Journal of Applied Research on Medicinal and Aromatic Plants 3 (2016) 112–119

Contents lists available at ScienceDirect

Journal of Applied Research on Medicinal and Aromatic Plants

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

Genetic variation in the endangered Indian sweet flag (*Acorus calamus* L.) estimated using ISSR and RAPD markers

Avani Kasture[a,*], Ramar Krishnamurthy[b], Katagi Rajkumar[c]

[a] C. G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Maliba Campus, Bardoli-Mahuva Road, Tarsadi Dist., Surat, Gujarat 394350, India
[b] C. G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Surat District, Bardoli 394 350, Gujarat, India
[c] Main Cotton Research Station, Navsari Agricultural University, Surat, Gujarat 395 007, India

ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 25 February 2016
Accepted 6 March 2016
Available online 25 March 2016

Keywords:
Acorus calamus
Genetic variation
Geographical locations
RAPD
ISSR

ABSTRACT

Sweet flag (*Acorus calamus* L.), is a critically endangered species of mountainous regions of India. In order to evaluate and preserve the endangered medicinally important plant Sweet flag. We investigated the variation by collecting the whole plant from different geographical regions. Genetic variability among 20 accessions of this species was assessed using random amplified polymorphic DNA (RAPD) (25 primers) and inter simple sequence repeat (ISSR) markers (17 primers).The results showed 33.7% of bands formed by RAPD markers and 63.7% for ISSR were polymorphic. The Shannon's indices (I) and Nei's genetic diversity (h) among all the accessions were estimated for RAPD and ISSR respectively at 0.58 (SD = 0.07), 0.57 (SD = 0.13) and 0.40 (SD = 0.06), 0.33 (SD = 0.11) respectively. The similarity coefficient ranged from 0.72 to 0.94. The results revealed that genetic variation is much low among accessions. Since genetic variation within collected accessions is homogenous, the pattern of low genetic diversity within the accessions specifies that they are monoclonal. Therefore, we proposed that studies of intraspecific variation could be utilized in the development of conservation strategies, by identifying appropriate units of *A. calamus* for conservation.

1. Introduction

The main objective in nature conservation is to the reservation as much as possible of the evolutionary potential of species through preserving as much genetic diversity as possible (Gaudeul et al., 2000).

Sweet flag (*Acorus calamus* L.) is an economically and medicinally important non-endemic, semi-aquatic medicinal herb found in temperate and subtropical wetlands (Abdul Kareem et al., 2012). The plant is generally distributed in temperate countries like North America, Canada and Europe. In India, *A. calamus* are found throughout, predominantly in Himalayan and sub-Himalayan regions (Rana et al., 2013). It occurs in the marshes lands of the mountainous regions of India at an altitude above 2000. It is cultivated widely in the states of Himachal Pradesh, Manipur, Uttarakhand, Jammu Kashmir, Nagaland, Uttar Pradesh, Tamil Nadu, Andhra Pradesh, Maharashtra, and Karnataka (Ogra et al., 2009). *A. calamus* plants display incredible variations in the chemical constitution of the essential oil and chromosome numbers. The chromosome number of *A.calamus* is $n = 12$ and there are generally four natural cytotypes *viz.*, diploid ($2n = 2x = 24$), triploid ($2n = 3x = 36$), tetraploid ($2n = 4x = 48$) and hexaploid ($2n = 6x = 72$) (Marchant, 1973). Thus, *A. calamus* has geographical outline of distribution with respect to ploidy levels. The plants found in North America are generally diploid, whereas those originate in Europe and temperate Asia are primarily triploid, and plants that arise in eastern and tropical Asia are mostly tetraploids (Rana et al., 2013). Most of the *A. calamus* found in the Indian subcontinent are mainly triploids with high β-asarone contents (Ogra et al., 2009). However, tetraploids and hexaploids are also reported from India (Ahlawat et al., 2010). The different parts of *A. calamus* like rhizome, roots and leaves have been used traditionally from ancient times for the treatment of various ailments and treatment of various disease such as of cough, fever, bronchitis, inflammation, depression, tumors, hemorrhoids, skin diseases, numbness, general debility and as antidotes for several poisons (Balakumbahan et al., 2010). However, due to the awareness on its many medicinal values and other benefits in the recent years, *A. calamus* has been extensively exploited from its native places and forest (Singh, 2013). Hence, the wild population levels are rapidly decreasing due to the indiscriminate collection

* Corresponding author.
E-mail addresses: avani.kasture@utu.ac.in, edlabadkar.avani@gmail.com (A. Kasture), krishnamurthy@utu.ac.in (R. Krishnamurthy), bkrajsan11@gmail.com (K. Rajkumar).

http://dx.doi.org/10.1016/j.jarmap.2016.03.001
2214-7861/

and use, which may probably lead to species extinction in the nearest future. Consequently, there is the need for an urgent action plan for plant conservation and sustainable use in order to stem the trend. Conventionally, identification and characterization of medicinal plants was mostly based on morphological, anatomical and chemical analysis but these could be influenced by environmental issues. More efforts are required to create the genetic framework and varietal relationships of Sweet flag (*A. calamus* L.) including its wild variety. Among the various molecular markers utilized to assess the genetic variability in plants, PCR—based molecular markers such as RAPD (Ahlawat et al., 2010; Priyanka and Gohar, 2012; Radhika et al., 2012) and ISSR are the most common, as their submission does not need any prior sequence information (Abdul Kareem et al., 2012; Rana et al., 2013). However, AFLP have been applied widely to identify various accessions, landraces, as well as population diversity and relatedness (Lee and Han, 2014). Among previous studies, only limited reports have described estimation of the genetic diversity of *A. calamus* using numerous markers. More elaborate research is required for the conservation of this endanger species (Bhagat, 2011).

The main goal of this work was to construct a molecular database using RAPD and ISSR markers for *A. calamus*, to obtain specific molecular markers for individual identification and to study their genetic variability and germplasm conservation.

2. Material and methods

2.1. Experimental material

Twenty accessions of various *A. calamus* whole plants were collected manually from different locations of East, South, West and North Eastern parts of India, representing four biogeographic zones *viz*., Western Himalaya, Central India, North East India and Eastern Ghats (Table 1). Six samples from Uttarakhand, five from Maharashtra, two from Karnataka, one sample each from Himachal Pradesh, Jammu, Odisha, Madhya Pradesh, Punjab and Chhattisgarh, were collected and deposited in the C. G. Bhakta Institute of Biotechnology. The accessions of *A. calamus* collected were conserved through clonal propagation from single mother plant for each accession.

2.2. Morphological characterization

Morphological characterization was carried out on 10 randomly chosen clonally propagated from single mother plant for each accession for characters such as leaf length, leaf width, total number of leaves per plant and total numbers of tiller per plant, and rhizomes characters such as length, width, distance between nodes, fresh and dry weight among others were studied to compare the morphological similarity of accessions.

2.3. DNA extraction

Fresh *Acorus* leaf sample was collected from one representative plant (out of 10) sample whose morphological characterization was already doneand then used for the DNA extraction. One gram of leaf tissue was frozen with liquid nitrogen and grounded into a fine powder and then total genomic DNA from individual accessions were isolated using DNeasy Plant Mini Kit (QIAGEN, USA), according to the manufacturer's instructions. The precipitated DNA was dissolved in 50 μl of elution buffer. The quality and quantity of the DNA were checked using a spectrophotometer and agarose gel (0.8%) electrophoresis, respectively. The absorbance ratio of DNA sample between 260 and 280 nm was recorded and the quality of the genomic DNA was confirmed. The purified DNA sample was stored at 4 °C for further analysis.

2.4. PCR amplification

RAPD amplification was performed as described by Sharma et al. (2008) using 25 decamer random primers (Bangalore GeNei, India, GeNei™). PCR was carried out in a volume of 25 μl containing 1x reaction buffer with 2.0 mM $MgCl_2$, 10 pM primer, 200 μM each of deoxynucleotides (dNTPs), 0.9 unit of *Taq* polymerase(Bangalore GeNei, India, GeNei™), and 50 ng of genomic DNA. Finally the total reaction mixture volume was made up to 25 μl the reaction tubes were placed in an Eppendorf Mastercycler gradient thermal cycler (USA) and the PCR mixture was subjected to initial denaturation at 94 °C for 5 min. The reaction was subjected to the 35 cycles of denaturation at 94 °C for 1 s, annealing at 35 °C for 1 s and extension at 72 °C for 1 min with a final extension at 72 °C for 10 min.

ISSR amplification was performed as described by Abdul Kareem et al. (2012) by using di- and tri- nucleotide repeats ISSR primers(Bangalore GeNei, India, GeNei™). Polymerase chain reaction (PCR) was carried out in a volume of 25 μl containing 1X Reaction buffer with 2.0 mM MgCl2, 10 pM primer, 200 μM each of deoxynucleotides (dNTPs), 0.9 unit of *Taq* polymerase (Bangalore GeNei, India, GeNei™), and 50 ng of genomic DNA. The PCR mixture was subjected to initial denaturation at 94 °C for 4 min. The reaction was subjected to the 35 cycles of denaturation at 94 °C for 1 s, annealing at 50–60 °C for 1 s and extension at 72 °C for 2 min with a final extension at 72 °C for 7 min.

After the completion of PCR amplification, amplified products along with external size standard were separated in a horizontal gel electrophoresis unit using 1.5% agarose gel. The banding patterns were visualized under UV light and photographed using a Gel Documentation System (Bio-Rad,USA). The analysis was performed for all the samples at least thrice with each selected primer. Twenty-five primers of RAPD and seventeen primers of ISSR were selected for analysis based on their ability to detect diverse, clearly resolved and polymorphic amplified products of the collected accessions of *A. calamus*. All RAPD and ISSR reactions were carried out with the same cycling circumstances and chemicals. Fragment sizes of the amplification products obtained using RAPD and ISSR primers were anticipated from the gel by comparison with standard molecular weight marker ladder – low range DNA Ruler Plus (3000 bp–100 bp) (Bangalore GeNei, India).

2.5. Data analysis

The amplified products were scored across the lanes comparing their respective molecular weights. Each band was treated as one marker. Scoring of bands was done from gel photographs. Homology of bands was based on a distance of migration in the gel. Each amplification fragment was named by the source of the primer. Kit letter or number, and its approximate size in base pairs. The bands were scored as 1 for present or 0 for absent across the genotypes and only those bands which were well defined and consistently reproducible in three independent amplifications were included in the final analysis. All clear and intense bands were scored for the construction of the data matrix. The data were scored in an excel sheet and was converted manually in a text format for SAHN (sequential, agglomerative, hierarchical and nested clustering method) module of NTSYS-PC. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA). Dendograms were constructed using the UPGMA algorithms in the MEGA 4.0 software. The binary matrix was used to determine the genetic diversity, genetic differentiation and gene flow using the software POPGENE (Radhika et al., 2012) ver. 3.2. (Nei, 1973; Saitou and Nei, 1987) and the Shannon's index (I) were estimated for the accessions and genetic diversity (h) using corrected allele frequency. Gene flow