Industrial Crops and Products 55 (2014) 75–82

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Correlation and functional differentiation between different markers to study the genetic diversity analysis in medicinally important plant *Plumbago zeylanica*

Raja Feroz Ahmad Haji[a], Mili Bhargava[b], Bashir A. Akhoon[c], Amandeep Kumar[d], Narshima B. Brindavanam[d], Vijeshwar Verma[a,*]

[a] Department of Biotechnology, Shri Mata Vaishno Devi University, Katra, Jammu and Kashmir 182320, India
[b] Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow 226015, India
[c] Department of Bioinformatics, System Toxicology Group, CSIR-Indian Institute of Toxicology Research, Lucknow 226001, India
[d] Dabur Research & Development Centre, Dabur India Ltd., Ghaziabad 201010, India

ARTICLE INFO

Article history:
Received 30 September 2013
Received in revised form 23 January 2014
Accepted 27 January 2014

Keywords:
Plumbago zeylanica
Genetic diversity
Phylogenetic analysis
SSR
SRAP
ITS

ABSTRACT

The plant species *Plumbago zeylanica* (*P. zeylanica*) is a multipurpose medicinal herb of family Plumbaginaceae. This plant is a natural gift to mankind credited with potential medicinal properties such as anti-cancer, anti-atherogenic, cardiotonic, hepatoprotective, anti-fungal, diabetes and neuroprotective assets to list of few. In this study, genetic diversity and relationships among various *Plumbago* accessions, collected from different geographical regions of India, was assessed using simple sequence repeat (SSR), sequence related amplified polymorphism (SRAP) and internal transcribed spacer (ITS) markers. SSR and SRAP markers showed highest values of Nei's genetic diversity and Shannon information index among populations. We also observed statistically significant genetic differentiations among and within populations ($P < 0.01$ in the AMOVA tests). Additionally, both Un-weighted paired group method with arithmetic average (UPGMA) and principal coordinate analysis (PCoA) grouped *P. zeylanica* populations into similar clusters which corroborate the above analysis to be useful for genetic diversity analysis of this plant. Our data signifies that SSR and SRAP are both reliable and effective tools for analyzing genetic diversity in *P. zeylanica*. However, based on our chosen dataset, we find ITS marker less significant for the genetic diversity analysis of this multifaceted plant. This information would be useful towards the identification, characterization and conservation of this species at the molecular level.

1. Introduction

Plumbago zeylanica is a perennial flowering plant (commonly known as white chitarak) that belongs to the family Plumbaginaceae. It is a fast growing, much branched evergreen shrub that reaches about 2 m and is native to warm temperate-tropical regions of the world. The plant is supposed to be originated in South-East Asia (Inamdar and Patel, 1970) and grows wild in India and Sri Lanka (Kirtikar et al., 1993). In India, the plant grows commonly wild or in cultivation due to its more therapeutic uses (Chetty et al., 2006) and is widely distributed from Central India to West Bengal, Maharashtra and various parts of Southern India. The plant has multidimensional uses and exhibit varying degrees of therapeutic values in the treatment of viral, fungal, malaria and bacterial pathogens. The plant also shows various other medicinal activities like anti-inflammatory, antioxidant, anti-invasive,

Abbreviations: AFLP:, Amplified Fragment Length Polymorphism; AMOVA:, Analysis of molecular variance; ARE:, Antioxidant responsive element; CTAB:, Cetyl trimethylammonium bromide; dNTPs:, Deoxynucleotide Triphosphates; EDTA:, Ethylenediaminetetraacetic acid; HGT:, Horizontal gene transfer; ISSR:, Inter-Simple Sequence Repeat; ITS:, Internal Transcribed Spacer; mTOR:, Mammalian target of rapamycin; NCBI:, National center for biotechnology information; NEB:, New England Biolabs; NRF:, Nuclear factor erythroid; PCoA:, Principal coordinate analysis; PCR:, Polymerase chain reaction; POP:, Population; PVP:, Polyvinylpyrrolidone; SAHN:, Sequential Agglome rative Hierarchical Nested; SIMQUAL:, Similarity for Qualitative Data; SRAP:, Sequence-related amplified polymorphism; SSR:, Simple sequence repeat; TE:, Tris-EDTA; UPGMA:, Un-weighted paired group method with arithmetic average; VEGFR:, Vascular endothelial growth factor receptor.

* Corresponding author. Tel.: +91 1991 285692.
E-mail addresses: nviq@yahoo.com, v.verma@smvdu.ac.in (V. Verma).

0926-6690/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.indcrop.2014.01.045

hepatoprotective, hyperlipidaemic and antiarthritic property including cancer (Mandavkar and Jalalpure, 2011) through a variety of mechanisms, including induction of reactive oxygen species (Wang et al., 2008), suppression of nuclear factor-kappaB (NF-κB) (Sandur et al., 2006), AKT/mTOR (Kuo et al., 2006) and Stat3 (Sandur et al., 2010); induction of p53 and c-Jun N-terminal kinase (Hsu et al., 2006) activation of the NRF2–ARE pathway (Son et al., 2010), inhibition of histone acetyltransferase p300 (Ravindra et al., 2009) and VEGFR2-mediated Ras signaling pathway (Lai et al., 2012). The roots of this plant have been used as an anti-atherogenic, cardiotonic, hepatoprotective and neuroprotective agent in Indian medicine from last few decades (Sand et al., 2012) and nowadays researchers all over the world are paying more attention to its anti-cancer activities. The plant has been reported to prevent non-small cell lung cancer (Hsu et al., 2006), melanoma (Wang et al., 2008), breast and gastric cancers (Kuo et al., 2006; Manu et al., 2011).

Molecular genetic markers represent one of the most powerful tools to evaluate the influence of various factors on genetic diversity and population structure (Englbrecht et al., 2000; Whitehead et al., 2003; Liu et al., 2006). There are various marker systems available viz Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Inter-Simple Sequence Repeats (ISSR), SSR, SRAP, and a few others. Among them, SRAP has been verified to be a useful tool for population genetic studies (Li and Quiros, 2001; Ferriol et al., 2003; Esposito et al., 2007) and SSR molecular markers are also often used to evaluate genetic variation within and between populations as they are highly polymorphic and may identify individuals with a unique marker fingerprint. Such molecular markers can be used to study genetic relationships within and among populations of *P. zeylanica*, thereby improving our understanding of the genetic variability in this multipurpose plant.

Another upcoming technique for assessing the genetic diversity employs molecular markers known as 'DNA barcodes'. DNA barcoding (Hebert et al., 2003) is a technique which is useful for discriminating morphologically similar organisms, thus giving an approximation of genetic diversity.

In this study, SSR, SRAP and Internal Transcribed Spacer (ITS) markers were employed to investigate the inter- and intra-populational genic variations among *P. zeylanica* accessions gathered from different geographic locations of India.

2. Materials and methods

2.1. Plant material

Twelve accessions of *P. zeylanica* chosen in this study were collected from different Indian geographic locations. Fours accessions (PZ-2, PZ-4, PZ-6 and PZ-7) were collected from Karnataka, two (PZ-8 and PZ-9) from Uttarakhand and one each from Coimbatore (PZ-1), Tamil Nadu (PZ-3), Mangalore (PZ-5), Gujarat (PZ-10), Chennai (PZ-11) and Jammu and Kashmir (PZ-12). All plants were maintained under *in vitro* and green house conditions.

2.2. DNA extraction

Genomic DNA was extracted from plantlets of each accession using Qiagen (GmbH) plant DNA extraction kit and CTAB method with some minor modifications. Approximately 0.5 g of leaves were crushed with liquid nitrogen using a mortar and pestle. The resultant powder was mixed with 500 ml of CTAB-based extraction buffer (2% Cetyltrimethylammonium bromide, 5 M NaCl, 100 mM Tris–HCl, pH 8, 50 mM EDTA, 1% PVP and 2% β-mercaptoethanol) and again crushed. The crushed outcome was poured into the 50 ml polypropylene tube where 20 μl (100 mg/ml) of proteinase and 10 μl (10 mg/ml) RNase was also added. The samples were then incubated in a water bath at 65 °C for 30 min. Following incubation period, samples were cooled to room temperature and 250 μl of 5 M potassium acetate was added. The incubation period was once again employed for 10 min on ice. The samples were then centrifuged at 17,000 g for 15 min and the supernatant was transferred to fresh tube. Subsequently 3 ml of chloroform: isoamyl alcohol (24:1) was added, mixed by inversion and centrifuged for 5 min at 17,000 g. The supernatant was transferred to a fresh tube and equal volume of ice-cold isopropanol was added and incubated on ice for 15 min, centrifuged for 5 min at 17,000 g to form a pellet. The pellet was washed twice, first in 80% ethanol and then in 70%. The pellet was then air dried and the final obtained DNA pellet was dissolved in 250 μl TE.

DNA quality was estimated by running 3 μl of the extraction product on a 0.8% agarose gel that was stained with ethidium bromide. Quantitative estimates of DNA samples were made by nanodrop method (Thermo Scientific). SSR primer sequences were chosen from the information available in the public domain of NCBI. The sequences and length of SSR primers were selected on the basis of their proper properties of their broad coverage of the genome and their high levels of polymorphism.

2.3. SSR amplification

The PCR amplification reactions were carried out in a 20 μl reaction consisting of 10 × PCR buffer (New England Biolabs), 2 mM dNTPs (New England Biolabs), 1 unit of Taq polymerase, 10 pmol of each primer and 30–40 ng of RNase H (NEB) treated DNA. The amplification conditions were 32 cycles at 94 °C for 5 min, 50 °C for 1 min, 72 °C for 1 min and a terminal extension step at 72 °C for 10 min. PCR was performed in a 36-well microtiter plate in Bio-rad thermal cycler. DNA samples were electrophoresed on 1.8% (w/v) agarose at a constant voltage of 65 V for 4 h and were visualized in a Bio-rad gel doc system.

2.4. SRAP assays

SRAP primer sequences were taken according to Li and Quiros (2001). The analysis was performed using the same reaction conditions that were chosen for SSR analysis. However, the amplification conditions were made according to Li and Quiros (2001).

2.5. Data analysis

DNA banding patterns generated by the markers were scored for the presence (1) or for absence (0) of each amplified bands. Some SSR and SRAP assays were repeated twice and only the reproducible bands were scored. Since both the markers used in this study were scored for presence/absence, the binary data set option was used in all the analyses. Data matrices were analyzed using POPGENE version 1.32 (Yeh et al., 1997) with the assumption that the populations were in Hardy–Weinberg equilibrium. The following parameters were determined: percentage of polymorphic loci (PPL), number of alleles per locus (na), effective number of alleles per locus (ne), genetic diversity (HE = expected heterozygocity), Shannon's index diversity (I) and genetic differentiation among populations (GST). AMOVA was performed using GenAlEx 6.41 (Peakall and Smouse, 2006) to examine the hierarchical genetic variation across the chosen accessions and the genetic