ISSR Marker Based Genetic Diversity in *Morinda* Spp. For Its Enhanced Collection, Conservation and Utilization

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Abstract

Morinda (Rubiaceae) is considerably recognized for its multiple uses *viz.* food, medicine, dyes, firewood, tools, oil, bio-sorbent etc. The molecular characterization of such an important plant would be very useful for its multifarious enhanced utilization. In the present study, 31 *Morinda* genotypes belonging to two different species *Morinda citrifolia* and *Morinda tomentosa* collected from different regions of India were investigated using Inter Simple Sequence Repeat (ISSR) markers. Fifteen ISSR primers generated 176 bands with an average of 11.7 bands per primer, of which (90.34%) were polymorphic. The percentage of polymorphic bands, mean Nei's gene diversity, mean Shannon's information index in *Morinda tomentosa* and *Morinda citrifolia* was [(69.89%, 30.68%); (0.21 ± 0.19, 0.12 ± 0.20); (0.32 ± 0.27 0.17 ± 0.28)] respectively, revealing higher polymorphism and genetic diversity in *Morinda tomentosa* compared to *Morinda citrifolia*. Structure, and UPGMA cluster analysis placed the genotypes into well-defined separate clusters belonging to two species *Morinda tomentosa* and *Morinda citrifolia* revealing the utility of ISSR markers in species differentiation. Distinct ecotypes within a particular species could also be inferred emphasizing the collection and conservation of *Morinda* genotypes from different regions, in order to capture the overall diversity of respective species. Further higher diversity of *M. tomentosa* must be advanced for its utilization in nutraceutical, nutritional and other nonfood purposes.

Introduction

Morinda genus (Rubiaceae) is distributed throughout the tropics and subtropics. It is an important underutilized fruit plant; *Morinda citrifolia* (commonly known as noni) and *Morinda tomentosa* are its two recognized species. Its multifarious uses as food, medicine, dyes, firewood, tools, toys, bio-sorbent, oil etc. are renowned. *Morinda citrifolia* have broad range of therapeutic effects (Wang and Su 2001; Duke et al. 2002; McClatchey 2002). West et al. 2008, reported the utility of oil (average oil content of 124.9 g/Kg) extracted from the seeds of *M. citrifolia* as a potential source of vegetable oil containing healthy linoleic (59.4 %) and oleic fatty acids. In a study by Palu et al. (2012), the utility of noni seed oil for human skin health was also reported. There is another study in which, potential application of noni oil as whipped-soft margarine or salad oil as well as in nonfood industries was reported (Lee et al. 2015). Regarding *Morinda tomentosa* (syn. *Morinda coreia* Buch; *M. tinctoria* Roxb.) its fruits are consumed; wood is useful for making dishes, plates and toys and a red dye is made from its root bark (Anonymous 1962; Jukema et al. 1991). *Morinda tomentosa* is also being fed to cattle and buffaloes to improve milk yield by livestock owners in tribal and semiarid belt of east of Gujarat (Rangnekar 1991). There are also reports available on its usage as an environmentally safe bio-sorbent (Suneetha and Ravindhranath 2012; Vijayalakshmi et al. 2013). Keeping in view such a wide significance of *Morinda* species, genetic diversity and species differentiation studies using molecular markers would give way for its superior conservation as well as utilization. Molecular markers like RAPD, ISSR, SCoT (Singh et al. 2011; Arya et al. 2013; Arya et al. 2014; Bordello et al. 2017) have been used to characterize the genetic diversity of *Morinda*.

In the present study, ISSR markers were used for molecular characterization of such an important plant in order to estimate the genetic variation among *Morinda* genotypes belonging to two different species

(*Morinda citrifolia* and *Morinda tomentosa*).

Materials And Methods

Plant material

The experimental material (leaves) used in the present study consisted of 31 *Morinda* genotypes collected from Gujarat, Rajasthan, Kerala and Dharampuri (Tamil Nadu) regions of India (Table 1). Genotypes collected from Kerala and Rajasthan (Jodhpur) belonged to *Morinda citrifolia* species, while genotypes from Gujarat, Rajasthan (Kota and Bundi) and Dharmpuri (Tamil Nadu) belonged to *Morinda tomentosa* species (Fig. 1A and Fig. 1B).

Table 1
List of *Morinda tomentosa* and *M. citrifolia* used in the present study

Name of sample	Species	Habitat	Place of Collection	State
Gujarat 1	*Morinda tomentosa*	Disturbed roadside	Dudhwa	Gujarat
Gujarat 1A	*Morinda tomentosa*	Disturbed roadside	Popatpura	Gujarat
Gujarat 2	*Morinda tomentosa*	Disturbed roadside	Moholia	Gujarat
Gujarat 3	*Morinda tomentosa*	Disturbed roadside	Panchkhobala	Gujarat
Gujarat 4	*Morinda tomentosa*	Disturbed roadside	Dang	Gujarat
Gujarat 5	*Morinda tomentosa*	Disturbed roadside	Simalia	Gujarat
Rajasthan 1	*Morinda tomentosa*	Partly disturbed	Nayapura, Kota	Rajasthan
Rajasthan 2	*Morinda tomentosa*	Partly disturbed	Nayapura, Kota	Rajasthan
Rajasthan 3	*Morinda tomentosa*	Partly disturbed	Nayapura, Kota	Rajasthan
Rajasthan 4	*Morinda tomentosa*	Partly disturbed	Nayapura, Kota	Rajasthan
Rajasthan 5	*Morinda tomentosa*	Partly disturbed	Nayapura, Kota	Rajasthan
Rajasthan 6	*Morinda tomentosa*	Partly disturbed	Nayapura, Kota	Rajasthan
Rajasthan 7	*Morinda tomentosa*	Partly disturbed	Roadside Bundi	Rajasthan
Rajasthan 8	*Morinda tomentosa*	Partly disturbed	Roadside Bundi	Rajasthan
Kerala 1	*Morinda citrifolia*	Saline habitat, Partly disturbed	Thambakkadavu,Thrissur	Kerala
Kerala 2	*Morinda citrifolia*	Saline habitat, Partly disturbed	Thalikkulam, Thrissur	Kerala
Kerala 3	*Morinda citrifolia*	Saline habitat, Partly disturbed	Natika, Thrissur	Kerala

Name of sample	Species	Habitat	Place of Collection	State
Kerala 4	*Morinda citrifolia*	Saline habitat, Partly disturbed	Valappadu, Thrissur	Kerala
Kerala 5	*Morinda citrifolia*	Saline habitat, Partly disturbed	Kerayamparambu, Thrissur	Kerala
Kerala 6	*Morinda citrifolia*	Saline habitat, Partly disturbed	Kerayamparambu, Thrissur	Kerala
Kerala 7	*Morinda citrifolia*	Saline habitat, Partly disturbed	Kerayamparambu, Thrissur	Kerala
Kerala 8	*Morinda citrifolia*	Saline habitat, Partly disturbed	Kerayamparambu, Thrissur	Kerala
Kerala 9	*Morinda citrifolia*	Saline habitat, Partly disturbed	Kerayamparambu, Thrissur	Kerala
Kerala 10	*Morinda citrifolia*	Saline habitat, Partly disturbed	Thambakkadavu, Thrissur	Kerala
Kerala 11	*Morinda citrifolia*	Saline habitat, Partly disturbed	Kothakulam, Thrissur	Kerala
Kerala 12	*Morinda citrifolia*	Saline habitat, Partly disturbed		Kerala
Dharampuri	*Morinda tomentosa*	Disturbed roadside	Near railway station, Dharmpuri, Salem	Tamilnadu
Mogra Kalav	*Morinda citrifolia*	Partly disturbed	Mogra Kalav, Jodhpur	Rajasthan
Jodhpur city1	*Morinda citrifolia*	Partly disturbed	Jodhpur	Rajasthan
Jodhpur city2	*Morinda citrifolia*	Partly disturbed	Jodhpur	Rajasthan
Kalau	*Morinda citrifolia*	Partly disturbed	Kalau, Jodhpur	Rajasthan

DNA extraction

DNA was extracted from 100 mg of leaf samples of *Morinda* spp. using AuPrep DNA easy plant mini kit. DNA quantification was done using NANODROP 1000 (Thermo Scientific) spectrophotometer. Stock DNA was stored at -20°C and 20 ng working DNA solution was prepared for ISSR profiling.

ISSR analysis

PCR amplification was carried out with 100 ng of genomic DNA, 2.5 mM $MgCl_2$, 1U *Taq* DNA polymerase, 1x PCR buffer without $MgCl_2$, 1.0 µM ISSR primer and 0.2 mM dNTP mix. The volume was made up to 25

µl with sterile distilled water. Thermocycling conditions used for PCR were as follows: denaturation at 94°C for 5 min; thirty-five cycles of denaturation at 94°C for 1 min, primer annealing at 48 to 55°C for 1 min and primer extension at 72°C for 2 min and final extension step at 72°C for 7 min. PCR products were run on 1.6 % agarose gel and photographs were taken on SYNGENE G: Box Chemi XT4 Gel Documentation unit.

Data analysis

ISSR bands were scored as absent (0) or present (1). Genetic similarity among genotypes was evaluated by calculating the Jaccard's similarity coefficient and cluster analysis was performed using the UPGMA (Unweighted Pair Group Method of Arithmetic Means) algorithm (Rohlf 1998). Genetic parameters were estimated by Nei's gene diversity statistics (Nei 1973) using POPGENE version 1.32 (Yeh et al. 2000). Structure 2.3.4 (Prichard et al. 2000) and Structure Harvester (Earl and vonHoldt 2012) were used to know the genetic structure existing among the *Morinda* genotypes at K ranging from 1 to 10 with five iterations each (burn-in period of 100000 and number of Markov Chain Monte Carlo (MCMC) repetitions of 100000) using the admixture model.

Results

ISSR analysis

Fifteen ISSR primers were used to profile 31 *Morinda* genotypes belonging to two different species and yielded a total of 176 clear and bright bands and the number of bands varied from 5 $(GT)_8YG$ to 22 $(AG)_8T$ with an average of 11.7 bands per primer. Of 176 bands, 159 bands (90.34%) were found to be polymorphic and average number of polymorphic bands was 10.6. In case of *Morinda tomentosa* and *Morinda citrifolia* the %polymorphism was 69.89% and 30.68% respectively showing higher polymorphism in *Morinda tomentosa*.

Genetic diversity and differentiation

The mean Nei's gene diversity value for all the 31 genotypes was 0.27 ± 0.18 and Shannon's information index ranged from 0.03 to 0.52 with a mean of 0.42 ± 0.24 (Table 2) revealing considerable genetic diversity in *Morinda* spp. This may be due to the reason that genotypes used in this study belonged to two different species and different geographical locations of India (Gujarat, Rajasthan, Tamil Nadu and Kerala). Averaged over all the markers and genotypes, *Morinda tomentosa* displayed higher genetic variation (0.21 ± 0.19) as compared to *Morinda citrifolia* (0.12 ± 0.20) and also higher mean Shannon's information index for *Morinda tomentosa* (0.32 ± 0.27) as compared to *Morinda citrifolia* (0.17 ± 0.28). The ISSR primers varied in their power to detect diversity and the primers $BDB(CA)_7$, $(GT)_6AY$, $(AC)_8YT$, $HVH(TG)_7$ and $(AC)_8YA$ were selected as the most informative markers based on high Nei's gene diversity. And the least informative primer was $(GT)_8YG$, as it showed very low Nei's gene diversity.

Table 2
Characteristics of ISSR markers used for diversity analysis in *Morinda* spp.

Primers	Total Bands (no.)	No. of Polymorphic bands	% Polymorphism	Size range of bands (bp)	Nei's gene diversity	Shannon's Information Index
$(AC)_8YT$	14	12	85.71	275–1500	0.33	0.48
$(GA)_9AT$	12	12	100.00	350–2500	0.29	0.43
$BDB(CA)_7$	17	17	100.00	250–950	0.35	0.53
$(AGC)_4Y$	14	14	100.00	250–2250	0.34	0.51
$HVH(TG)_7$	9	9	100.00	300–1000	0.36	0.53
$(CA)_6$ RG	14	13	92.86	275–1800	0.27	0.41
$(AG)_8T$	22	20	90.91	275–2000	0.21	0.34
$VHV(GT)_7$	11	10	90.91	260–1100	0.29	0.44
$(GT)_8YG$	5	2	40.00	260–500	0.01	0.03
$(GT)_6AY$	6	6	100.00	450–1250	0.35	0.52
$(GA)_8T$	8	7	87.50	260–950	0.23	0.36
$(AC)_8YA$	9	7	77.78	250–750	0.32	0.46
$AC)_8T$	11	7	63.64	300–3000	0.14	0.23
$(GA)_8C$	6	5	83.33	325–900	0.23	0.37
$(AG)_8C$	18	18	100.00	250–2000	0.25	0.39
Average	11.7	10.6	90.34		0.27 ± 0.18	0.42 ± 0.24

Cluster Analysis

Genetic similarity was calculated among the 31 *Morinda* genotypes belonging to *Morinda citrifolia* and *Morinda tomentosa* species based on 176 scored bands. Genetic similarity coefficient between pairs of genotypes was obtained from the marker data based on Jaccard's coefficients using NTSYS-pc. ver. 2.1 software. Jaccard's similarity coefficients among the 31 genotypes ranged from a maximum of 1.0 ('Kerala 2' and 'Kerala 4') to a minimum of 0.186 (Dharampuri and Jodhpur City 2) with an average of

0.55. Two Kerala samples of *Morinda citrifolia* were showing maximum similarity and genotypes from Tamil Nadu (*Morinda tomentosa*) and Rajasthan (*Morinda citrifolia*) were found most distant.

Jaccard's similarity coefficients generated from the ISSR marker data were used to construct a dendrogram. UPGMA clustering reflected the grouping of 31 genotypes into two clusters (Fig. 2). The cluster 'I' consisted of 15 genotypes, including Dharampuri (Tamil Nadu) genotype as outliers of cluster I. Cluster I genotypes belonged to *Morinda tomentosa* species. Cluster I was further subdivided into two subgroups Ia and Ib. All the genotypes except one from Gujarat were present in subgroup Ia and genotypes from Rajasthan were placed in subgroup Ib and further in subgroup Ib also two genotypes from Bundi were present as outliers of Ib and all the genotypes from Kota were grouped together in Ib. The cluster 'II' consisted of 16 genotypes, from *Morinda citrifolia* species. Cluster II was further subdivided into two subgroups IIa and IIb. In subgroup IIb all the genotypes were from Jodhpur, Rajasthan. In subgroup IIa all the genotypes were from Kerala.

Structure Analysis

STRUCTURE analysis revealed two groups G1 and G2 based on delta K value which was settled at 2. G1 (Ia and Ib of UPGMA cluster) contained all the genotypes from *Morinda tomentosa* and G2 (IIa and IIb of UPGMA cluster) genotypes were from *Morinda citrifolia* (Fig. 3). These results were consistent with UPGMA cluster analysis.

Discussion

Morinda tomentosa and *Morinda citrifolia*, the flowering plant species of the genus *Morinda* are known for their immense health benefits because of the presence of secondary metabolites of medicinal importance and nutritional value due to excellent source of minerals and vitamins. Other than this, these species also provide wood, dye, oil etc. and can be used as bio-sorbents. Molecular characterization of such valuable species is essential for making strategies for their collection and conservation followed by its utilization. Molecular markers (RFLPs, AFLPs, RAPD, ISSR, SCoT, SSRs etc.) are very well recognized for assessing the level of genetic diversity (Powell et al. 1996). RAPD, ISSR and SCoT markers were used earlier to find the level of genetic diversity in *Morinda* spp. also (Singh et al. 2011; Singh et al. 2012; Arya et al. 2013; Arya et al. 2014). These studies predicted the level of genetic diversity in the samples collected from Andaman and Nicobar Islands, Tamil Nadu, Karnataka, Gujarat and Madhya Pradesh.

The present study was carried out to assess a) the level of genetic diversity between and within *Morinda tomentosa* and *Morinda citrifolia* collected from diverse geographical locations of India using ISSR markers b) use of ISSR markers in species differentiation in *Morinda*. ISSRs were chosen for the present study as these are simple to perform, more reproducible, stable than RAPD, multi-locus dominant marker system and have been used for diversity analysis, population structure analysis, DNA finger printing, phylogenetic analysis etc. in different plant species (Ansari et al. 2012; Zhang et al. 2015; Kumar et al. 2016; Ana-Cruz et al. 2017).

In the present study, ISSR markers revealed 90.34% polymorphism among the 31 *Morinda* genotypes belonging to *Morinda tomentosa* from Gujarat, Rajasthan and Tamil Nadu and *Morinda citrifolia* from Kerala and Rajasthan. The mean Nei's gene diversity and Shannon's information index values also revealed considerable genetic diversity in our genotypes.

In an earlier study by Singh et al. 2011 in 22 accessions of *M. citrifolia*, *M. tinctoria* and *M. pubescens* based on ISSR markers, polymorphism level of 56.02% was reported, which is very less than the polymorphism level reported in the present study. Our results indicated higher genetic variability in the *Morinda* genotypes collected from Gujarat, Rajasthan, Tamil Nadu and Kerala. So these regions must be explored thoroughly to collect the maximum diversity available in these regions for conservation, characterization and utilization.

Genetic relatedness analysis based on Jaccard's similarity coefficients values revealed maximum similarity between the two genotypes of *M. citrifolia* from Thalikkulam and Valappadu, Thrissur, Kerala and maximum dissimilarity was observed between genotypes from Tamil Nadu (*Morinda tomentosa*) and Rajasthan (*Morinda citrifolia*).

Coming to species level, the two species *M. tomentosa* and *M. citrifolia* were grouped separately based on UPGMA as well as Structure analysis and clearly distinguished based on ISSR markers thereby confirming the utility of ISSR markers in phylogenetic analysis of *Morinda* genus. A recent study by Kumar et al. 2016 found ISSR markers better than psbA-trnH sequences in phylogenetic studies of *Ocimum* L. genus and thereby supports our study confirming the role of ISSR markers in species differentiation. Further, *M. tomentosa* showed higher polymorphism and genetic variation as compared to *M. citrifolia.* So the advantage of higher diversity in *M. tomentosa* must be advanced for its utilization in nutraceutical, nutritional and other purposes.

Within respective species viz. *Morinda tomentosa* the genotypes from Gujarat, Rajasthan and Dharampuri (Tamil Nadu) were clustered as distinct ecotypes, with Dharampuri genotypes as the most diverse among the three regions. Similarly, within *Morinda citrifolia* most of the genotypes from Kerayamparambu, Thrissur were separately grouped, emphasizing the utility of ISSR markers in genetic relatedness, species differentiation and geographic patterning studies. So in order to conserve the overall diversity of both the species, genotypes from all the regions should be collected. *In-situ* farm conservation should be promoted in the respective regions, including a substantial number of genotypes from that region.

Conclusion

Unique ISSR profiles were generated for genotypes from *M. tomentosa* and *M. citrifolia* species. Higher genetic variability and polymorphism was observed in *M. tomentosa* as compared to *M. citrifolia*. Further genotypes collected from different eco-geographical regions were clustered in well-defined groups. ISSR markers proved as excellent, informative and effective marker system for species differentiation, geographic patterning, genetic diversity and relatedness studies of *Morinda* spp. The information

generated during the present study will certainly aid in proper management of *Morinda* genetic resources in terms of exploration, conservation and utilization.

Declarations

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Conflict of interest: The authors declare that they have no conflict of interest.

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Figures

M. tomentosa from Dharampuri, Tamil Nadu

M. tomentosa from Gujarat

M. tomentosa from Bundi, Rajasthan

M. tomentosa from Kota, Rajasthan

M. citrifolia from Thrissur, Kerala

M. citrifolia from Thrissur, Kerala

Fig. 1A.

M. tomentosa fruits from Gujarat

M. tomentosa fruits from Rajasthan

M. citrifolia fruits from Thrissur, Kerala

M. citrifolia fruits from Thrissur, Kerala

Fig. 1B.

Figure 1

1A. Leaf mophology of M. tomentosa and M. citrifolia collected from different regions of India 1B. Fruit mophology of M. tomentosa and M. citrifolia collected from different regions of India

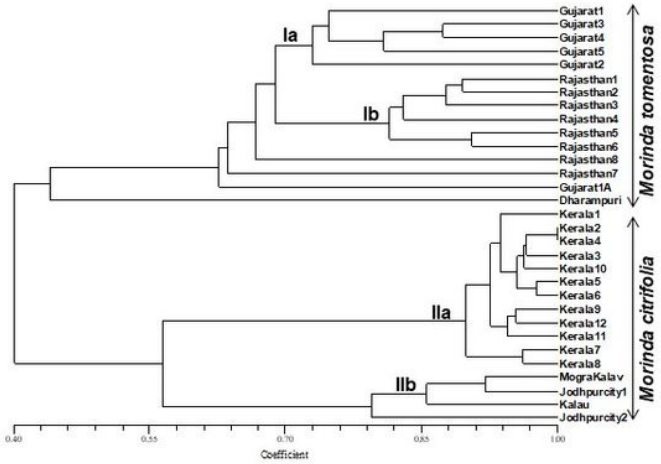

Fig. 2

Figure 2

UPGMA cluster analysis of Morinda tomentosa and Morinda citrifolia

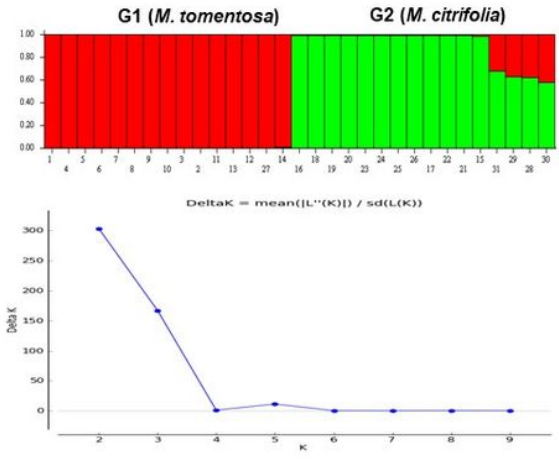

Fig. 3.

Figure 3

Structure Analysis of Morinda tomentosa and Morinda citrifolia