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UNMASKING GENETIC RELATIONSHIPS AMONG *Corypha umbraculifera* POPULATIONS FROM KARNATAKA IN INDIA USING SIMPLE SEQUENCE REPEAT MARKERS

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Abstract

Corypha umbraculifera L., is a monocarpic, endangered plant and show endemism to India and Sri Lanka. Natural population of this species occurs in semi-wild conditions in Karnataka and Kerala regions of Western Ghats, India and in addition it is found as a cultivated ornamental plant in other parts of India. Simple sequence repeat (SSR) analysis was used to determine the genetic diversity and relatedness among 14 C. umbraculifera populations collected from different locations within Karnataka, India. Among the 10 SSR primer pairs used for the study, 9 could amplify polymorphic SSRs from all the 14 populations. 168 alleles were detected at 22 loci ranging from 1 to 14 alleles, with average score of 7.63 alleles per locus. The statistical analysis of the results revealed that allelic frequency ranged from 7.1% to 100% with a mean of 54.51%. Polymorphism of alleles and genetic diversity measured by polymorphic information content (PIC) revealed that PIC of the loci was ranged from 0.23 to 0.83 with an average value of 0.61. The UPGMA-based clustering analysis showed the genetic similarity index ranged from 0.316-0.917. The similarity coefficients were used as input data for cluster analysis performed by a dendrogram construction utility (DendroUPGMA online server, Garcia-Vallve et al., 1999) and similarity matrix was calculated based on Jaccard index. The dendrogram divided the collected 14 populations into 2 major groups as the first group, i.e. cluster A included 7 populations, viz. Dndi, Manr, Sond, Nari, Bakl, Mtrd and Bltd, whereas cluster B included 7 populations, viz. Yana, Ytgd, Chpi, Honr, Bdag, Uppi and Udne. Thus, results indicated the presence of genetic diversity among the plant samples. These results suggest that SSR markers are efficient for determining genetic diversity and relatedness in C. umbraculifera. The present study endorses further the use of several other molecular marker systems that will help in germplasm characterization of semi-wild populations of C. umbraculifera for further conservation and sustainable utilization.

Key words*: Corypha umbraculifera, genetic diversity, Karnataka, SSR markers, UPGMA Dendrogram.*

INTRODUCTION

Western Ghats is recognized as one of the richest mega biodiversity centres of the world, wherein 30% of the area is covered by forest and considered as richest ecological region. Nearly more than 15,000 plant species have been documented until now, of which more than 4,000 plant species exhibit endemism (Chenniappan and Kadarkarai, 2010). Several tribal communities inhabit in this region because of rich natural diversity and abundance found across Western Ghats (Khan et al., 2008). Several prominent plant species that are medicinally and economically important have been reported from this ecologically sensitive region (Pushpangadan and Atal, 1984).

Palms are the third most economically important plants; they are extensively used as ornamental plants and also for making furniture and thatching material for roof as they are rigid, and exhibit ultimate tensile strength. Palm oil is an important commodity across the globe, which is used for daily cooking as it is known for its health benefits (Johnson, 1996; Renuka, 1992, 1998, 2001). Various palm species are found in their natural habitats that are attractive and represents as a unique niche among the wide diversity of different plant species. Karnataka region of Western Ghats comprises wide distribution of palms with about 21 species belonging to 7 genera. *Corypha umbraculifera* is one among them and is commonly called as centurion palm belonging to *Arecaceae* family that requires moist climate and commonly found across coastal plains, the unique feature of this palm is flower sets once in its lifetime. This palm is endemic to India and Sri Lanka. In India, it is commonly found across coastal regions of Kerala, Karnataka and Maharashtra. Previously it has been reported in Kozhikode, Kottayam and Palakkad districts of

Kerala (Renuka, 1999) and also distributed in forests of North Canara and South Canara districts of Karnataka including *Yana*, *Arebail*, *Udane*, *Naravi* and *Hosmar*. In Karnataka it has limited population and locally called as *Panolimara* or *Pane* (Bhat, 2011). Prehistorically, leaves of *C. umbraculifera* were used as thatching material for constructing houses and the leaves were used as manuscripts because of their distinctive characters such as size, shape, rigidness and greater strength and life. Further it yields a greater amount of starch which is widely utilized for multiple purposes locally (Chandran, 1996). However, according to previous reports, due to urbanization and shear lack of knowledge, this plant faces a serious threat and has been considered as one of the endangered species.

Recent advancement and innovations in molecular biology tools and technologies has enabled the researchers to find the variation within and between the different species of an organism (Robarts and Wolfe, 2014). A molecular marker acts as an enhanced tool for distinguishing variations and genetic distances in a population. Utilization of suitable molecular markers is dependent on DNA polymorphism (Ouborg et al., 1999). Genetic markers are best tools for efficient diversity evaluation and selection. Molecular markers exhibit distinctive properties and these genetic markers as single or two gene loci are not affected by the environment. They are polymorphic, co-dominant, occur repetitively and abundantly in a genome (Morgante et al., 2002). Apart from these inherent properties, they are also considered as best tools as they show better reproducibility, reliability and easy sharing of information between research laboratories.

The microsatellites are popularly known for identification of genetic diversity and relatedness among closely associated individuals. Microsatellite markers are commonly referred as short tandem repeats (STR) or simple sequence repeats (SSR) (Vincent et al., 2016). They are considered to be most efficient molecular markers, because they confer better understanding of population structure there by offering high degree of variability and also favours in the identification of closely linked species (Smith and Devey, 1994). In the present study, we attempted an SSR based molecular analysis in order to evaluate the genetic diversity or relatedness among the 14 semi-wild populations of *C. umbraculifera* collected from different locations of Karnataka, India.

MATERIALS AND METHODS

Plant material

The plant, being an endemic species has a limited distribution range and only available in selected regions. The plant samples were collected from along the Western Ghats area of Karnataka covering Uttara Kannada, Shimoga, Mangalore and Udupi districts. The young leaf samples of *C. umbraculifera* (14 populations) were collected in wild condition from their natural habitats in a zip pouch bags and transported to the laboratory in icebox. Upon arrival to the laboratory the samples were washed in 70% alcohol, blotted with tissue paper, quickly frozen by dipping in liquid nitrogen and stored at -80ºC for further use. The details of plant collection, their accession name and location of collection is given in Table 1.

DNA extraction and quantification

CTAB method was followed for the extraction and isolation of DNA as prescribed by Doyle and Doyle (1987). Several modifications were made to the original protocol in order to suit our experimental conditions. The 2× CTAB lysis buffer was prepared using 2% cetyltrimethylammonium bromide, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 2% β-mercaptoethanol and 2% PVP. About 2 g leaf sample was ground in to fine powder using liquid nitrogen in a mortar and pestle and 8 ml of preheated 2× CTAB lysis buffer at 65ºC to make the paste. The mixture is incubated at 65ºC for 60 min for lysis and lysate was extracted with phenol:chloroform:isoamylalcohol (25:24:1) upon cooling to room temperature. The DNA is precipitated by adding equal quantity of prechilled isopropanol. The DNA pellet is washed in 70% alcohol, air dried, dissolved in $T_{10}E_1$ and stored at −20ºC until further use.

The isolated DNA was profiled using gel electrophoresis unit (Mini Sub System, Bio-Rad, India) and quantified by Nano Drop technique using bioanalyzer (Q3000, Quawell

Technology Inc., San Jose, CA, USA). Gel electrophoresis of the genomic DNA was done with 0.8% agarose gel in 1× TAE buffer using horizontal gel electrophoresis unit. Gel imaging was performed using gel documentation system (Infinity-1000/26M, Vilber Lourmat, France).

SSR primers

A total of ten palm specific SSR markers (Sigma-Aldrich, Bangalore) were selected based on literature and was screened to assess the genetic diversity among the 14 accessions of *C. umbraculifera* considered for this study. SSR primer selection was made based on the reports of Ngoot-Chin et al. (2010) and Noorhariza et al. (2012). SSR analysis was performed following the protocol of Singh et al. (2008) with necessary minor modifications.

PCR amplification

PCR amplification was carried out in a 20 µl reaction volume containing 100ng of genomic DNA, 5 pM of forward primer, 5 pM of reverse primer, 100 µm of dNTP mix, 1× Taq buffer, 1U Taq DNA polymerase and total volume is adjusted using molecular biology grade water. The PCR amplification was performed using Eppendorf MasterCycler Gradient 5331 Thermal Cycler (Germany) under the initial denaturation temperature set at 95°C for 30 sec followed by 40 cycles of denaturation temperature set at 95°C for 15 sec. The annealing temperature was set depending on the standardized annealing temperature of each SSR primer, with common duration of 15 sec for all primers. The extension temperature was set at 68°C for 1 min and the final extension for 5 min temperature was set at 68°C.

Agarose gel electrophoresis

Agarose gels were prepared using 1.5% (for PCR products) dissolved in 1× TAE buffer. 0.6µl of ethidium bromide (10mg/ml) was added to stain bands for better visualization. The PCR products were loaded along with the loading dye bromophenol blue. The PCR amplicons (bands) were visualized using UV transilluminator.

Statistical scoring of amplified fragments

Only those fragments that could be clearly scored were used in the data analysis. Total number of alleles profiled was documented along with the number of alleles per locus, average number of alleles per loci, frequency of alleles and average frequency of alleles. Polymorphism information content (Botstein et al., 1980; Anderson et al., 1993) value calculation was performed as described by Sharma et al. (2009). Each band thus generated was considered as a single unit and the populations were scored for their presence (1) or absence (0) of a band on a gel (Botstein et al., 1980; Anderson et al., 1993) and the cluster analysis was performed. The dendrogram was constructed using a dendrogram construction utility (DendroUPGMA online server, Garcia-Vallve et al., 1999) and similarity matrix was computed with Jaccard index (Tanimoto).

RESULTS AND DISCUSSIONS

The present study was undertaken to evaluate genetic relationship among the 14 accessions of *C. umbraculifera*. Plant samples of *C. umbraculifera* were collected from 14 different geographic locations of Karnataka State in India (Table 1, Figure 1). It is very important to understand the genetic diversity and relatedness of *C. umbraculifera* in order to work on genetic improvement and germplasm conservation of this palm. Thus, in this study, we have made an attempt to unmask the genetic diversity of this plant by using SSR markers. A total of 10 SSR primers that were reported earlier to be specific for palm species, were selected and tested for their ability to generate expected polymorphic SSR bands in collected palm species (Singh et al., 2008; Ngoot-Chin et al., 2010; Noorhariza et al., 2012). The detailed information about the primers used is tabulated in Table 2. Among them 9 primers successfully demonstrated the ability to generate amplicons of expected DNA band sizes. The primers sEg00090, sEg00113, sEg00036, sEg00066, sMo00020, sEg00038, sMo00130, sEg00067 and sMo00154 were able to produce robust and reproducible bands in tested 14 populations of *C. umbraculifera* (Figure 2).

Among the analysed primers, primer sEg00067 amplified highest polymorphic bands as compared with the rest of the primers screened.

Figure 1. *Corypha umbraculifera.* A - young plant; B – habitat; C – leaf; D - fruits

Table 1. List of regions of plant collection and their accession names

Collection site	Accession name
Uppinangadi, Dakshina Kannada	Uppi
Udane, Dakshina Kannada	Udne
Honnavara, Uttar Kannada	Honr
Badabag, Uttar Kannada	Bdag
Yaana, Uttar Kannada	Yana
Yattin gudda, Dharwad	Ytgd
Chipgi, Sirsi, Uttar Kannada	Chpi
Mantraddi, Dakshina Kannada	Mtrd
Beltangadi, Dakshina Kannada	Bltd
Naravi, Dakshina Kannada	Nari
Dandelli, Uttar Kannada	Dndi
Bakkal, Sirsi, Uttar Kannada	Bakl
Sonda, Sirsi, Uttar Kannada	Sond
Mansur, Dharwad	Manr

The above mentioned primers were efficient in amplifying a total of 168 alleles from a total of 22 loci with a range of 01 to 14 alleles and an average of 7.63 alleles per locus. Similar investigation was carried out by Rupp et al. (2009) on sweet corn using SSR markers. Vincent et al. (2016) have also reported the importance of SSR markers is detecting the genetic diversity among *Triticum aestivum* cultivars. In this study, SSR markers proved to be an efficient tool for detecting genetic variation in genotypes of *C. umbraculifera* based on their habitat and regional soil.

In this study, only those fragments that could be clearly scored were used in the statistical data analysis. The statistical analysis of the results revealed that polymorphic frequency of the amplified alleles ranged from 7.1 to 100% with a mean of 54.51%, indicating efficiency of the selected SSR primers. Genetic diversity or relatedness among the 14 populations of *C. umbraculifera* was calculated at each locus for allelic PIC based on allele frequencies at 22 loci amplified among all 14 populations that were analyzed following standardized methods (Botstein et al., 1980; Anderson et al., 1993). According to the prescribed standards, the PIC value must be almost zero (0) if the primer is not demonstrating any allelic variation; however PIC value may be one (1) at the maximum depending upon the allelic variation indicating the diversity of a gene or DNA segment in a gene pool of *C. umbraculifera.* The polymorphism of alleles and genetic diversity revealed that PIC of the loci ranged from 0.23 to 0.83 with a mean of 0.61. Mean PIC value >0.5 indicates the efficiency of selected primer. Thus the primer used in this study demonstrated efficient polymorphism among the related populations of *C. umbraculifera.*

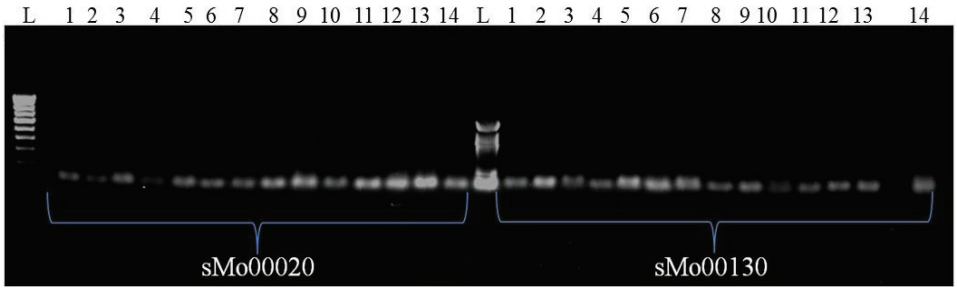

Figure 2. Gel profile showing the amplicons produced by two primers (sMo00020 and sMo00130): 1 – Uppi; 2 – Udne; 3 – Honr; 4 - Bdag; 5 – Yana; 6 – Ytgd; 7 – Chpi; 8 – Mtrd; 9 – Bltd; 10 – Nari; 11 – Dndi; 12 – Bakl; 13 – Sond; 14 - Manr

Table 2. List of SSR primers used for amplification with their SSR motif, number of alleles and annealing temperature

SSR primer	Sequence 5′–3′	SSR motif	Number of alleles	Annealing temperature (°C)
sEg00090	F: TCAGAAATGCCTACATCAAAC R: AGGGACACGAGAATACATACA	(AT)9	1	62.3
sEg00113	F: GTCACCGAACCCTAATAAAAT R: ATGCAGTTGAGGACAAAAAG	(CT)15	1	62.3
sEg00036	F: GGACCCTTTTTGTTACTGTTT R: AGCCTACCACAACTTCCTTT	(AG)9	1	62.3
sEg00066	F: ACTGATGCAGGAAAGAGGAA R: GAAGTACACAAGGTAAGTTCATAG	(AT)8	1	65.4
sMo00020	F: CCTTTCTCTCCCTCTCCTTTTG R: CCTCCCTCCCTCTCACCATA	(AG)15	1	60.7
sEg00038	F: ATCAAGCGGCAGTTATGAGAT R: ATACATTATTCCCACCACCA	(AAT)9	6	60.7
sMo00130	F: TAAGCAAAAGATCAGGGCACTC R: GGCTGGTGAAAATAGGTTTACAAAG	(AAG)11	1	60.7
sEg00067	F: GATTAAGTCCCAACCGTCTC R: TAAGAGAGCACGCAGTTCAG	(TGTA)6	2	60.7
sMo00154	F: CAAAAGGGTTGTTTGTATACGTG R: TGCATGAATATCCTCTCAAAGTTAC	(TG)7cgcgcgt gtgcgcgtg(TA)8	4	60.7
sMo00138	F: AGGGTTGTCGCTCCAATTTAT R: GGCATCTTTTTGACCTGTAGAAG	(TTTTC)6	-	66.7

Table 3. The degree of similarity index among 14 populations of *C. umbraculifera*

	Uppi	Udne	Honr	Bdag	Yana	Ytgd	Chpi	Mtrd	Bltd	Nari	Dndi	Bakl	Sond	Manr
Uppi	1	0.909	0.643	0.667	0.625	0.471	0.600	0.571	0.533	0.714	0.714	0.643	0.571	0.533
Udne		1	0.692	0.727	0.667	0.500	0.643	0.615	0.571	0.769	0.769	0.692	0.615	0.571
Honr			1	0.750	0.688	0.625	0.667	0.533	0.500	0.562	0.562	0.500	0.438	0.412
Bdag				1	0.600	0.643	0.692	0.538	0.500	0.571	0.571	0.500	0.429	0.500
Yana					1	0.706	0.750	0.444	0.421	0.556	0.556	0.500	0.444	0.421
Ytgd						1	0.800	0.389	0.368	0.421	0.421	0.368	0.316	0.444
Chpi							1	0.500	0.471	0.529	0.529	0.471	0.412	0.471
Mtrd								1	0.917	0.846	0.714	0.917	0.833	0.643
Bltd									1	0.786	0.786	0.846	0.769	0.714
Nari										1	0.857	0.923	0.846	0.667
Dndi											1	0.786	0.714	0.786
Bakl												1	0.917	0.714
Sond													1	0.643
Manr														1

Based on the generated SSR profiles across all the examined populations, cluster analysis of the genetic diversity was calculated using Jaccard index and unweighted pair group method with arithmatic mean (UPGMA) was used to plot a dendrogram that represented overall genetic diversity among the different populations (Figure 3). The intrapecific genetic similarity indices ranged from 0.316 to 0.917 (Table 3). Further, cluster analysis categorised the total populations into two major groups. Cluster A consists of seven populations, *viz*. Dndi, Manr, Sond, Nari, Bakl, Mtrd and Mltd1, whereas cluster B also comprises seven

populations, *viz*. Yana, Ytgd, Chpi, Honr, Bdag, Uppi and Udne. Among the accessions grouped in cluster I, high level of genetic similarity is evident between Dndi (accession from Dandeli) and Manr (Mansur) while the least genetic similarity was recorded between Udne (Udane) and Manr (Mansur). The cluster II consists of seven accessions, of which the Ytgd (Yattin gudda) and Chpi (Chipgi) were more closely related followed by Honr (Honnavara) and Bdag (Badabag). The least genetic similarity was observed between Yana (Yaana) and Udne (Udane), whereas Sond (Sonda) and Yana (Yaana) both from different cluster appear to belong to different lineage. This study revealed the presence of genetic diversity among the populations used for analysis, suggesting that SSR markers are the important tool to assess the genetic diversity and relatedness in *C. umbraculifera* semi wild populations.

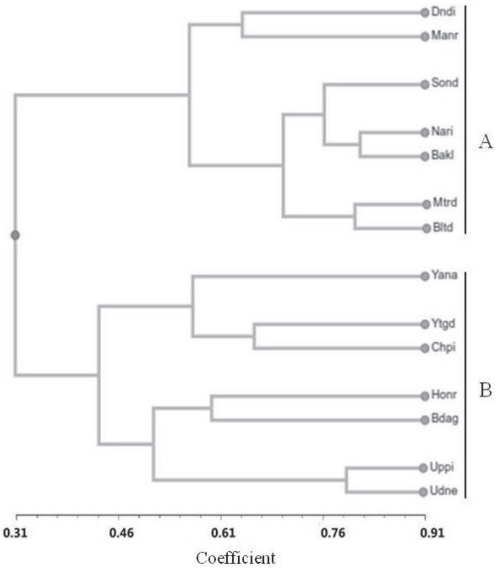

Figure 3. Dendrogram of fourteen *C. umbraculifera* populations constructed using UPGMA cluster analysis based on Jaccard similarity index

CONCLUSIONS

In this study, SSR markers were used to assess the molecular diversity among 14 populations of *C. umbraculifera* collected across different geographic locations. Results observed in the present study reflect the level of polymorphism within the populations and strongly suggest that SSR markers can be solely used to study the genetic polymorphism among the populations because of their high specificity and reproducibility. Despite the use of morphological tools, there are limited reports on molecular phylogeny in relation to the genus *Corypha*. Hence, SSR molecular markers can be efficiently used to evaluate the genetic polymorphism and to establish the conservation strategy for endemic palm species like *C. umbraculifera*.

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