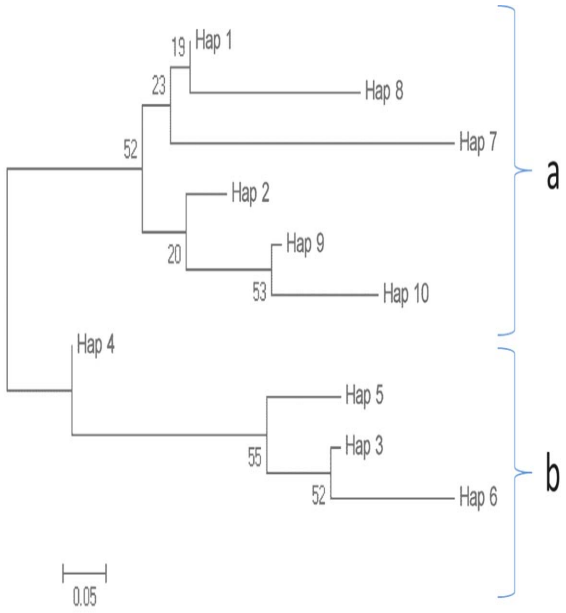

Fig. **1.** Neighbor-joining tree of *B. salicina* based on haplotypes sequences of the *psbA-trnH* noncoding spacers of cpDNA. Numbers above branches indicate the bootstrap values of 1000 replicates.

Like ISSR, the haplotypes analysis of molecular variance (AMOVA) was performed twice. In the first case all populations were used without grouping according to geographical area, and in the second case two main regions based on geographic distance was determined. In this case, population 1 was in the first region (Jizan); populations 2 and 3 were in the second region (Asir), while Al-Baha individual was excluded to avoid statistical error.

When analyzing without grouping, AMOVA showed that the proportion of genetic variation attributable to differences among populations was null, with all genetic variation residing within populations (Table 5). Based on geographic origin, the hierarchical AMOVA showed that genetic variation among regions was very low (6%), while among populations within regions was absent, a larger significant amount (94% of the total) is due to differences within populations.

Discussion

Genetic diversity detected by *psbA-trnH* intergenic spacer: In comparison to haplotype diversity values previously found in some endangered plants reported in different studies (Su *et al.,* 2004, 2005; Huang *et al.,* 2001, 2005), the value of haplotype diversity obtained by *psbA-trnH* in this study was reasonably high. However, the level of nucleotide diversity within the whole species (Pi = 0.0058) of *B. salicina* was lower than that of the same endangered plants (Su *et al.,* 2004, 2005; Huang *et al.,* 2005) and in other woody species [*Cycas taitungensis* "Pi = 0.01268" (Huang *et al.,* 2001), *Eucalyptus loxophleba* "Pi = 0.088" (Byrne & Hines, 2004), *Eucalyptus nitens* "Pi = 0.084" (Byrne & Moran 1994), *Lambertia orbifolia* "Pi = 0.072" (Byrne *et al.,* 1999), *Melaleuca alternifolia* "Pi = 0.017", *Melaleuca linariifolia* "Pi = 0.15" (Butcher *et al.,* 1995)] and these results are in congruence with that obtained from Yemeni population.

In the case of high haplotype diversity with low nucleotide diversity in this study, Zhou *et al.* (2010) suggested that most polymorphic sites could have emerged during a demographic expansion, and haplotype polymorphism has been accumulated by mutations, but it failed to accumulate nucleotide sequence diversity (Avise, 2000). In addition, the low nucleotide diversity of the species also may be due to habitat fragmentation (Zhou *et al.,* 2010).

Genetic structure detected by *psbA-trnH* intergenic spacer: Essentially null population differentiation was found by hierarchical analysis via AMOVA, which is concordant with the results of the NJ tree, in which the distribution of haplotypes was mixed and did not cluster according to populations or least clustered to geographic regions. This level of population differentiation may have resulted from an abundance of ancestral haplotype sharing. All populations of *B. salicina* shared one haplotype (H1). According to coalescence theory (Posada & Crandall, 2001), the common haplotype most likely represents the oldest lineage. While haplotype H1 was subsided in Yemeni population being only in two accessions, but the most common one was Haplotype H3.

Moreover, the low nucleotide diversity in this case could be due to habitat fragmentation and through the presence of H1 as the co-ancestor in all populations. The low genetic differentiation among the populations examined in the present study supports the hypothesis that all these populations were initially one.

Although approximately the whole genetic diversity resides within populations studied in the present study that is about more than 80% of the total genetic diversity within populations, so maintenance of five populations is enough to preserve most of genetic diversity of the species under study (Hamrick, 1993). Whereas losses of individual trees at specific locations may not cause an immediate decrease in genetic diversity, so such losses may have dangerous long-term consequences. The critically endangered state of this plant including low population size and the presence of a small number of remaining populations requires on-site preservation of all present populations to maintain the population size and even restoring suitable habitats to a certain extent in order to attain efficient population size (Carson, 1990). Furthermore, reinforcing off-site conservation must be established by transplanting seedlings or collecting seeds from different populations.

The presence of AT-rich regions in *psbA-trnH* locus which tends to stutter around mono-nucleotide repeats with an extremely indel rich nature (24 indel characters in this study). These mono-nucleotide repeats are disreputable for higher rates of evolution than normal indels, which make homology assessment via alignment difficult, and congruence between data sets was rejected when mononucleotide repeat data were included (Oberlander *et al.,* 2012). We recommend supporting results of *psbA-trnH* locus in genetic diversity with other cpDNA markers with longer variable sequence, which is considered as more reliable (Dong *et al.,* 2012) in order to provide enough informative characters. Finally, the *psbA-trnH* intergenic spacer was suggested as a candidate DNA barcode early due to the presence of this high variability (Kress *et al.,* 2005), but has not been supported in further studies. Although, *psbA-trnH* locus was useful in studying the genetic diversity in this study, it did not produce enough informative results because of its short length. Moreover, *psbA-trnH* locus has more inversions or mononucleotide repeats which may cause incorrect alignments or bring sequencing difficulties.

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