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MOLECULAR DIVERSITY IN WILD POPULATIONS OF *Pinanga dicksonii* (Roxb.) Blume *(Arecaceae)* FROM WESTERN GHATS OF KARNATAKA USING MICROSATELLITE MARKERS

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Abstract

A precise understanding of genetic diversity and relatedness of Pinanga dicksonii (Roxb.) is an important component in its genetic improvement and germplasm conservation. P. dicksonii is an understory endemic palm of Western Ghats of Karnataka. The genetic diversity analysis among nine genotypes of P. dicksonii has been carried out using SSR markers. Among the 10 tested SSR markers, 9 successfully revealed polymorphism, SSRs demonstrating 123 alleles in total, with a range of 20 to 22 alleles at each primer. Allele frequency at each locus ranged from 22.22% to 100% with a mean of 71.92%. The PIC of each primer varied from 0.25 to 0.87 with an average of 0.38. The UPGMA-based clustering analysis performed by NTSYS pc program (version 2.0) revealed that among the 9 studied genotypes, there was a high level of polymorphism among some genotypes, as well as genetic similarities, with index values ranging from 0.473 to 0.928.

Key words*: genetic diversity, Pinanga dicksonii, SSR markers, UPGMA, dendrogram.*

INTRODUCTION

Palms are one of the significant economically essential classes of tropical and subtropical plants which play a vital role in food and raw material. Although they are under-explored species, they certainly amplify the chances of survival for people in developing countries. Throughout the world, they are represented by 212 genera encompassing 3000 species. Whereas, India it is very well represented by 22 genera with 105 species. Small quantities of palm species occur elsewhere in India, predominantly in Gangetic plains and in the lower hill valleys of Northern India (Bhat, 2011; Renuka & Sreekumar, 2012).

P. dicksonii (Roxb.) Blume is an endemic palm of the Western Ghats of Karnataka and Kerala, commonly recognized as Ivory crown shaft palm (Bhat, 2011). It is commonly grown across the moist area, as an understory-solitary palm. Usually, stem emerges beneath stolon. It is found as colonizer under favorable conditions in the evergreen forests of Western Ghats. The plant grows up to 5-7 meters height. It is widely distributed in Puttur Ghat, Kerekatte, Gerusoppa, Kuduremukha and Agumbe forests at an elevation of 250-1000 m. Some local tribes consume the fruits of this plant as a substitute for betel nuts. The stem is used to make walking sticks and as building material for huts (Renuka, 1998).

Molecular markers are relevant for genetic diversity assessments being able to define population relationships. They are not harmful to the environment and can be detected in all stages of plant growth and development (Mondini et al., 2002). But isozymes markers are not adequately variable due to their low polymorphism (Ghesquiere, 1985; Purba et al., 2000). In genetic diversity analysis, Random Amplified Polymorphism DNA (RAPD) has certain limitations, like poor reproducibility of amplification (Rafalski, 1997). Restriction fragment length polymorphic DNA (RFLP) is another vigorous molecular marker which requires a relatively large amount of purified DNA with high molecular weight, which is a

time-consuming and overlong protocol (Maizura et al., 2006). Ultimately, Amplified fragment length polymorphism (AFLP) is scored either as a presence/absence polymorphism. Simple sequence repeats (SSRs) are most commonly used molecular markers in studying interspecies and intraspecies genetic diversity of the microsatellite loci. Microsatellites can be implemented as mono locus co-dominant markers by amplifying single microsatellite loci into molecular markers by designing primers with the aid of specific sequence flanking (Kumar et al., 2009). Microsatellites offer a high level of polymorphism (Ellegren, 2004; Moges et al., 2016). As a result, they act as unique markers for studying population genetics, and gene mapping (Hearne et al., 1992; Jarne & Lagoda, 1996). Among all types of PCR based molecular markers, the SSRs are more informative, efficient and also cost-effective. The factor of abundance, co-dominance, highly reproducible and interspersed nature of microsatellite loci throughout the genome make the SSR marker the most potential and highly applicable in genetic diversity studies (Matin et al., 2012).

Several previous reports suggest that SSR markers are suitable for studying genetic variation and relationship among different genotypes, thus finds its application as a potent molecular marker in studying genetic variation (Eujayl et al., 2001; Russell et al., 2004). SSRs markers are widely used for studying the genetic diversities of different plant species, viz., maize (Garcia et al., 2004; Afaf et al., 2009), *Medicago sativa* L. (Wang et al., 2014), *Coffea canephora* Pierre ex A. Froehner (Hendre et al., 2014); raspberry and blackberry (Eric et al., 2005), *Myrica rubra* (Yun et al., 2012), cucumber (Hu et al., 2011), *Cynodon transvaalensis* (Tan et al., 2014), garlic (Meryem et al., 2015) and rice (Matin et al., 2012; Shahriar et al., 2014; Nivedita et al., 2016). Many investigators have also demonstrated the use of SSR markers (Powell et al., 1996; Afaf et al., 2009).

In the present study, we used SSR markers to analyze the intraspecies genetic diversity and relationship among the 9 wild genotypes of *P. dicksonii* from different locations of Karnataka region of Western Ghats.

MATERIALS AND METHODS

Plant Material

Nine genotypes of *P. dicksonii* were considered for this study. This plant is an endemic species for Karnataka and Kerala. It has a limited distribution range and is available only in the specified zones (Figure 1). The leaf samples were collected from forest areas of Uttara-Kannada, Shivamogga, Dakshina Kannada, Chikmagalur and Udupi districts of Karnataka. Collected plants are listed in Table 1. Young leaves were collected from the wild habitat and stored at -80°C prior to DNA extraction.

DNA Isolation and Purification

The DNA extraction followed the CTAB method described by Doyle and Doyle (1987) with certain modifications. CTAB lysis buffer (2×) was prepared by mixing 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-40. An amount of 7.5 ml 2× CTAB lysis buffer was preheated at 65ºC and mixed with 2 g leaf powder, prepared by grinding the leaf samples in liquid nitrogen. The mixture was then incubated for lysis at 65ºC for 60 min. The lysate was extracted with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) and DNA were precipitated by adding an equal quantity of pre-chilled isopropanol. The DNA pellet was washed in 70% alcohol, air dried and dissolved in $T_{10}E_{1.}$ The DNA samples were stored at -20ºC until further use.

DNA Quantification

The genomic DNA was profiled using 0.8% gel electrophoresis in 1× TAE buffer (Mini Sub System, BioRad, India). Gel imaging was performed using UV trans-illuminator (VilberLourmat Infinity-1000/26 M). Genomic DNA was quantified using NanoDrop Spectrophotometer (Quawell 3000-UV Spectrophotometer). The genomic DNA samples having absorbance ratios of 260/280 between 1.8-1.9 were used for SSR amplification.

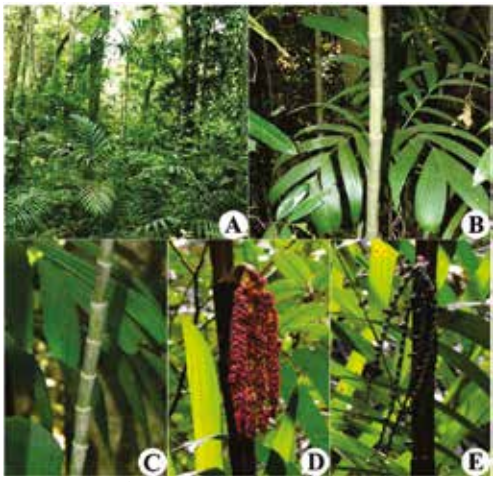

Figure 1. *P. dicksonii.* A – habit; B – leaves; C – trunk; D – inflorescence; E – fruit

SSR Primers

A total of 10 palm specific microsatellite markers (Table 3) were used to estimate the genetic diversity among 9 genotypes of *P. dicksonii* considered in this study. SSR primers were purchased from Sigma Aldrich, Bangalore.

SSR-PCR Amplification

The SSR primers were chosen selectively based on those which yielded amplification of microsatellite loci in the genome of the studied plant species. PCR amplification was carried out in a 20 µl reaction volume containing Taq buffer 1×, dNTP mix 2 mM, Forward Primer 5 pM, Reverse Primer 5 pM, Taq Polymerase 1 U, genomic DNA 100 ng. Amplification was carried out using The Master Cycler Gradient thermal cycler (Eppendorf AG 22331, Hamburg, Germany) under the following conditions: initial denaturation at 95ºC for 30 sec, and 40 cycles of denaturation at 95ºC for 15 sec, specific primer annealing temperature for 15 sec, primer extension at 68ºC for 1 min and a final extension at 68ºC for 5 min.

Agarose Gel Electrophoresis

The PCR products were visualized on a 2% agarose gel prepared in 1× TAE buffer, with a standard 50 bp ladder. A concentration of 0.05 µg/ml of ethidium bromide was added to the gel for visualization of the bands. The PCR products were mixed with loading dye 0.25% bromophenol blue in 40% of sucrose solution. The gel profile of each SSR primer amplicons was visualized in a UV trans-illuminator Gel documentation system (Infinity-1000/26M, VilberLourmat, France).

Scoring of Amplified Fragments

The best gel profile showing the perfectly resolved bands of each allele was considered for further analysis. Clear PCR products were transcripted in binary code, where each amplicon was recorded as one (1) for presence, and as zero (0) for the absence of DNA band in the gel. The number of alleles per loci was documented; accordingly average allele frequency was estimated. Polymorphism information content value was calculated by following a standardized method for each primer in order to evaluate the primer efficiency (Botstein et al., 1980; Anderson et al., 1993). Cluster analysis and dendrogram were drawn using NTSYS-pc software version 2.0 by UPGMA method, and the similarity matrix was deduced using Dice coefficient (Garcia-Vallvé et al., 1999).

RESULTS AND DISCUSSIONS

In the present investigation, 9 genotypes of *P. dicksonii* were studied for their intra-species genetic diversity and clustering. The OD values ranged between 1.82 and 1.97 (Table 2). High-quality DNA was obtained from all the *Pinanga* leaf samples and high amount of DNA was isolated, between 110.0 µg/ml and 498.5 µg/ml. A total of 10 SSR markers reported in earlier studies were selected for generation of polymorphic SSR in the present study (Table 3) (Singh et al., 2008; Ngoot-Chin et al., 2010; Noorhariza et al., 2012). Among the SSR markers used, 9 primer pairs showed amplification of DNA fragments. These markers were coded as sEg00090, sEg00113, sEg00036, sMo00020, sMo00130, sEg00067, sMo00154, sMo00138 and sEg00038. Primer pair sEg00090 revealed the most relevant results among all tested primers, as this primer pair amplified the highest number of polymorphic bands (Figure 2).

Table 1. Plant sampling locations and their accession names

Collection area	Accession name	Latitude (°N)	Longitude (°L)
PutturGhat, Puttur, Dakshina Kannada District	Putghat	12° 50′	75° 31′
Bhagavathi, Kuduremukha, Chikmagalur District	Bgvti	13° 11′	75° 11′
Jamble, Kuduremukha, Chikmagalur District	Jmble	13° 13′	75° 15′
Gerusoppa, Uttara Kannada District	Grspa	14° 13′	74° 39′
Agumbe, Thirthahalli, Shivamogga District	Agmbe	13° 30′	75° 05′
SK Border, Sringeri, Chikmagalur District	Skbrdr	13° 17′	75° 08′
GanapathiKatte, Sringeri, Chikmagalur District	Gptkte	13° 25′	75° 19′
Jamble, Koppa, Chikmagalur District	Jmbko	13° 31′	75° 21′
Gulaganjimane, Sringeri, Chikmagalur District	Glgjmne	13° 20′	75° 10′

Table 2. Nano-drop values for genomic DNA quantification of *P. dicksonii*

Samples	260/280	ng/ml
Putghat	1.88	110.0
Bgvti	1.83	159.5
Jmble	1.83	120.1
Grspa	1.85	169.1
Agmbe	1.97	199.4
Skbrdr	1.82	271.9
Gptkte	1.86	183.5
Jmbko	1.92	498.5
Glgjmne	1.91	240.7

Nine tested markers efficiently produced 123 amplicons with a range of 20 to 22 alleles. The gel profile of each primer pair was analyzed and the allele frequency at each locus was estimated ranging from 22.22% to 100% with a mean of 71.92%, demonstrating the efficiency of each primer based on allele frequency. Genetic diversity or similarities among the 9 genotypes were calculated at each locus for allelic Polymorphism Information Content (PIC). The calculation for each SSR primer pair used in the study was made following the standardized method (Botstein et al., 1980; Anderson et al., 1993), by analyzing the allele frequencies of 9 primers among all 9 genotypes. According to the prescribed standards, the PIC value must be between zero (0) and one (1) depending upon primer efficiency in order to demonstrate the allelic variation. In this study, the PIC of the loci ranged from 0.25 to 0.87 with a mean of 0.38. PIC value for primer pairs sEg00090, sEg00113, sEg00036, sMo00020, sEg00038, sMo00130, sEg00067, sMo00138 and sMo00154 was 0.277778, 0.623457, 0.876543, 0.277778, 0.0, 0.255144, 0.592593, 0.0 and 0.598765, respectively, where primer sEg00036 showed highest PIC and sMo00020 showed lowest PIC value among all primers used. This indicates the efficiency of the selected markers in presenting the polymorphism among related genotypes of *P. dicksonii.* Afaf et al. (2009) have reported that SSR markers are twice more informative than AFLP and RAPD in terms of revealing alleles per locus. The alleles were scored individually based on the comparison with molecular weight ladder. The similarity matrix was derived based on Jaccard-coefficient and the dendrogram was constructed by Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrogram showed two distinct groups (Figure 3) in which the genotypes Puttur Ghat (Putghat), Bhagavathi (Bgvti) and Gerusoppa (Grspa) formed a cluster A whereas genotypes Agumbe (Agmbe), Jamble (Jmble), SK Border (Skbrdr), Ganapathi Katte (Gptkte), Jamble-Koppa (Jmbko), and Gulaganjimane (Glgjmne) formed a distinctive cluster B. The cluster A was further divided into 2 subgroups, with Puttur Ghat (Putghat) and Bhagavathi (Bgvti) as one subgroup with the highest similarity index of 0.928, and Gerusoppa (Grspa) formed an individual subgroup with a similarity index

with its neighboring subgroup of 0.733. Cluster B possessed two closest subgroups, with one subgroup formed between SK Border (Skbrdr) and Ganapathi Katte (Gptkte) genotypes with a similarity index of 0.875, indicating their genetic relationship and the other subgroup formed between genetically closely related Jambli-Koppa (Jmbko) and Gulaganjimane (Glgjmne) genotypes as revealed by their similarity index of 0.882. Whereas Agumbe (Agmbe) and Jamble (Jmble) genotypes appeared to form separate individual lineages in cluster B, showing their genetic divergence from the other genotypes under study.

Table 3. List of SSR primer pairs sequences and annealing temperature used in the present study

SSR Markers	Sequence 5′→3′	Repeated motif	Amplicon (bp)	Annealing temperature (°C)
sEg00090	F- TCAGAAATGCCTACATCAAAC R- AGGGACACGAGAATACATACA	(AT)9	230-250	62.3
sEg00113	F- GTCACCGAACCCTAATAAAAT R- ATGCAGTTGAGGACAAAAAG	(CT)15	100-110	62.3
sEg00036	F- GGACCCTTTTTGTTACTGTTT R- AGCCTACCACAACTTCCTTT	(AG)9	115-125	62.3
sMo00020	F- CCTTTCTCTCCCTCTCCTTTTG R- CCTCCCTCCCTCTCACCATA	(AG)15	100-110	60.7
sEg00038	F- ATCAAGCGGCAGTTATGAGAT R- ATACATTATTCCCACCACCA	(AAT)9	140-150	60.7
sMo00130	F- TAAGCAAAAGATCAGGGCACTC R- GGCTGGTGAAAATAGGTTTACAAAG	(AAG)11	170-178	60.7
sEg00067	F- GATTAAGTCCCAACCGTCTC R- TAAGAGAGCACGCAGTTCAG	(TGTA)6	145-155	60.7
sMo00138	F- AGGGTTGTCGCTCCAATTTAT R- GGCATCTTTTTGACCTGTAGAAG	(TTTTC)6	70-78	60.7
sMo00154	F- CAAAAGGGTTGTTTGTATACGTG R- TGCATGAATATCCTCTCAAAGTTAC	(TG)7cgcgcgtgtgcgcgtg(TA)8	168-175	60.7
sEg00066	F- ACTGATGCAGGAAAGAGGAA R- GAAGTACACAAGGTAAGTTCATAG	(AT)8	-	-

F - Forward; R - Reverse

Table 4. Similarity matrix index of 9 wild genotypes of *P. dicksonii*

	Putghat	Bgvti	Jmble	Grspa	Agmbe	Skbrdr	Gptkte	Jmbko	Glgjmne
Putghat	1								
Bgvti	0.928	1							
Jmble	0.533	0.571	1						
Grspa	0.733	0.666	0.500	1					
Agmbe	0.687	0.625	0.692	0.562	1				
Skbrdr	0.611	0.555	0.600	0.500	0.750	1			
Gptkte	0.705	0.647	0.600	0.500	0.866	0.875	1		
Jmbko	0.578	0.526	0.562	0.555	0.705	0.823	0.823	1	
Glgjmne	0.578	0.611	0.562	0.473	0.705	0.823	0.823	0.882	1

The similarity index values ranged from 0.473 to 0.928. The highest similarity index value observed was 0.928 which was shown by several samples.

The *P. dicksonii* genotypes Putghat and Bgvti showed highest similarity index value of 0.928, the same was revealed in the dendrogram, indicating they are the closely related among all the genotypes, possessing the fewest divergence between each other.

On the contrary, the lowest similarity index value observed was 0.473 between Glgjmne and Grspa genotypes, indicating the highest genetic divergence between these genotypes. A high genetic similarity was also seen between Glgjmne and Jmbko (0.882).

The similarity matrix index is given in (Table 4). These values suggest that the genetically related plants subjected to this study have some variability at DNA level.

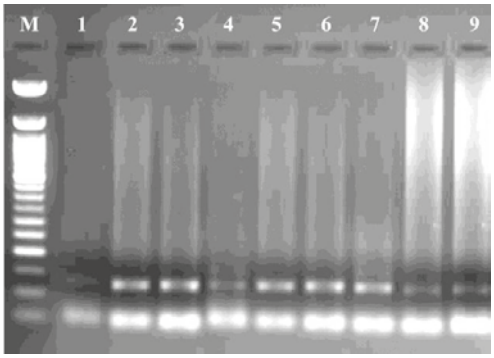

Figure 2. Gel profile showing the amplicons produced by primer pair sEg00090. 1 – Putghat; 2 – Bgvti; 3 – Jmble; 4 – Grspa; 5 – Agmbe; 6 – Skbrdr; 7 – Gptkte; 8 – Jmbko; 9 – Glgjmne

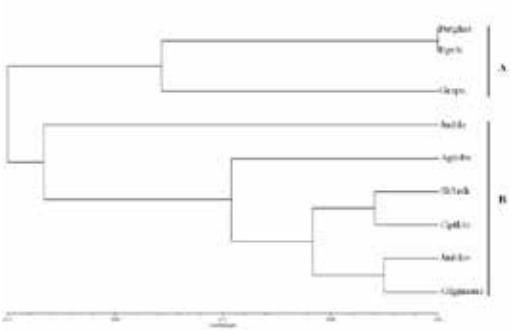

Figure 3. Dendrogram indicating phylogenetic relationships among 9 *P. dicksonii* genotypes constructed using UPGMA cluster analysis based on Jaccard similarity index obtained from 9 SSR markers

Reports indicate the immense application of SSR markers in various studies on plant interspecies or intraspecies genetic variation. Zhao et al. (2012) identified and categorized gene based SSR markers in date palm (*Phoenix dactylifera* L.). The genetic structure and diversity of *Acrocomia emensis* (Arecaceae) using SSR molecular markers showed low level of genetic variability among the populations and high, positive and significant inbreeding quality, which determines the level of homozygotes (Neiva et al., 2016). Such studies lay a strong platform for improvement of plant quality for better of human welfare and also in the conservation of respective germplasm. SSR markers are most widely and commonly used tool in studying the genetic diversity among plants (Eric et al., 2005; Afaf et al., 2009; Hu et al., 2011; Matin et al., 2012; Yun et al., 2012; Hendre et al., 2014; Shahriar et al., 2014; Tan et al., 2014; Wang et al., 2014; Meryem et al., 2015; Nivedita et al., 2016). Various studies have demonstrated the importance of SSR markers (Powell et al., 1996; Afaf et al., 2009). The molecular evaluation of genetic diversity within the 9 studied genotypes of *P. dicksonii* showed high genetic similarity in plants from nearby locations, while plants from different geographic location exhibit a low level of genetic similarity.

CONCLUSIONS

This study throws the light on genetic diversity of the endemic plant species, *P. dicksonii* found to grow in the wild. This species is not very well known and needs to be studied and conserved to maintain its' germplasm. The present study provides evidence on the genetic variation observed in the collected plant samples from the wild condition of Karnataka and Kerala. The present study also reveals the ability of SSR markers to generate polymorphic bands within *P. dicksonii* palm species. Among the 10 SSR markers used in this study, 9 have successfully amplified the plant genomic DNA fragments. This study reveals the existence of genetic differentiation among the collected plant species. Among the analysed plant samples, there was a high level of polymorphism as well as genetic similarity. This variation is indicative of the evolution of the species in their natural habitat. The plants from different geographical locations exhibited less genetic similarity while species from the same localities showed more genetic similarity. Thus more studies of this nature need to be conducted to assess the phylogeny of the plant species at the molecular level and hence aid in the conservation of their germplasm.

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