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Diversity Analysis of Arrowroot (*Maranta arundinacea* L.) Germplasm using ISSR Markers

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Abstract

Arrowroot (*Maranta arundinacea* L.) is an underutilized tuber crop having great potential to be developed as a carbohydrate source and functional food. Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. ISSR markers provide more sophisticated analysis of genetic diversity, cultivar identification, population genetic structure and other events of evolutionary biology processes. The present work is aimed at the molecular characterization of the seven arrowroot (*Maranta arundinacae* L.) accessions maintained at ICAR-Central Tuber Crops Research Institute field genebank using 12 ISSR primers and to estimate the extent of diversity using the similarity index. DNA isolation was done using DNeasy® Plant Mini Kit method. Out of the 13 ISSR markers screened, 12 markers gave polymorphic bands in the accessions screened. The 12 ISSR markers produced a total of 124 bands across the seven samples of which 66 were polymorphic. The phylogenetic tree generated using UPGMA cluster analysis revealed that diversity exists in *M. arundinacea* unlike previously reported. The accessions formed two major clusters with two outliers in the grouping. The Cluster 1 consisted of two accessions i.e. Acc. 1 (M1) and Acc. 6 (M6) while Cluster 2 consisted of 3 accessions viz. Acc. 2, 4 and 3 (M2, M4 and M3). The two outliers that got separated were accessions 5 and 7 (M5 and M7). No duplicates were identified in the present study. The results indicated that the primers selected for the present study will be useful for future genetic diversity studies and would provide breeders with a genetic base for selection of diverse parents for crop improvement programmes in arrowroot.

Key words: Arrowroot, ISSR, genetic diversity, polymorphism, cluster analysis, molecular characterization

Introduction

Maranta arundinacea L. known as West Indian arrowroot or arrowroot belonging to the family Marantaceae is an erect herbaceous perennial large herb having 60-80 cm height and with large white flowers arranged in twin clusters, which very rarely produce seeds. The stems are slender, finely hairy and tumid at the joints. The leaves are alternate with long leafy hairy lanceolate sheaths and slightly hairy underneath. The plant has a perennial fibrous starchy rhizome producing numerous fusiform fleshy, scaly tubers from its crown. The fleshy, white and cylindrical rhizome is covered with regular scale leaves and grows approximately 2.5 to 5.0 cm thick and 20-45 cm in height. It is used for the extraction of a very fine easily digestible starch known as the arrow root starch. Yields of rhizomes normally average about 12 to 31 t ha^{-1} and the normal commercial yield of starch is 8-16 %. The very fine, easily digestible starch from rhizomes is valued as a food product, particularly for infants and invalids and is used in biscuits, cakes and puddings. It is suitable for infants as a substitute for breast milk. The rhizomes are sometimes eaten, boiled or roasted.

Arrowroot is nutritive, and is used as an agreeable, non-irritating diet in certain chronic diseases, during convalescence from fevers, in irritations of the alimentary canal, pulmonary organs or of the urinary organs. Arrowroot starch possesses demulcent properties and is sometimes used in the treatment of disorders of the intestine. It may also be employed in the preparation of barium meals and in the manufacture of tablets where rapid disintegration is desirable. In the West Indies, the pounded rhizomes are used for poulticing wounds and ulcers. The leaves of the plant are used as local packing material while the fibrous material remaining after the extraction of starch can be used as cattle feed or manure. The starch is also used as a base for face powders, in the preparation of certain specialised glues and in the manufacture of carbonless paper for computers (Kay, 1987).

The crop is native to Mexico, Central America, the West Indies and South America. It is widely cultivated in many warm countries and is considered naturalized in countries like Jamaica, Bahamas, Bermuda, the Netherlands, India, Sri Lanka, China, Mauritius, Equatorial Guinea, Gabon, Florida, Cambodia, Indonesia and the Philippines. Arrowroot is indigenous to Tropical America and has long been cultivated in the West Indies, particularly St. Vincent, which produces about 95% of the world's commercial supply. The plant grows wild in some parts of India and is sporadically cultivated in Uttar Pradesh, Bihar, Orissa, West Bengal, Assam and Kerala. The name arrowroot is primarily due to the shape of the tuber and also due to the fact that the pulp from the fresh rhizome was used by the Indians in the Caribbean to treat wounds inflicted by poison arrows. *Maranta indica* Tussac, *M. nobilis* Royle. and *M. ramosissima* Wall. are the other species of *Maranta* producing starch. The improvement of *Maranta* as a cultivated species was started in West Indies with the development of improved varieties that exhibited smooth leaves. As there is no seed setting reported in the crop, the possibilities of the presence of natural hybrid and propagation of the hybrid varieties are practically absent. Difficulty in seed setting is a major setback in this crop for improvement programmes. Thus research needs to be conducted for evolving high yielding varieties in this crop using tissue culture or other breeding techniques (Mathew, 2007).

Arrowroot grows well under Indian conditions, especially Kerala, since it prefers a tropical climate. Arrowroot with the name Koova or Kochikuva in Malayalam is reported to be cultivated in the districts like Kottayam, Kasaragod, Kollam, Thiruvananthapuram, Malappuram, Kozhikkode and Wayanad of Kerala (Sasidharan, 2011). Existence of the three forms of peroxidises viz., SoPOD, IoPOD and CoPOD in the leaves of the arrowroot contributing to the disease and pest resistant nature of the plant was reported by Pradeepkumar et al. (2008).

A variety of DNA based molecular marker techniques has been developed in higher plants for assessing genetic diversity. The use of markers such as RAPD (Welsh and Mc Clelland, 1990; Williams et al., 1990), ISSR (Blair et al., 1999), RFLP, SSR, and AFLP (Vos et al., 1995) have been very popular, are technically simple and hence have been extensively used for genetic diversity assessment in many plants.

The aim of this study was to estimate the genetic diversity among seven accessions of arrowroot using ISSR markers and pair-wise distance (similarity) index and dendrogram generation based on UPGMA.

Materials and Methods

Source of plant materials

Leaf samples of seven accessions of arrowroot maintained in the field genebank of the ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram served as the source material for the present study (Fig.1; Table 1).

Isolation of DNA

Tender leaves of the seven arrowroot accessions were collected and 100 mg each was weighed out. Grounded the leaf tissue in liquid nitrogen using mortar and pestle. Further steps were followed as per the instructions in the DNeasy® Plant Mini Kit (Qiagen). Quality of DNA was

Table 1. Arrowroot accessions subjected for the study

Sl. No.	Sample Name	Identity Number	Place of collection
1	Acc. 1	M-1	Orissa
2	Acc. 2	M-2	Assam
3	Acc. 3	M-3	Tamil Nadu
4	Acc. 4	M-4	Bihar
5	Acc. 5	M-5	Maharashtra
6	Acc. 6	M-6	Madhya Pradesh
7	Acc. 7	M-7	Kerala

Fig.1. Field genebank of arrowroot at ICAR-CTCRI

checked in an agarose gel and then quantified by measuring the OD at 260 nm. OD at 280 nm was also recorded to check the purity of DNA. Purity of DNA sample was calculated from OD at 260/280 ratio.

Concentration of DNA was derived using the formula,

Concentration of DNA (µg/ml) = OD_{260} x dilution factor x 50

Dilution of DNA samples

All the DNA samples were uniformly diluted to 10 ng/µl irrespective of their variable concentrations calculated spectrophotometrically.

ISSR primer details

In the present work, a total of 13 ISSR primers were tested initially for analysis. Out of these 13 primers, 12 were used for further analysis as they gave distinct bands on agarose gel. The primers $(ACC)_6Y$, UBC 811, UBC 810, UBC 809, UBC 817, UBC 824, UBC 827, $(GA)_9AC$, UBC 818, UBC 836, UBC 825, UBC 808 and $(GA)_9AT$ were used (Table 3).

PCR amplification

The extracted DNA was amplified in a Thermal cycler (Bio Rad). The reaction mixture consisted of 2.0 µl of buffer, 0.2 µl dNTP mix (2.5 mM each), 1.0 µm of each primer, 1 U of Taq polymerase, and 20 ng of DNA. The total reaction volume was made up to 20 µl using sterile distilled water. PCR was carried out in a master cycler gradient with the following profile - An initial heating at 94 °C for 10 min followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C - 56 °C for 1 minute, extention at 72 °C for 1 minute followed by final extention at 72 °C for 10 minutes. The annealing temperature was standardized for each primer. A total of 13 primers were screened on DNA samples of arrow root and 12 primers were selected based on the number of polymorphic bands produced, band size, amplification intensity and reproducibility. The amplification products were separated by gel electrophoresis in 2 % agarose gel with 1 X TBE buffer stained with ethidium bromide (10 mg/ml) and was imaged using a gel documentation unit (Alpha Imager HP).

Data analysis

The bands obtained by electrophoresis were labelled as present (1) or absent (0). This binary data was scored and statistically analyzed using NTSYS-pc. Pair wise distance (similarity) matrix was computed using sequential hierarchical and nested (SAHN) clustering option of the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Biostatics, New York, USA, soft ware version 2.02 package) (Rohlf, 1998). The programme also generated a dendrogram, which grouped the accessions on the basis of Nei's Genetic distance using unweighted pair group method with arithmetic average (UPGMA) cluster analysis (Sneath and Sokal, 1973).

Results and Discussion

The ICAR-Central Tuber Crops Research Institute (ICAR-CTCRI) under the Indian Council of Agricultural Research (ICAR) is the only research organization in the world dedicated solely to the research on tropical tuber crops. The centre has been identified as the 'National repository for tropical tuber crops' and maintains a large collection of the variability on tropical tuber crops collected from different parts of India as well as the world. Genetic

resources form the backbone of any crop improvement programme.

Tubers are modified plant structures enlarged to store nutrients that are used by plants to survive the winter or dry months which provide energy and nutrients for re-growth during the next growing season, and also a means of asexual reproduction. In this study, seven accessions of arrowroot germplasm maintained at ICAR-Central Tuber Crops Research Institute, Sreekariyam, Thiruvananthapuram were subjected to genetic diversity analysis using ISSR markers. Absence of variation among the arrowroot accessions based on molecular characterisation using five RAPD primers was reported earlier (CTCRI, 2007). DNA was extracted from fresh leaves of the seven accessions of arrowroot using DNeasy ® kit method (Qiagen). Quantification of DNA was performed to determine the concentration, as well as its purity. The ratio of DNA absorbance at 260 nm and 280 nm (A 260/280) was used to assess the purity of DNA. For pure DNA, $A_{260/280}$ is 1.8 - 2.0. In the present study, the quality of the DNA for seven accessions of arrowroot tested by spectrophotometery and agarose gel electrophoresis (1 %) indicated that protein contamination was there, however, the primer amplification was good and hence was used for further studies.

To assess the quantity and quality of the entire genomic DNA, samples were run on 1 % agarose gel, stained with Ethidium bromide and the bands were visualised and documented using a gel documentation system (Alpha Imager). Good quality of DNA was observed.

ISSR analysis

Inter Simple Sequence Repeats (ISSR) are DNA fragments of about 100 – 3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSR are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors in to the no repeat adjacent regions (16 - 18 bp). About 10 - 60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored on the presence or absence of fragments of particular size. The main advantage of ISSR is that no sequence data for primer synthesis is needed. The analytical procedures included PCR which requires only low quantities of template DNA. Furthermore, ISSRs are randomly distributed throughout the genome. ISSR being a multilocus technique, disadvantages include the possible non-homology of similar sized fragments. Moreover, ISSRs, like RAPD can have reproducibility problems, because of the multilocus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clones and strain identification, as well as taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies.

Of the thirteen ISSR primers tested, 12 gave clear, reproducible bands and were used for further studies. All the 12 primers used in the study produced scorable bands. These primers were tested against seven accessions of arrowroot genotype and produced well defined products which were visualized in EtBr stained agarose gel (Fig.2). The details of amplified products by different ISSR primers are furnished in Table 3. The ISSR primers produced a total of 12 markers across 7 samples of arrowroot accessions all of which were polymorphic.

The quantity of DNA from different samples varied from 280 - 1205 ng/µl (Table 2). The variation was due to the quality of leaf sample taken for DNA extraction. From the calculated ratio of absorbance it was noticed that the sample contained good quantity of DNA. After quantification, all the samples were diluted to a final volume of 10 ng/µl and the same was used for further analysis.

Table 2. Concentration of DNA sample

Sample Name	OD 260	OD 280	260/280	Conc. (ng/µl)	Vol. of DNA for 10 ng/µl 100 µl stock	Vol. of SDW for 10 ng/µl 100 µl stock
Acc. 1	0.0056	0.0057	0.982	280	3.57	96.43
Acc. 2	0.0241	0.0236	1.021	1205	0.83	99.17
Acc. 3	0.011	0.0117	0.940	550	1.82	98.18
Acc. 4	0.0211	0.0192	1.099	1055	0.95	99.05
Acc. 5	0.0148	0.0152	0.974	740	1.35	98.65
Acc. 6	0.0236	0.0234	1.009	1180	0.85	99.15
Acc. 7	0.0125	0.0126	0.992	625	1.60	98.40

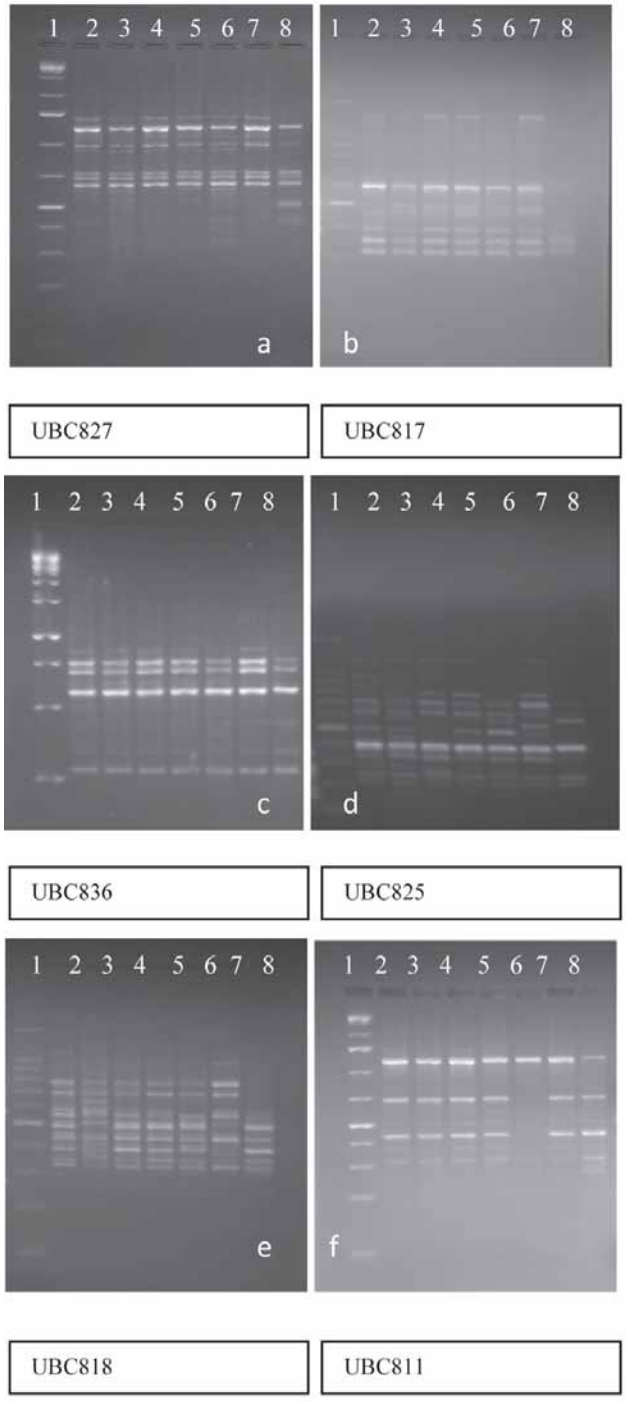

Fig. 2. (a-f): Representative figures showing amplification of 7 accessions of arrowroot (M1 to M7) using ISSR primers. Lane 1 - Marker [1kb plus ladder (Fermantas), figs. a,c,f; 100 bp ladder (Fermantas), figs. b,d,e]; lanes, 2-8 M1 - M7 in all the figures

Diversity analysis of the 7 accessions of arrowroot done using 12 ISSR markers produced an average of 10.33 bands of which 5.5 were polymorphic with 86.7 % polymorphism (Table 3). In the present study, high polymorphisms among the 7 accessions indicated a high degree of genetic variability between the lines of *Maranta arundinacea*. Polymorphism arise because sequence variations in the genome alter the primer binding sites. A similar result of high polymorphism suggesting intra species variability was suggested among 18 culti-varieties/ species of *Dioscorea* (Lay et al., 2001). Genetic diversity studies on 27 *Piper* species using ISSR markers and grouping them into six clusters was reported by Sheeja et al. (2013). Intervarietal and interspecific polymorphisms of RAPD data enabling reliable discrimination of Jamaican cultivars of *Dioscorea* were reported by Asemota et al. (1996). ISSR markers are mostly dominant markers and have higher reproducibility as compared to RAPD (Meyer et al., 1993) which involve PCR amplification of DNA using single primers composed of microsatellite sequences. These primers target microsatellites that are abundant throughout the eukaryotic genome (Tautz and Renz et al., 1984) and evolve rapidly. Even though both methods are PCR-based molecular markers, RAPD relies on the amplification of genomic DNA using short primers (10 nucleotides) with a random sequence, whereas ISSR is based on the amplification of regions flanked by repeating sequences (microsatellites or SSR), so the primers used contain those 2-6 nucleotides repeats with usually 2 varying nucleotides to the 3' end. RAPD markers are considered to be uniformly distributed along the genome, whereas ISSR are found only between microsatellite loci making them more reliable than RAPD.

Similarity index

The similarity index values obtained for each pair wise comparison among the 7 arrowroot accessions based on ISSR marker data is given in Table 4. The similarity coefficient based on ISSR markers ranged from 0.61 to 0.88. Among the 7 accessions, the lowest similarity indices (0.61 and 0.62) were observed among the accessions Acc. 4 and Acc. 7 as well as Acc. 1 and Acc. 7, respectively, whereas, the highest similarity index (0.88) was observed between accessions Acc. 2 and Acc. 4 as well as Acc. 3 and Acc. 4.

A dendrogram generated using UPGMA separated the 7 arrowroot accessions into two major clusters with two outliers (Fig. 3). The Cluster 1 consisted of 2 accessions (Acc. 1 and Acc. 6) with around 85% similarity, while Cluster 2 consisted of 3 accessions (Acc. 2, Acc. 4 and

Table 3. Number of amplified bands and polymorphism (%) per primer

Primer name	Sequence (5' -3')	Number of bands	Number of polymorphic bands	% polymorphism
$(ACC)_6Y$	ACCACCACCACCACCACCY	10	5	50.0
UBC 811	GAG AGA GAG AGA GAG AC	11	9	81.8
UBC 810	GAG AGA GAG AGA GAG AT	5	1	20.0
UBC 817	CAC ACA CAC ACA CAC AA	10	6	60.0
UBC 824	TCT CTC TCT CTC TCT CG	4	2	50.0
UBC 827	ACA CAC ACA CAC ACA CG	13	7	53.8
$(GA)_9AC$	GAGAGAGAGAGAGAGAGAAC	8	2	25.0
UBC 818	CAC ACA CAC ACA CAC AG	17	13	76.4
UBC 836	AGA GAG AGA GAG AGA GYA	12	5	41.6
UBC 825	ACA CAC ACA CAC ACA CT	18	12	66.6
UBC 808	AGA GAG AGA GAG AGA GC	8	1	12.5
$(GA)_9AT$	GAGAGAGAGAGAGAGAGAAT	8	3	37.5
	MEAN	10.33	5.5	86.7

Table 4. Similarity coefficient of 7 arrowroot accessions using 12 ISSR markers

	Acc. 1	Acc. 2	Acc. 3	Acc. 4	Acc. 5	Acc. 6	Acc. 7
Acc. 1	1						
Acc. 2	0.82	1					
Acc. 3	0.84	0.82	1				
Acc. 4	0.81	0.88	0.88	1			
Acc. 5	0.73	0.73	0.70	0.75	1		
Acc. 6	0.85	0.79	0.85	0.81	0.70	1	
Acc. 7	0.62	0.70	0.66	0.61	0.65	0.66	1

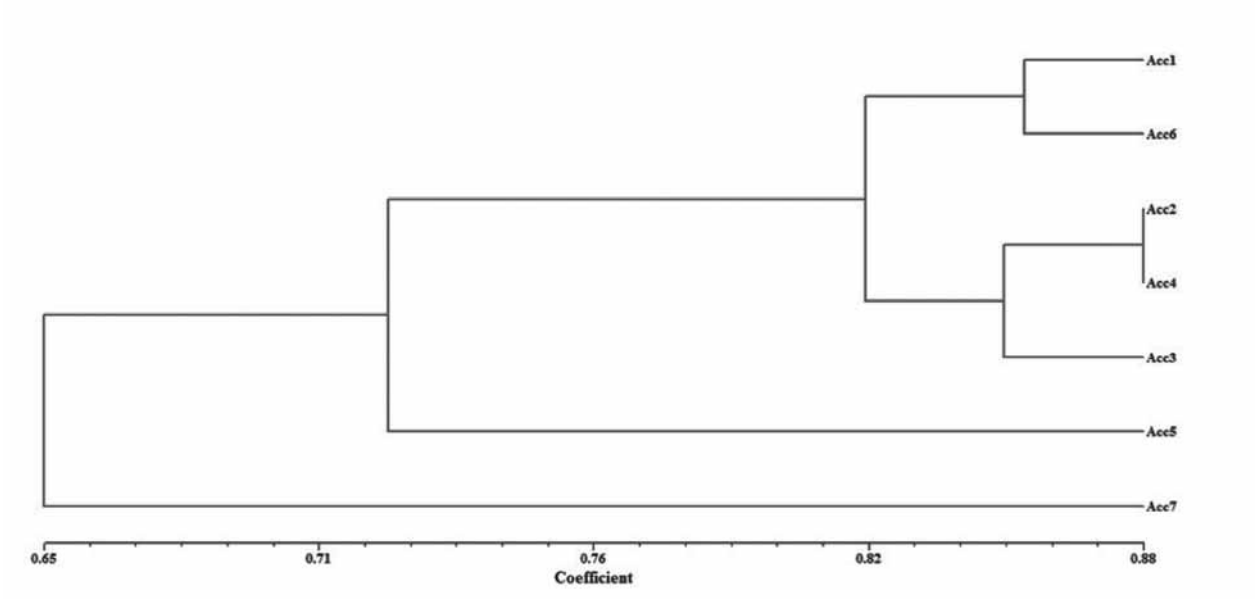

Fig. 3. Dendrogram showing the clustering pattern in 7 arrowroot accessions using 12 ISSR markers

Table 5. Details of 7 arrowroot accessions grouping under the major clusters

Cluster	Accessions
Cluster I	Acc. 1 (M1) and Acc.6 (M6)
Cluster 2	Acc. 2 (M2), Acc. 4 (M4) and Acc. 3 (M3)
Outliers	Acc. 5 (M5) and Acc. 7 (M7)

Acc. 3). The accessions 5 and 7 remained as two outliers in the grouping (Table 5). The accessions 2 and 4 were the most similar accessions with a similarity coefficient of 0.88, whereas Acc. 7 was the most distinct with only 65% similarity.

The present results of molecular study on 7 accessions of arrowroot have shown that variation exists among the accessions collected from different states of the country even though the morphological variation is very less. In the present study, the 12 ISSR primers tested against seven accessions of arrowroot genotype produced well defined products. The similarity coefficient based on ISSR markers ranged from 0.61 to 0.88. Most of the similarity coefficients ranged between 0.66 and 0.88. Among the 7 arrowroot accessions, the lowest similarity index (0.61) was observed for Acc. 4 and Acc.7, whereas, the highest similarity index (0.88) was observed between Acc. 3 and Acc. 4 as well as Acc. 2 and Acc. 4. Molecular markers are highly heritable, available in high number and exhibit enough polymorphism to discriminate the closely related genotypes. ISSR is fairly recent genetic marker that overcomes many of the technical limitations of methods like RFLP and RAPD analysis. ISSR markers have higher reproducibility than RAPDs and have been successfully used to estimate the extent of genetic diversity at intra and inter-specific levels in a wide range of crop species. Hence, these markers were used in the present study with the aim to assess the extent of genetic diversity present in this crop.

Conclusion

The present work aims at assessing the molecular diversity of 7 accessions of arrowroot (*Maranta arundinacea* L.) collected from different locations in India using 12 ISSR markers and also to estimate the extent of variability present genetically. Morphological variation is less in this important crop and earlier studies using RAPD have shown that no genetic variability existed in *Maranta*. However, results from this study proved that variability exists at the genetic level. This result will help the breeders in exploiting this variability for further improvement of the crop through selection procedure or hybridization.

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