Journal of Genetic Engineering and Biotechnology xxx (2017) xxx–xxx

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Contents lists available at ScienceDirect

Journal of Genetic Engineering and Biotechnology

journal homepage: www.elsevier.com/locate/jgeb

Original Article

Evaluation of genetic diversity in wild populations of *Peganum harmala* L., a medicinal plant

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ARTICLE INFO

Article history:
Received 26 August 2017
Received in revised form 11 November 2017
Accepted 20 November 2017
Available online xxxx

Keywords:
Peganum harmala
Genetic diversity
ISSR
rDNA-ITS
SSR

ABSTRACT

Peganum harmala L. is a perennial herbaceous plant and can be a future drug due to its wide medicinal purposes. Despite its economic importance, the molecular genetics of *P. harmal* have not yet been studied in detail. Genetic diversity of 12 *P. harmala* genotypes were investigated by using Inter-Simple Sequence Repeats (ISSR), PCR-RFLP of rDNA-ITS, PCR-SSCP of rDNA-ITS and Simple Sequence Repeat (SSR) markers. The level of polymorphism revealed by ITS-SSCP is the lowest, followed by ITS-RFLP then ISSR and the highest polymorphism level was reported for SSR marker. The AMOVA analysis implied that most of the variation occurred within the Populations. A value of inbreeding coefficient F_{is} estimated by the three co-dominant markers was nearly equal and offer an indication of the partial out-crossing reproductive system of *P. harmala.* Principal Coordinate Analysis (PCOA) plot revealed a clear pattern of clustering based on the locations of collected plants which coincide with the isolation by distance. The study revealed that ITS-SSCP and ISSR markers respectively were more informative than the other used markers in the assessment of genetic diversity of *P. harmala.* The results reflect the great diversity of *P. harmala* and data obtained from this study can be used for future collecting missions.

1. Introduction

Peganum harmala L. (Peganaceae) is a perennial herbaceous plant and it has long been used for medicinal purposes as herbicide due to the presence of harmine [19]. It is one of the most frequently used medicinal plants for the relief of pain and as an antiseptic agent to treat hypertension, cardiac disease, some nervous system disorders such as Parkinson's disease, Lumbago asthma, colic, jaundice and as a stimulant emmenagogue [1,5,15,33].

P. harmala is propagated by seeds, which have a very short span viability [28]. Because of increasing exploitation of *P. harmala* natural populations, there is a need to conserve the genetic stock and facing the problem of extinction [13,28]. Despite, the medicinal uses of this species little is known about the breeding system, population genetic structure. Thus, the assessment and maintenance of its genetic diversity is prerequisite. Molecular techniques can play a role in uncovering the history, estimating the diversity, distinctiveness and population structure, and understanding of the distribution and extent of genetic variation within and between species [32]. So far there are no reports of using molecular markers for characterization of accessions of *P. harmala.*

In the present study, ISSR, ITS-RFLP, ITS-SSCP and SSR markers were used to assess the genetic diversity in *P. harmala.* Inter Simple Sequence Repeats (ISSR) markers have proved their effectiveness for population genetic studies and for studying taxonomic relationships at or below the species level [7,14,16].

The Internal Transcribed Spacer (ITS) of the nuclear ribosomal DNA region is a large segment consisting of 18S rDNA, 5.8S rDNA and 26S rDNA, congregated together with internal transcribed spacers, ITS1 and ITS2, between genes. ITS-rDNA region has been extensively used to determine genetic diversity and to classify several plants species because they are highly variable [2,24,27]. In this respect, two techniques are used to detect polymorphism in ribosomal DNA region; PCR-RFLP and PCR-SSCP. The PCR-RFLP technique detects polymorphisms in DNA regions, which have been amplified by specific oligonucleotide primers and restricted with different endonucleases. ITS-RFLP is a potent tool for the taxonomic study, population heterogeneity and the identification and monitoring of specific accession [18,24]. Single strand

Peer review under responsibility of National Research Center, Egypt.
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https://doi.org/10.1016/j.jgeb.2017.11.007
1687-157X/

conformation polymorphism (SSCP) detects single-base sequence changes by forming a different banding pattern dependent on its size and structure on a non-denaturing polyacrylamide gel [10].

Microsatellites (SSRs) are highly abundant, co-dominant inheritance, and have enormous extent of allelic diversity depending on the number of motif repetitions [34]. They have high potential for use in genetic diversity and evolutionary studies and determine self-pollination easily and accurately [23,30,39].

The ribosomal markers are small-sized molecules and have a low level of recombination compared with genomic markers. Using several marker techniques increase the sensitivity and resolution of detection genetic distinctiveness and revealing genetic variation through increased genome coverage. The present study aimed to compare the effectiveness of ISSR, ITS PCR-RFLP, ITS PCR-SSCP and SSRs markers, in assessing and analyzing the nature and the extent of genetic diversity of wild *P. harmala* populations collected from a narrow geographical region.

2. Materials and methods

2.1. Plant materials

A total of 12 accessions of *A. halimus* were collected from four populations. Three populations (1, 2 and 3) grow naturally in the Western Mediterranean desert of Egypt and population 4 grow inland at El-Katamya Plateau (Table 1). Samples were very scarce so three individuals were collected from each population. Genetic diversity of 12 *P. harmala* genotypes were investigated by using one dominant marker; Inter-Simple Sequence Repeats (ISSR), and three co-dominant markers; PCR-RFLP of rDNA-ITS, PCR-SSCP of rDNA-ITS and Simple Sequence Repeat (SSR) markers.

2.2. DNA extraction and ISSR-PCR amplifications

DNA was extracted from 100 mg of seeds using I-Genomic Plant DNA Extraction Mini Kit (INRTON), following the manufacturer's instructions. The genomic DNA was used as a template in PCR amplification using standard ISSR-PCR protocol, with 25 μL sample volumes consisting of: 50 ng of genomic DNA, 5 μL of 5× amplification buffer; 0.5 μL of 25 mM MgCl; 2 μL of 10 mM of each dNTP; 6 μL 10 pmol of ISSR primer 0.1 μL Taq (Promega, USA), the volume was completed up to 25 ul with sterile distilled water. Three anchored inter-SSR primers were used (Table 2). The thermal cycling for the inter-SSR primers was as follows: denaturation at 95 °C for 2 min; 40 cycles at 95 °C for 15 s, 30 °C for 15 s, 72 °C for 1 min, a final elongation step of 5 min at 72 °C. The amplified products were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide and photographed under UV illumination.

Table 1
Location of the collection sites of *P. harmala* populations and their respective geographic coordinates in Egypt.

Populations	Site	No	Longitude (N) Latitude (E)
Population 1	Alexandria, Borg Al-arab, Egypt	3	30°54′33.50″ 29°31′2.70″
Population 2	Alexandria, Borg Al-arab, Egypt	3	30°54′7.50″ 29°32′42.20″
Population3	Alexandria, Omayed, Egypt	3	30°47′3.31″ 29°12′9.30″
Population4	Cairo, Al-Kattamya mountain, Egypt	3	29°58′22.90″ 31°48′36.20″

No = Sample size.

2.3. Amplification ITS1-5.8S-ITS2 rDNA gene

The internal transcribed spacer ITS1 and ITS2 regions and the 5.8S ribosomal DNA (rDNA) regions were amplified by using universal primers ITS1 (5′-TCCGTA GGTGAACCTTGCGG-3′) and ITS4 (5′-TCCTCC GCTTATTGATATGC-3′) [36]. Amplifications were performed in 25 μL volumes containing 50 ng of template DNA, 12.5 μl of PCR master mix buffer (2×) (Bioline, Germeny), and 10 pmol for each primer. PCR cycles were as follows: initial denaturation for 3 min at 95 °C followed by 30 cycles of 1 min at 95 °C, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and 5 min at 72 °C. PCR products were then separated electrophoretically on agarose gel using 2% (w/v) agarose in 0.5 × TBE buffer [29]. The gel was stained with ethidium bromide.

2.4. ITS PCR-RFLP amplification

The PCR product (approx. ≥850 bp) was digested using three restriction enzymes *Eco*RV, *Hae*III and *Alu*I (Table 2). Reactions contained 5 μl of amplified PCR product and 1 μl of restriction enzyme, 2.5 μl enzyme buffer, the volume was completed up to 20 ul with sterile distilled water. Digestions were incubated 45–60 min at 37 °C and stored at −20 °C until use.

2.5. ITS PCR-SSCP amplification

A 3 μl of the PCR product (approx. ≥850 bp) was added to 10 μl of denaturation solution (94% formamide, 0.05% xylene cyanol) and heated at 95 °C for 5 min then suddenly placed on ice. Samples were loaded on a nondenaturing polyacrylamide gel (GeneGel Excel 12, 5/24; Amersham Pharmacia Biotech). Electrophoresis was performed in a temperature-controlled electrophoresis system (GenePhor; Amersham Pharmacia Biotech) at 6 °C with a first run at 600 V, 25 mA, and 15 W for 10 min and then at 600 V, 37 mA, and 21 W for 3 h. Gel was stained with ethidium bromide and photographed under UV illumination.

2.6. SSR-PCR amplification

Amplification of DNA used standard Polymerase Chain Reaction (PCR) protocols, with 25 μL sample volumes consisting of: approximately 50 ng of genomic DNA, 5 μL of 5× amplification buffer; 1.5 μL of 25 mM MgCl; 2 μL of 10 mM of each dNTP; 0.5 U Taq (Promega, USA); and 1 μL 10 pmol of primer. Four SSR primers were selected based on microsatellite polymorphism according to HAN & AN [11] (Table 2). The thermal cycling SSR primers was as follows: 30 cycles at 95 °C for 2 min, 53 °C for 1 min, 72 °C for 1 min, a final elongation step of 10 min at 72 °C. The amplified products were separated on 2% agarose gel, stained with ethidium bromide and photographed under UV illumination.

2.7. Statistical analysis

Alleles were sized using TotalLab version 1.11 software (Nonlinear Dynamics Ltd., Durham, USA) in the presence of a 1.5 kb DNA ladder. Bands were binary scored presence (1) or absence (0) characters to assemble the matrix of the ISSR phenotypes and the number of total bands (TB), the number of polymorphic bands (PB) and the percentage of polymorphic bands (PB%) were calculated. Two parameters: polymorphic information content (PIC) and marker index (MI) were calculated to measure the performance of the used markers according to Roldan-Ruiz et al. [25] and Varshney et al. [35].

The parameters of genetic diversity were calculated using POPGENE 3.2 software [38]. The observed heterozygosity (H_o) is available for co-dominant markers; ITS-RFLP, ITS-SSCP and SSR.

Please cite this article in press as: EL-Bakatoushi R, Ahmed DGA. Evaluation of genetic diversity in wild populations of *Peganum harmala* L., a medicinal plant-Peganum harmala L. –>. Journal of Genetic Engineering and Biotechnology (2017), https://doi.org/10.1016/j.jgeb.2017.11.007