GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Assessment of phylogenetic relationship of rare plant species collected from Saudi Arabia using internal transcribed spacer sequences of nuclear ribosomal DNA

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Genet. Mol. Res. 12 (1): 723-730 (2013)
Received September 11, 2012
Accepted December 18, 2012
Published March 11, 2013
DOI http://dx.doi.org/10.4238/2013.March.11.21

ABSTRACT. The rare and endangered plants of any country are important genetic resources that often require urgent conservation measures. Assessment of phylogenetic relationships and evaluation of genetic diversity is very important prior to implementation of conservation strategies for saving rare and endangered plant species. We used internal transcribed spacer sequences of nuclear ribosomal DNA for the evaluation of sequence identity from the available taxa in the GenBank database by using the Basic Local Alignment Search Tool (BLAST). Two rare plant species viz, *Heliotropium strigosum* claded with *H. pilosum* (98% branch support) and *Pancratium tortuosum* claded with *P. tenuifolium* (61% branch support) clearly. However, some species, viz *Scadoxus multiflorus*, *Commiphora myrrha* and *Senecio hadiensis* showed close relationships with more than one species. We conclude that nuclear ribosomal internal transcribed spacer sequences are useful markers for phylogenetic study of these rare plant species in Saudi Arabia.

Key words: nrDNA; Conservation measures; BLAST; Rare species

INTRODUCTION

The Arabian Peninsula of Saudi Arabia harbors herbs, shrubs and trees, and it is also a hot spot for medicinal herbs. The flora of Saudi Arabia is faced with various threats such as deforestation, climate change, invasive species, altered soil pH and composition, stress due to various heavy metals, drought, salinity, high and low temperatures, different altitudes, and increased habitat fragmentation (Khan et al., 2012). The percentage of rare and endangered species is very high in Saudi Arabia as compared to other Middle East countries. Of 2250 plant species in the Kingdom, 600 species are rare and endangered in their natural habitat (Collenette, 1998; NCWCD, 1998; Al-Farhan, 2000). Saudi Arabia is going through a series of socio-economic changes as part of its development programs, and therefore, large areas of natural habitat in the mountainous regions and the range lands in the Northern, Eastern and Central regions are being turned into urban and agricultural lands.

Heliotropium strigosum is an important medicinal plant of the family Boraginaceae. Traditionally, this plant is used as a laxative and diuretic. The juice of the plant is used to treat gum boils, sore eyes, stings from nettles and insects and snake bite, as it has antimicrobial and antioxidant activities (Nasir, 1970; Hussain et al., 2010). *Pancratium tortuosum* is also a medicinal plant of the family Amaryllidaceae. *Senecio hadiensis* belongs to the family Asteraceae and has potential medicinal value due to the presence of pyrrolizidine alkaloids (Were et al., 1993). *Commiphora myrrha* (Burseraceae) has important terpenoids, which are the characteristic constituents of this plant species (Su et al., 2009), and sesquiterpenes, which show neuroprotective effects (Xu et al., 2012). *Scadoxus multiflorus* is also a medicinal plant and its extract has antimycobacterial, antibacterial and antifungal properties (Mariita et al., 2011). All these plant species are rare in the Kingdom of Saudi Arabia and have small population sizes; therefore, their molecular characterization and phylogenetic study are necessary.

A number of strategies have been implemented for the conservation of critically endangered species such as botanical gardens, GenBank, pots, seed banks, semi-natural environments, or *in vitro* culture. Rare and endangered plants may have potentially important genes such as for resistance to disease and tolerance to drought, salinity and other abiotic stresses, and measures should be taken for their conservation. Molecular tools are potential tools, and have been used for the assessment of genetic diversity and evaluation of the phyogenetic relationship within and between species. Therefore, such molecular tools should be implemented for the conservation of various rare and endangered plant species of Saudi Arabia in their natural habitat.

However, the assessment of genetic diversity of rare and endangered plant species is very important for a conservation strategy aimed at long-term survival. Genetic variation is a prerequisite for the short- and long-term survival of a species (Schonewald-Cox et al., 1983; Lande, 1988), and the importance of preserving genetic diversity of wild and domesticated species is extensively acknowledged today (Karron, 1991). There are several molecular markers available based on sequencing and non-sequencing for the evaluation of the phylogenetic relationship within and between the various species of the same genus. However, nuclear genomes are more reliable as compared to the chloroplast genome for markers, since the former remains more or less unchanged. The introgression rate is more frequent in chloroplast DNA markers than in the nuclear DNA markers, as reported in angiosperms (Rieseberg and Soltis, 1991). Hence, nuclear ribosomal DNA (nrDNA) was selected for the evaluation of the phylogenetic relationship of rare plant species using internal transcribed spacer (ITS) sequences. However, in most species, high homogeneity exists among the copies. Therefore, the ITS1-5.8S-ITS2 region has been extensively

used for phylogenetic analyses in plants and animals (Baldwin et al., 1995; Alvarez and Wendel, 2003), establishment of phylogeny between closely related taxa and identification of species or strains (Hillis and Dixon, 1991). The ITS sequences (mainly ITS 2) of nrRNA genes may be used as a potential DNA barcode in highly variable plants for establishment of molecular phylogenetic relationships at the species level (Shneer, 2009; Chen et al., 2010).

MATERIAL AND METHODS

Germplasm

The plant materials were collected from different geographical regions of Saudi Arabia. These materials were morphologically identified by a taxonomist in the Botany and Microbiology Department, King Saud University. Five rare species were selected for the evaluation of their phylogenetic relationship to the other species of the same genus available in the GenBank database (Table 1).

Table 1. Plant collection and internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA-ITS) sequence characteristics.

Taxon	Family	Place of collection	Status	Size of ITS locus (ITS1-5.8S-ITS2)	%GC
Scadoxus multiflorus	Acanthaceae	Fayfa	Rare	721	63
Pancratium tortuosum	Amaryllidaceae	Al-Hayl	Rare	723	67
Senecio hadiensis	Asteraceae	Jizan	Rare	595	49
Commiphora myrrha	Burseraceae	Jizan	Rare	735	64
Heliotropium strigosum	Boraginaceae	Jizan	Rare	659	66

Genomic DNA isolation and purification

DNA was isolated and purified by a modified CTAB method (Khan et al., 2007). The material (0.2 g) was ground with liquid nitrogen with a pestle and mortar. The fine powder was transferred to a 2-mL Eppendorf microtube along with 600 µL extraction buffer. At this step, 20 µL β-mercaptoethanol, 3% polyvinyl pyrrolidone and 4 µL 10 mg/mL RNase were added and the tube vortexed. The mixture was kept in a water bath at 65ºC for 20 min. The mixture was then taken from water bath after incubation, and an equal volume of chloroform:isoamyl alcohol (24:1) was added. The mixture was mixed for 20 min and afterwards centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was transferred to another 1.5-mL tube along with 2/3 volume ice-cold isopropanol. The mixture was kept at -20°C for 30 min. Next, the mixture was centrifuged at 8000 rpm for 10 min at 4°C. The pellet was washed with 80% alcohol, dried at room temperature, and then dissolved in TE buffer. The isolated DNA was directly used for the amplification of the nrDNA-ITS sequence.

PCR

The ITS primer (S1/S4) was used for the amplification of the nrDNA-ITS locus. The reaction was carried out in a Techni thermal cycler (UK). First, denaturation was performed at 94°C for 4 min. This was followed by 40 cycles of denaturation at 94°C for 1 min, annealing

at 49°C for 1 min, and extension at 72°C for 1 min. Final extension was completed at 72°C for 5 min. The amplified PCR products were stored at 4°C. The size of the amplicon was checked by 1.2% agarose gel electrophoresis and comparison with a DNA ladder.

Sequencing and data analysis

Prior to sequencing the PCR products, they were purified with the SolGent PCR Purification Kit-Ultra (SolGent, Daejeon, South Korea). Each sequencing reaction was performed with the BigDye Terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems) by following manufacturer instructions, using an ABI PRISM 3730XL DNA Analyzer (Perkin-Elmer, Applied Biosystems). Each sample was sequenced in the sense and antisense directions and analyzed with the ABI Sequence Navigator software (Perkin-Elmer/Applied Biosystems). The more highly similar sequences were retrieved from the GenBank database (NCBI) for the evaluation of the phylogenetic relationship of these rare plant species. The phylogenetic tree was constructed from an aligned data matrix of combined ITS1-5.8S-ITS2 using MEGA version 5 (Tamura et al., 2011). Based on species availability at GenBank database, some taxa were selected for the evaluation of their phylogenetic relationship. Five rare species were used for the evaluation of their phylogenetic relationship with the other species of the same genus retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/). Sequence alignment was performed using the ClustalX program (Thompson et al., 1997) with default settings. The boundary of each sequence was determined by comparing them with earlier sequences available in the GenBank database. All generated sequences in the present study have been deposited at GenBank database (accession Nos. KC311151, KC311152, KC311153, KC311154, and KC311155).

RESULTS AND DISCUSSION

nrDNA-ITS has been used for the identification and authentication of plant species (Al-Qurainy et al., 2011). Besides, this locus has also been used for the evaluation of the phylogenetic relationship between many species. We studied some rare plant species of Saudi Arabia, which are faced with harsh conditions such as high temperature, salinity, heavy metal stress, high and low altitude, etc. We used the nrDNA-ITS locus as a genetic marker for the evaluation of the phylogenetic relationship with the other species of the same genus. All retrieved sequences were sorted for ITS1-5.8S-ITS2. All five species showed their respective genus/species when performing BLAST at the NCBI database (http://www.ncbi.nlm.nih.gov/). We studied the phylogenetic relationship for each rare species with other species with the same genus as shown in the phylogram (Figures 1, 2, 3, 4, and 5). The outgroups selected for *H. strigosum* (KC311152), *P. tortuosum* (KC311153), *S. multiflorus* (KC311154), *C. myrrha* (KC311151), and *S. hadiensis* (KC311155) were *Cerinthe major* (FJ763244) and *Glandora diffusa* (FJ763246), *Zephyranthes cearensis* (AF223507) and *Rhodophiala araucana* (JX464308), *Angkalanthus oligophylla* (EU087478) and *Chorisochora transvaalensis (*EU087474), *Santiria griffithii (*JF919031) and *Beiselia mexicana* (JF919030), *Hieracium aurantiacum* and *Pilosella serbica*, respectively. All positions containing gaps and missing data were eliminated from the dataset (Liu and Sun, 2008; Liu and Chen, 2010; Liu et al., 2005, 2008, 2012; Zeng et al., 2012; Yuan et al., 2012). There were a total of 592 positions (10 sequences), 630 (8 sequences), 503 (7 sequences), 481

(14 sequences), and 481 (29 sequences) in the final dataset when *H. strigosum*, *P. tortuosum*, *S. multiflorus*, *C. myrrha*, and *S. hadiensis* were respectively analyzed with the other sequences retrieved from GenBank database. Some taxa showed a close phylogenetic relationship, where *H. strigosum* claded with *H. pilosum* and *P. tortuosum* claded with *P. tenuifolium* as shown in the phylogenetic tree (Figures 1 and 2, respectively). However, some rare species showed their phylogenetic relationship with more than one taxon as shown in Figures 3, 4 and 5. Thus, nrDNA-ITS, is a potential marker for the phylogenetic study as it has been used in many taxa such as Silene section *Melandrium* (Rautenberg et al., 2010), *Crocus* (Harpke et al., 2012), *Ficus* (Kusumi et al., 2012), etc. The cladding of these taxa depended on the sequence similarity to those sequences retrieved from the GenBank database. However, the rare plant species have a small genetic base, which depends on the population sizes of that species. Some rare species maintain a large population size, but the majority occur in small-size populations, often with decreasing numbers, which has been supported by several reviews of large numbers of isozyme studies (Gitzendanner and Soltis, 2000).

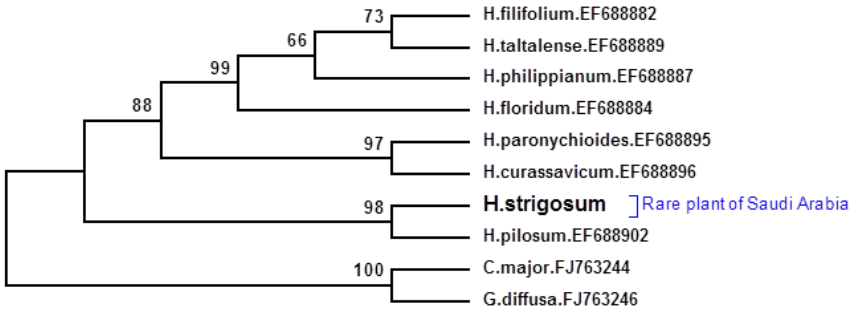

Figure 1. Gene tree of the ITS1-5.8S-IT2 sequences for *Heliotropium strigosum*.

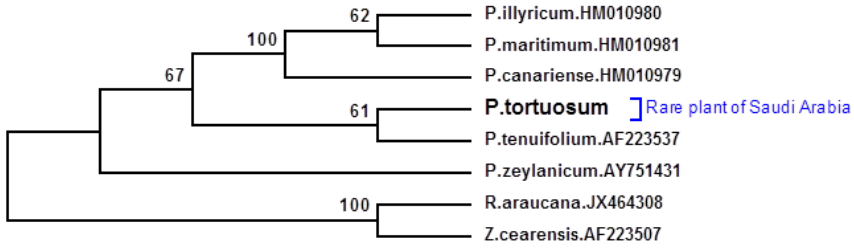

Figure 2. Gene tree of the ITS1-5.8S-IT2 sequences for *Pancratium tortuosum*.

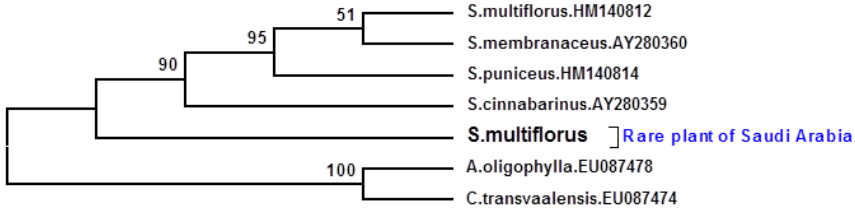

Figure 3. Gene tree of the ITS1-5.8S-IT2 sequences for *Scadoxus multiflorus*.

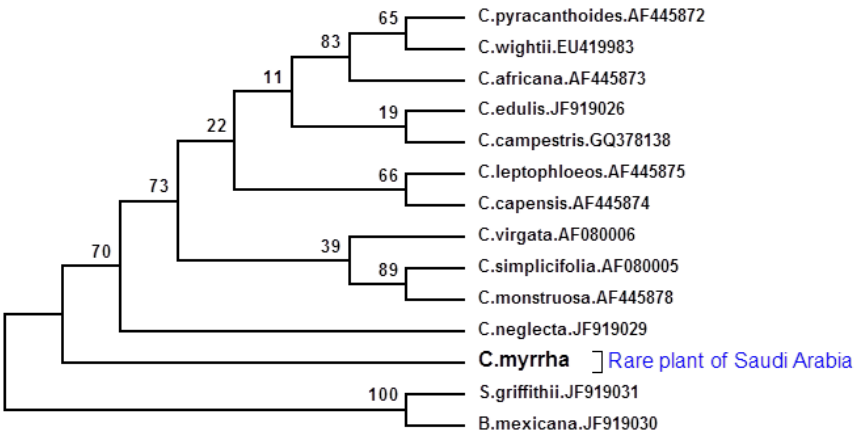

Figure 4. Gene tree of the ITS1-5.8S-IT2 sequences for *Commiphora myrrha*.

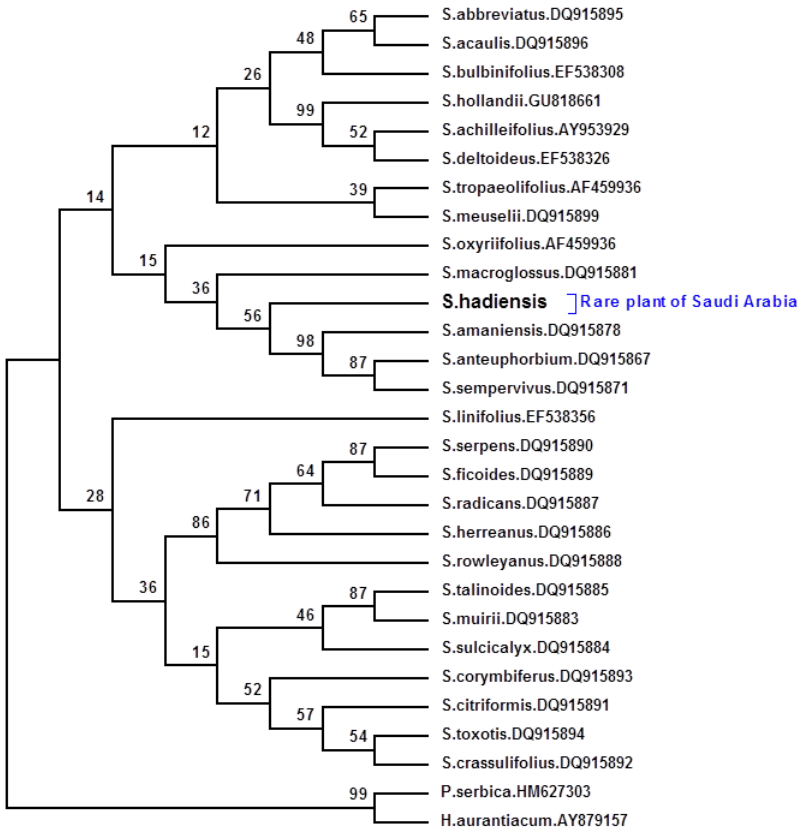

Figure 5. Gene tree of the ITS1-5.8S-IT2 sequences for *Senecio hadiensis*.

CONCLUSION

Based on a BLAST search and phylogenetic study using nrDNA-ITS sequences, we conclude that the taxa studied are close to their various species of the same genus. In addition, the possible populations will be collected from the different geographical regions of Saudi Arabia for identification of genetic diversity so that their genetic base could be improved by using various biotechnological approaches, because once these plant species disappear from the natural habitat, they cannot be re-grown in the same habitat.

ACKNOWLEDGMENTS

Research supported by the Deanship of Scientific Research at King Saud University (Research group project #RGP-VPP-014).

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