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Inter and intra-population genetic variability in *Calamus guruba* Buch.-Ham. (Arecaceae)-an economically important rattan species, using RAPD and ISSR molecular markers

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ABSTRACT

The genetic variability among nine natural populations and 45 accessions of *Calamus guruba* Buch.-Ham. (Arecaceae)- a dioecious and economically important Indian rattan species, was assessed using Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) molecular markers. Twenty RAPD and twenty ISSR primers were used to study the genetic diversity within and among populations of *C. guruba* collected from six districts of Odisha state. Out of 121 amplified loci with RAPD primers, 82 bands were polymorphic and 39 monomorphic in nature; no private band was detected. Similarly, with ISSR analysis, a total of 138 bands were generated which included 87 polymorphic and 51 monomorphic bands. The genetic similarity among all the accessions studies varied in the range of 65% to 99% indicating considerable variability within the species and close similarity among individuals of a particular population. Maximum genetic diversity was detected in the population collected from Chandaka Wildlife Sanctuary, Khurda district with an average polymorphism of 37.50% and minimum (18.98%) in the population from Khallikote, Ganjam district of Odisha. Use of *Calamus guruba* population from Chandaka with higher genetic variability as source of seed and other planting materials is suggested for maintaining the genetic stock and raising plantations under forestry programmes.

1. Introduction

Cane and rattans are spiny climbing palms belonging to the tribe Calameae of the subfamily Calamoideae under the family Arecaceae or Palmae (Uhl and Dransfield, 1987). They comprise about 600 species under 13 genera, which are distributed in equatorial Africa, South Asia, Southern China, the Malay Archipelago, Australia and the Western Pacific. Rattans and canes with solid stem are principal non-timber forest products in international trading and are highly valued and have social and economic importance because of their unique characteristics such as strength, durability, looks and bending ability. Due to overexploitation, habitat degradation and low regeneration capacity, the rattan resources of the world are under serious threat. About 117 species of rattans are considered as threatened to some degree (Walter and Gillett, 1998).

Of the 13 recognized genera of rattans and canes, *Calamus* is the most widespread, occurring in both the tropical and subtropical regions of Africa and south-east Asia (Uhl and Dransfield, 1987). A recent census suggests that the genus is represented by 374 species (Govaerts & Dransfield, 2005) in the world. Several species of canes are used for furniture, fancy items in cottage industries and basketry, and fruits of some are also consumed as a delicacy in parts of the world including India. There is extensive global demand for both raw and processed canes making it a valuable item for international trade.

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The species of *Calamus* are dioecious and wind-pollinated, with their phonological behaviour being influenced by climatic, topographical and edaphic factors. The low frequency of male plants and wastage of pollen during rains have led to decreased pollination efficiency and low seed set. It has been observed that adverse climatic factors influence the phenology of certain canes and cause variations in time and space of maturity and receptivity of stigma as well as production of pollen grains (Manohara *et al*., 2007). Overexploitation and forest fire pose serious threats to the survival of *Calamus* species in their natural habitats. Due to habitat degradation and overexploitation for trade purposes, the natural reserves of rattan are fast declining, causing genetic erosion of the existing resources.

In India, the genus *Calamus* is represented by 32 species (Karthikeyan *et al.,* 1989) and 22 species are reported to occur in Peninsular India alone (Anto *et al*., 2001). In Odisha, six species have been reported to occur in the wild till date (Mahapatra *et al*., 2012). Of these, *Calamus guruba* Buch.-Ham. ex Mart. (Kanta Beta) is the most common and economically important species occurring in several districts of Odisha. It's culms are used in making handicrafts, house construction, umbrella handles, basketry, ropes, mats etc. The fruits with fleshy pulp are edible and are often pickled. The tender shoots before emergence taste sweet and are eaten raw or after cooking as vegetable. Immature seeds are also used as beads. Besides, the roots have several medicinal uses.

In view of the extraction pressure and dwindling populations of *Calamus guruba* coupled with problems relating to reproductive biology and inherent genetic bottle-necks, understanding of genetic diversity is essential for developing appropriate conservation strategies. Different molecular markers have been used to assess the genetic variation and phylogenetic relationship of palms using PCR based markers and cpDNA studies (Wilson *et al*., 1990; Uhl *et al*., 1995; Baker *et al*., 1999; Asmussen *et al.,* 2000; Asmussen and Chase, 2001; Baker *et al*., 2000). The present study aims at evaluating the genetic diversity among and within populations of *Calamus guruba* occurring in Odisha using RAPD and ISSR markers with a view to identify populations with higher genetic variability for breeding and conservation programmes.

2. Materials and Methods

2.1. Plant materials

Forty five (45) individuals representing 9 different populations of *Calamus guruba* (CG1-CG45) were studied. Tender leaves from five different plants of each population were collected at random and were pooled together for genomic DNA extraction. The details of collection of samples are provided in Table 1**.**

2.2. Genomic DNA isolation

DNA was isolated from freshly collected young leaves by CTAB method as described by Doyle and Doyle (1990). RNA was extracted with RNaseA (Quiagen) treatment: @ 60 µg for 1 ml of crude DNA solution at 37 ^{0}C followed by two washings with phenol/chloroform/iso-amyl-alcohol (25:24:1 v/v/v) and subsequently two washings with chloroform/iso-amyl-alcohol (24:1 v/v). After centrifugation, the upper aqueous phase was separated, 1/10th volume of 3 M sodium acetate (pH 4.8) was added and DNA precipitated with 2.5 volume of pre-chilled absolute ethanol. The extracted DNA was dried and then dissolved in 10 mm Tris-HCl [tris(hydroxyl amino methane]/1 mm EDTA (ethylene diamine tetra acetic acid disodium salt) ($T_{10}E_1$ buffer, pH 8). Quantification was made by running the dissolved DNA in 0.8% agarose gel alongside uncut λ DNA of known concentration. The DNA was diluted to 25 ng/µl for RAPD or ISSR analysis**.**

Table 1

Details of accessions of *Calamus guruba* collected from different localities of Odisha with their geographical co-ordinates and identification code

Species Code	District	Location	Latitude	Longitude	Altitude
CG 1	Berhampur	Khallikote	N 19^0 36.503'	E 085^0 06.096'	141 ft
CG 2			N 19^0 36.505'	E 085^0 06.093'	142 ft
CG 3			N 19^0 36.500'	E 085^0 06.097'	140 ft
CG 4			N 19^0 36.507'	E 085^0 06.092'	139 ft
CG 5			N 19^0 36.509'	E 085^0 06.089'	145 ft
CG 6	Khurda	Barunei	N 20^0 09' 43.6"	E 085^0 39'01.7"	392 ft

CG 7			N 20^0 09' 43.4"	E 085^0 39’01.4"	390 ft
CG 8			N 20^0 09' 43.2"	E 085^0 39’01.6"	432 ft
CG 9			N 20^0 09' 43.4"	E 085^0 39’01.5"	435ft
CG 10			N 20^0 09' 43.0"	E 085^0 39’00.7"	437 ft
CG 11	Jagatsinghpur	Jasabantapur	N 20^0 13' 38.3"	E 086^0 09 ’35.2"	144 ft
CG 12			N 20^0 13' 38.1"	E 086^0 09 ’35.2"	145ft
CG 13			N 20^0 13' 37.9"	E 086^0 09 ’35.1"	141ft
CG 14			N 20^0 13' 38.1"	E 086^0 09 ’35.0"	136ft
CG 15			N 20^0 13' 38.3"	E 086^0 09 ’34.9"	133ft
CG 16	Cuttack	Odopada,	N 20^0 25’20.1"	E 086^0 04’05.8"	189 ft
CG 17		Kisannagar	N 20^0 25’20.2"	E 086^0 04’05.5"	159 ft
CG 18			N 20^0 25’20.0"	E 086^0 04’04.8"	160 ft
CG 19			N 20^0 25’20.0"	E 086^0 04’05.1"	156 ft
CG 20			N 20^0 25’20.6"	E 086^0 04’05.8"	157ft
CG 21	Khurda	Bhatapada,	N 19^0 49' 54.5"	E 085^0 01' 30.1"	346ft
CG 22		Balugaon Range	N 19^0 49' 54.8"	E 085^0 01' 30.0"	303ft
CG 23			N 19^0 49' 54.9"	E 085^0 01' 30.2"	280ft
CG 24			N 19^0 49' 55.1"	E 085^0 01' 30.1"	279ft
CG 25			N 19^0 49' 55.2"	E 085^0 01' 30.1"	280ft
CG 26	Khurda	Chandaka	N 20^0 18' 32.2"	E 085^0 48' 20.1"	294 ft
CG 27		Wildlife	N 20^0 18' 32.4"	E 085^0 48' 20.0"	297 ft
CG 28		Sanctuary	N 20^0 18' 33.0"	E 085^0 48' 20.6"	293 ft
CG 29			N 20^0 18' 33.5"	E 085^0 48' 20.2"	293 ft
CG 30			N 20^0 18' 34.7"	E 085^0 48' 20.6"	283 ft
CG 31	Keonjhar	Kodapada,	N 21^0 17' 15.5"	E 086^0 05' 21.8"	163 ft
CG 32		Anandapur	N 21^0 17' 15.2"	E 086^0 05' 21.5"	155 ft
CG 33		Wildlife Division	N 21^0 17' 15.4"	E 086^0 05' 21.4"	154 ft
CG 34			N 21^0 17' 15.5"	E 086^0 05' 21.0"	153 ft
CG 35			N 21^0 17' 15.4"	E 086^0 05' 21.2"	154 ft
CG 36	Cuttack	Nuapada,	N 20^0 31' 16.9"	E 085^0 49' 48.2"	119 ft
CG 37		Athagarh	N 20^0 31' 16.8"	E 085^0 49' 48.3"	120 ft
CG 38			N 20^0 31' 16.6"	E 085^0 49' 48.9"	122 ft
CG 39			N 20^0 31' 16.9"	E 085^0 49' 48.7"	121ft
CG 40			N 20^0 31' 16.7"	E 085^0 49' 48.8"	123 ft
CG 41	Bolangir	Gandhamardhan	N 20^0 50' 54.1"	E 082^0 51' 57.0"	1344 ft
CG 42		hills, Harishankar	N 20^0 50' 54.1"	E 082^0 51' 56.6"	1317 ft
CG 43			N 20^0 50' 53.9"	E 082^0 51' 56.7"	1310 ft
CG 44			N 20^0 50' 53.8"	E 082^0 51' 56.8"	1309 ft
CG 45			N 20^0 50' 53.9"	E 082^0 51' 56.9"	1305 ft

2.3 Random Amplified Polymorphic DNA (RAPD) analysis

Prior to polymerase chain reaction (PCR) for RAPD analysis random decamer Operon Primers (Operon Tech., Alameda, USA) were dissolved in double sterilized $T_{10}E_1$ buffer, pH 8.0 to the working concentration of 25 ng/ml. Out of Twenty five primers, twenty best selected primers as per the reproducibility and amplification pattern from A, C, D, N and AF series OPC-05, OPA-03, OPA-04, OPA-05, OPN-06, OPA-10, OPA-16, OPA-20, OPC-02, OPN-04, OPN-03, OPN-05, OPA-02 , OPN-08, OPAF-14, OPD-02, OPD-05, OPD-08, OPD-18& OPD-20 (Operon Tech. Alameda, CA) were used for RAPD analysis . The RAPD analysis was performed as per the methodology described by Williams *et al*. (1990). Each amplification reaction mixture of 25 ml volume contained 2.5 ml of 10X assay buffer (100 mM Tris-Cl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin), 200 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MBI Fermantas, Lithuania) 15 ng of primer, 0.5 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd, Bangalore, India) and 25 ng of template DNA. The amplification reaction was carried out in GeneAmp PCR System 9700 (Applied Biosystems, Germany). The amplification was performed in three steps PCR. Initial denaturation of the template DNA was carried out at 94 ^{0}C for 5 min for one cycle. The second step was carried out for 42 cycles and each cycle consisting of three temperature steps i.e. one min at 92 ^{0}C for denaturation of template, one min at 37^{0}C for primer annealing followed by two min at 72^{0}C for primer extension. The Final step consisted of only one cycle i.e. 7 min at 72 ^{0}C for complete polymerization. The soak temperature was 4^{0}C. After completion of the PCR, 2.5 ml of 6X loading dye (MBI Fermentas, Lithuania) was added to the amplified products and were stored at –20^{0}C till further use.

2.4 Inter simple sequence repeat (ISSR) analysis

Inter Simple Sequence Repeats were used for PCR amplification. Out of twenty five primers screened, best twenty anchored and non-anchored microsatellites were used as primers. These simple sequence repeats were synthesized and procured from Genei (Bangalore Genei Pvt. Ltd, Bangalore, India). Those primers were (PCP1, PCP2, PCP3, PCP5 ,PCP6, PCP7, PCP8, PCP9, PCP12, Oligo 1(b), Oligo 2(b), Oligo 3(b), Oligo 4(a), Oligo 4(b), Oligo 5(a), Oligo 5(b), Oligo 8(a), Oligo 9(a), Oligo 11(a) & Oligo 11(b)). The ISSR analysis was performed as per the methodology given by Zietkiewicz *et al*. (1994). Each amplification reaction mixture of 25 ml contained 20ng of template DNA, 2.5ml of 10X assay buffer (100mM Tris-HCl pH 8.3, 0.5M KCl and 0.01%gelatin), 1.5mM $MgCl_2$, 200mM each of dNTPs, 44ng of primer and 0.5U Taq DNA polymerase (Bangalore Genei, Bangalore, India). The amplification was carried out in a thermal cycler. The first cycle consisted of denaturation of template DNA at 94^{0}C for 5 min, primer annealing at specific temperature for particular primer for 1 min and primer extension at 72 ^{0}C for 2 min. In the subsequent 42 cycles the period of denaturation was reduced to 1 min while the primer annealing and primer extension time was maintained same as in the first cycle. The last cycle consisted of only primer extension at 72^{0}C for 7 min.

The amplified products were resolved in 1.5% agarose gel stained with ethidium bromide . Standard DNA ruler (GeNei Medium Range DNA Ruler, Merk Millipore, Merk specialities Private Limited, Mumbai) was used. The electrophoresis was performed in a constant voltage at 60°C for two hours. The amplicons were visualized under the UV light and photographed. The gel was also documented by Gel Doc 2000(Bio Rad, USA) for scoring the bands. The size of the amplicons was determined by comparing them with that of ladder. The entire process was repeated at least three times to study the reproducibility.

2.5 Data analysis

The presence/absence of bands in RAPD and ISSR analysis was recorded in binary (0, 1) form. All the bands (polymorphic and monomorphic) were taken into account for calculation of similarity with a view to avoid over-/ underestimation of the distance (Gherardi *et al*., 1998). Jaccard's coefficient of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients generated by the un-weighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973) and SHAN clustering. The statistical analysis was done using the computer package nNTSYS-PC (Rohlf, 1997). Resolving power (Rp) of the RAPD primer was calculated according to Prevost and Wilkinson (1999) as Rp = ΣIB, where IB (band informativeness) = 1 D [2 \ (0.5 D *P*)], *P* being the proportion of the 5 species containing the band.

3. Results

3.1 DNA isolation

The concentration of the total genomic DNA isolated from different species varied from 60 ng μl^{-1} to 1.8 µg μl^{-1} as can be seen from the agarose gel analysis (Fig. 1).

3.2 RAPD analysis

Out of 25 RAPD primers screened, 20 primers produced distinct reproducible bands (Table 2). A total of 121 amplified loci were generated including 82 polymorphic and 39 monomorphic bands. No unique band was observed. The RAPD banding patterns using three random primers

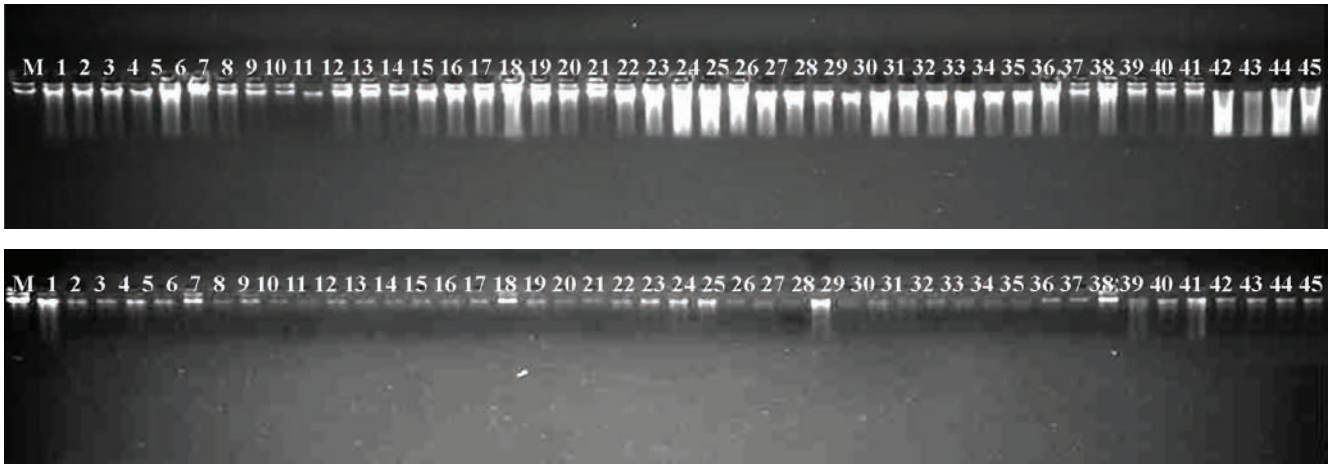

Fig. 1. Qualitative and quantitative analysis of total genomic DNA by 0.8% agarose gel. M, uncut phage λ DNA (600ng); lanes 1 to 45 *Calamus guruba* (CG1-CG45).

Table 2

Details of 20 RAPD primers used, bands amplified, percent polymorphism, primer index and resolving power

Sl No.	Primer Name	Primer Sequences	Range of amplicons in bp	Total No. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	No. of Unique Bands	% Polymorphic Bands (PPB)	Resolving Power	Primer Index
1	OPC-05	5'-GATGACCGCC-3'	800-3000	4	2	2	0	50%	6.57	0.79
2	OPA-03	5'-AGTCAGCCAC-3'	1000-2500	6	3	3	0	50%	10.88	0.81
3	OPA-04	5'-AATCGGGCTG-3'	400-1200	4	2	2	0	50%	6.53	0.91
4	OPA-05	5'-AGGGGTCTTG-3'	580-2800	5	3	2	0	60%	8.62	0.97
5	OPA-10	5'-GTGATCGCAG-3'	380-3300	10	9	1	0	90%	13.64	2.87
6	OPN-06	5'-GAGACGCACA-3'	850-2300	5	3	2	0	60%	8.62	0.92
7	OPA-16	5'-AGCCAGCGAA-3'	680-3000	8	6	2	0	75%	12.71	1.67
8	OPA-20	5'-GTTGCGATCC-3'	600-2100	6	3	3	0	50%	10.88	0.83
9	OPC-02	5'-GTGAGGCGTC-3'	450-2600	6	5	1	0	83.33%	8	1.55
10	OPN-04	5'-GACCGACCCA-3'	580-2000	7	4	3	0	57.14%	12.35	1.24
11	OPN-03	5'-GGTACTCCCC-3'	700-2500	7	6	1	0	85.71%	7.86	2.45
12	OPN-05	5'-ACTGAACGCC-3'	750-2900	8	5	3	0	62.5%	13.2	1.55
13	OPA-02	5'-TGCCGAGCTG-3'	650-1900	9	8	1	0	88.88%	14.22	2.54
14	OPN-08	5'-ACCTCAGCTC-3'	500-2000	8	5	3	0	62.5%	11.37	1.68
15	OPAF-14	5'-GGTGCGCACT-3'	800-3000	5	4	1	0	80%	8.57	1.01
16	OPD-02	5'-GGACCCAACC-3'	980-1480	3	2	1	0	66.66%	4.84	0.77
17	OPD-05	5'-TGAGCGGACA-3'	400-1400	4	3	1	0	75%	5.77	0.84
18	OPD-08	5'-GTGTGCCCCA-3'	800-3000	6	3	3	0	50%	10.6	1.02
19	OPD-18	5'-GAGAGCCAAC-3'	560-2200	4	2	2	0	50%	6.31	0.64
20	OPD-20	5'-ACCCGGTCAC-3'	350-1820	6	4	2	0	66.66%	8.66	1.65

are represented in Fig. 2. The resolving power (Rp) of primers ranged from 1.81 (OPA3 and OPA 20) to 1.33 (OPC2) where as the primer index (Pi) varied from 0.35 (OPN3) to 0.13 (OPA3 and OPA20). The primers OPA10 produced highest number of amplified products (10), whereas OPD2 produced least number of amplified loci (03). Percent polymorphism with the primer OPA2 was found to be 88.88% and with OPC5, OPA3, OPA4, OPA20, OPD8, OPD18 the range of polymorphism varied between 50.00-85.71 %. The average number of bands and polymorphic bands per primer was 6.05% and 4.1%. The genetic similarity between accessions CG1 & CG45 and CG5 &CG7 was high (95%) with Jaccard's similarity coefficient (Jaccard, 1908) of 0.95. However, lowest similarity (52%) was observed between *Calamus guruba* accessions CG1 & CG45 and CG36 & CG 42. Average genetic similarity among all accessions was found to be 0.76.

The dendrogram constructed using RAPD data (Fig. 4) divided the 45 accessions of 9 populations of *Calamus guruba* into two clusters; one with 5 accessions collected from Harishankar, Bolangir district and the other with 40 accessions from 8 different populations. Both the clusters shared a node at 62% level of similarity. Similarly, all 5 accessions (CG 31-CG 35) from Anandapur, Keonjhar district formed a single clade and got separated from 2 accessions collected from Chandaka Wildlife Sanctuary, Bhubaneswar (CG 28 and CG 29) with 77% genetic similarity between them. In the dendrogram, all other 35 accessions did not form clear clusters according to the population to which they belonged; and were found intermingled with accessions of other populations.

3.3. ISSR analysis

For analyzing the genetic diversity of 45 accessions of *Calamus guruba* representing 9 widely distributed natural populations, 25 ISSR primers were screened, of which 20 primers responded well resulting in amplification of reproducible bands. A total 138 bands were amplified which include 87 polymorphic and 51 monomorphic ones (Table 3). The ISSR banding pattern is shown in Fig. 3. The primer PCP2 produced highest number of amplified loci (11), whereas least number (4) of bands were amplified with primers Oligo2 (b) and Oligo5(b). The primer PCP1 and PCP9 were responsible for amplification of highest number of polymorphic bands (88.88%). The primer PCP2 amplified maximum number of 5 monomorphic loci.

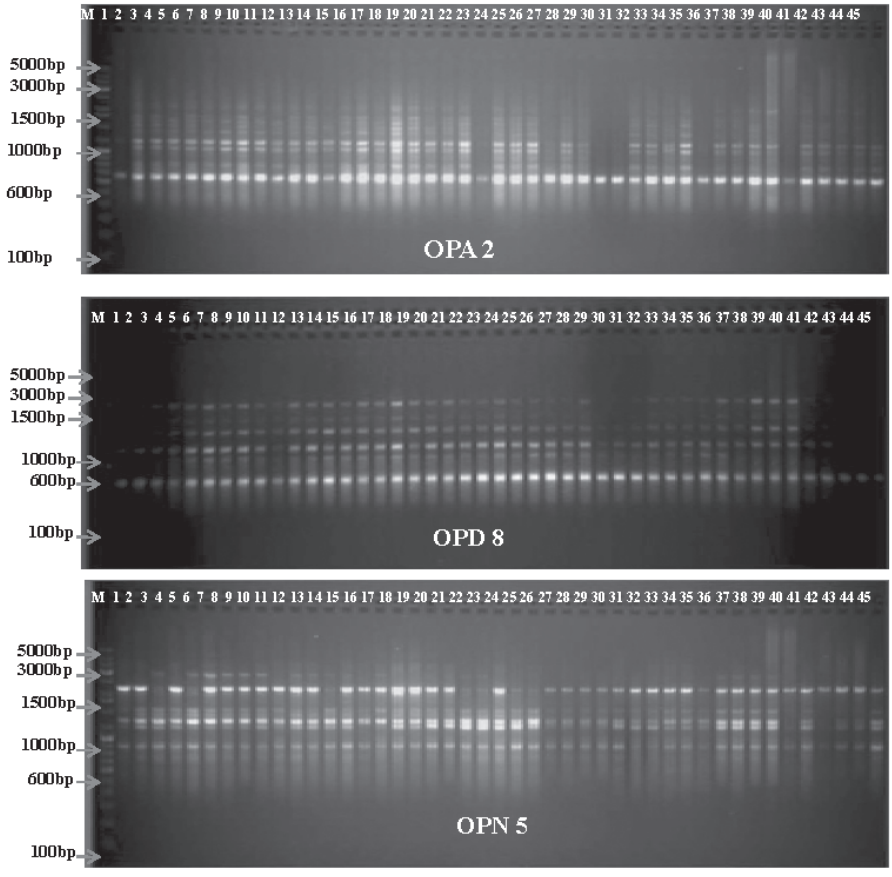

Fig.2. RAPD banding patterns of 45 accessions of *Calamus guruba* with primers OPA 2, OPD8 and OPN5; M-Genei ruler (Medium range) (100bp-5kb); Lanes 1 to 45, (CG1-CG45).

Table 3

Details of 20 ISSR primers used, bands amplified, percent polymorphism, primer index and resolving power

Sl No.	Primer Name	Primer Sequences	Range of amplicons in bp	Total No. of Bands	No. of Polymo-rphic Bands	No. of Mono-morphic Bands	No. of Unique Bands	% Polymo-rphic Bands (PPB)	Resolving Power	Primer Index
1	PCP-1	5'-GACGACGACG ACGAC-3'	200-1800	9	8	1	0	88.88%	14.48	2.35
2	PCP-2	5'- AGGAGGAGG AGGAGGAGG-3'	320-3050	11	6	5	0	54.54%	18.44	1.16
3	PCP-3	5'-GTCGGTGCGTG CGTGC-3'	300-1750	8	5	3	0	62.5%	9.86	1.16
4	PCP-5	5'-GAGAGAGAGAG AGAGAGAT-3'	620-1760	7	5	2	0	71.42%	10.62	1.24
5	PCP-6	5'-GACAGACAGA CAGACA-3'	620-1815	6	2	4	0	33.33%	10	0.16
6	PCP-7	5'-GGAGGAGGA GGA-3'	670-1950	6	5	1	0	83.33%	10.44	1.22
7	PCP-8	5'-GTGGTGGTGG TGGTG-3'	500-1830	7	3	4	0	42.85%	12.4	0.58
8	PCP-9	5'-GACACGACACG' ACACGACAC-3	250-1500	9	8	1	0	88.88%	9.73	1.67
9	PCP-12	5'-GACAGACAGACA GACAGACAGACAG ACAGACAG-3'	480-1600	8	7	1	0	87.5%	5.37	1.53
10	Oligo 1(b)	5'-AGAGAGAGAG AGAGAGG-3'	480-1200	6	4	2	0	66.66%	11.02	0.68
11	Oligo 2(b)	5'-GAGAGAGAG AGAGAGAG-3'	500-1050	4	2	2	0	50%	6.13	0.55
12	Oligo 3(b)	5'-GACAGACAG ACAGACA-3'	480-2600	7	3	4	0	42.85%	9.68	0.53
13	Oligo 4(a)	5'-GACAGACAG ACAGACAT-3'	480-2000	9	6	3	0	66.66%	12.9	1.01
14	Oligo 4(b)	5'-TGACAGACAG ACAGACA-3'	600-1900	7	4	3	0	57.14%	10.84	1.43
15	Oligo 5(a)	5'-GGACAGACAG ACAGACA-3'	600-1600	5	2	3	0	40%	9.6	0.34
16	Oligo 5(b)	5'-GACAGACAG ACAGACAG-3'	700-1815	4	0	4	0	0%	8	0
17	Oligo 8(a)	5'-CTCTCTCTC TCTCTCTG-3'	400-1900	8	5	3	0	62.5%	12.13	1.71
18	Oligo 9(a)	5'-GCTCTCTCT CTCTCTCT-3'	700-2000	6	4	2	0	66.66%	7.91	1.44
19	Oligo 11(a)	5'-GCTGTCTG TCTGTCTGT-3'	900-3200	5	4	1	0	80%	4.93	1.65
20	Oligo 11(b)	5'-CTGTCTGTC TGTCTGTC-3'	800-3000	6	4	2	0	66.66%	8.8	1.06

It could be derived from the similarity matrix (Jaccard, 1908) that accessions CG43 and CG44 of *Calamus guruba* collected from Harishankar hills of Bolangir district had maximum genetic similarity of 99%; lowest similarity (65%) was observed between accessions CG1 (Khallikote, Berhampur) and CG45 (Harishankar, Bolangir) and between CG30 (Chandaka WL sanctuary) and CG39 (Nuapada, Athgarh district). All the accessions of the nine populations of *Calamus guruba* had an average similarity of 0.82.

The dendrogram constructed on the basis of ISSR data divided the 45 accessions from 9 individual population of *Calamus guruba* divided them into two clusters; one with CG29 and CG30 both collected from Chandaka sanctuary having a similarity of 74% with the rest 43 accessions (Fig. 5). Further, out of the second major clade, 5 accessions of Harishankar (Bolangir) population (CG41- CG45) got segregated sharing a node at 75% level of similarity. In many cases, accessions of a particular population formed compact and clear groups in the dendrogram demonstrating their genetic relatedness as can be seen among the individuals of populations from Khallikote, Ganjam (CG1-CG5), Anandapur, Keonjhar (CG31-CG35), Nuapada, Athgarh (CG36-CG40) and Harishankar, Bolangir (CG41-CG45).

3.4. Combination of RAPD and ISSR analysis

Using pooled data of both ISSR and RAPD analysis, a total of 259 bands were observed in the 45 accessions of *Calamus guruba* collected from 9 different localities of Odisha. Of these, 169 bands were polymorphic and 90 were monomorphic in nature. However, no unique band was detected. The accession CG17 (Odapada, Cuttack district) produced the highest number of bands (229) taking all primers in to consideration and lowest number of bands (155) in the accession CG 42 (Harishankar, Bolangir).

The Jaccard's similarity coefficient (Jaccard, 1908) was maximum (0.94) between accessions CG1 and CG2 (Khalikote, Ganjam) and CG11 and CG12 (Jasabantpur, Jagatsinghpur) and the lowest (0.62) between CG23-CG42, CG29-CG39 and CG29-CG40 representing different locations. All the accessions of *Calamus guruba* had an average similarity of 0.79. The dendrogram constructed using the combination of RAPD and ISSR data (Fig. 6) placed five accessions of many populations together in single cluster as in case of CG1-CG5 (Khallikore, Berhampur), CG21-

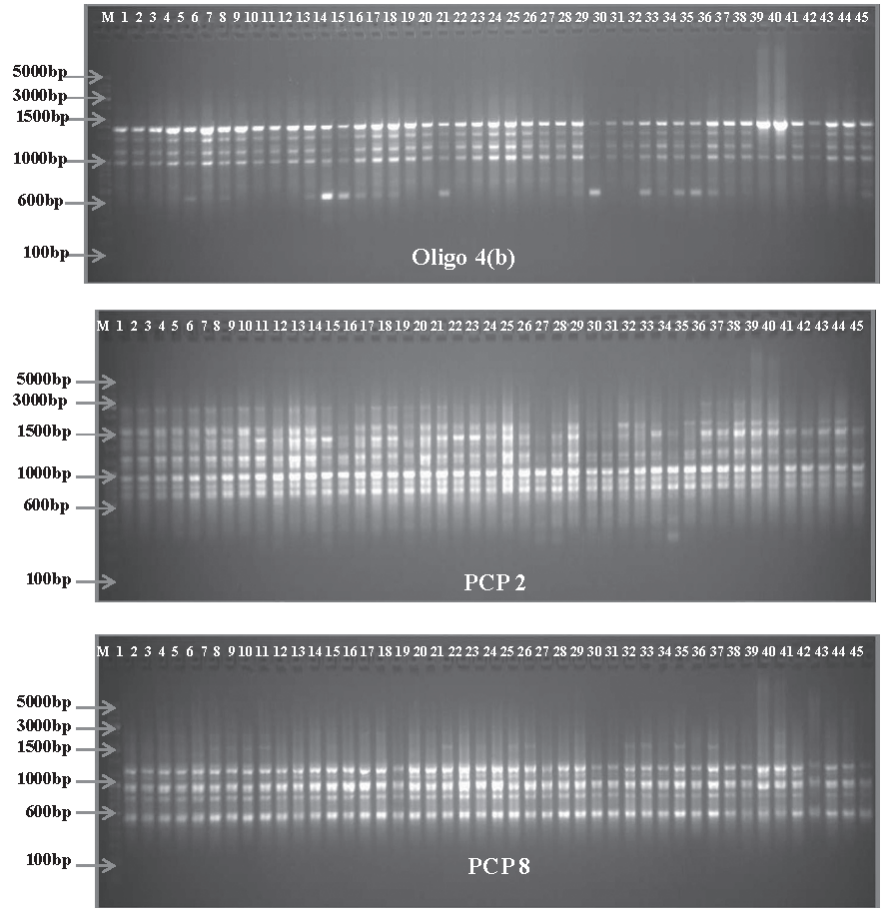

Fig. 3. ISSR banding patterns of 45 accessions of *Calamus guruba* with primers Oligo 4(b), PCP2 and PCP 8; M-Genei ruler (Medium range) (100bp-5kb); Lanes 1 to 45, (CG1-CG45).

CG25 (Bhatapada, Balugaon), CG31-CG35 (Anandapur, Keonjhar) and CG41-CG45 (Harishankar, Bolangir). Interestingly, the five accessions (CG41-CG45) collected from Harishankar (Bolangir) formed a segregated cluster from the rest 40 accessions at 70% level of similarity in the dendrogram.

4. Discussion

Molecular characterization of 45 individuals belonging to 9 populations of *Calamus guruba* collected from 7 districts of Odisha was made with a view to establish genetic relatedness with in individuals of a particular population and among populations and also identify populations with maximum genetic diversity to serve as source of seeds and other planting materials. Genetic richness can be assessed by estimating the genetic diversity parameters (viz. percentage of polymorphic loci and gene diversity index (Yeh, 2000) and in rattans and palms, high levels of genetic polymorphism have been documented (Bon *et al*., 1996; Cardoso *et al*., 2000). In the present study, two molecular markers (RAPD and ISSR) and their combination were used to study the inter- and intra-population genetic variability in *Calamus guruba* Buch.-Ham., a species with dwindling populations and genetic bottle-necks requiring conservation intervention and genetic improvement. The RAPD and ISSR technique has been successfully used in a variety of taxonomic and genetic relatedness studies on palms (Witono *et al*., 2006; Loo *et al*., 1999; Thawaro and Te-chato, 2009; Sathish and Mohankumar, 2007, Karim *et al*., 2015; Adawy, 2002) and the same is also applicable to assess genetic diversity of *Calamus guruba* as it has the ability to generate reproducible polymorphic markers. The results of the present study demonstrated the utility of RAPD and ISSR markers to characterize genetic relatedness and diversity among population of the studies species.

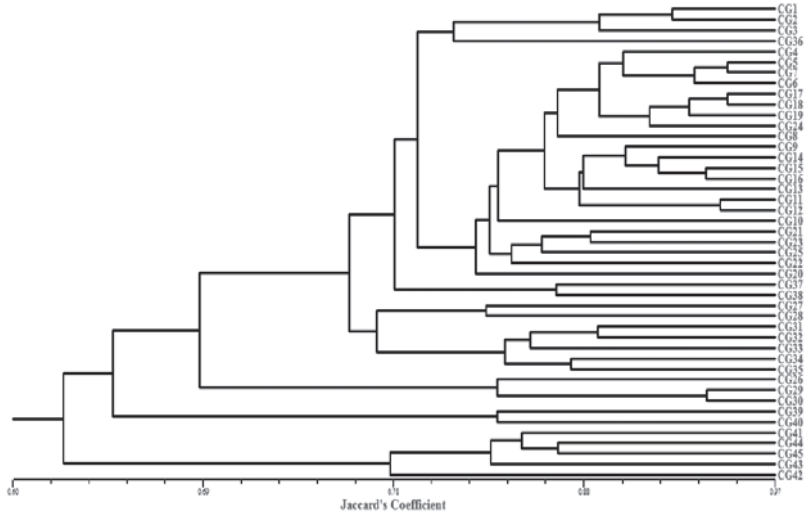

Fig. 4. Dendrogram showing genetic relationship among 45 accessions of *Calamus guruba* (CG1-CG45) using RAPD markers

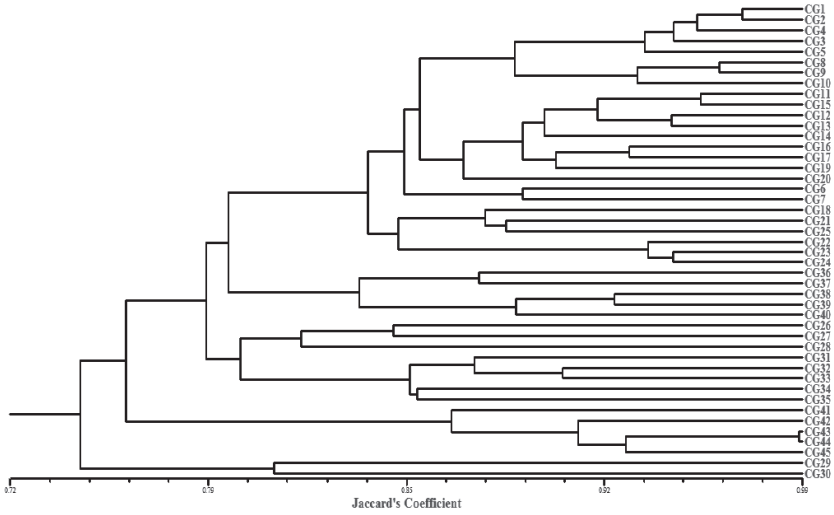

Fig. 5. Dendrogram showing genomic relationship among accessions of *Calamus guruba* (CG1-CG45) using ISSR markers

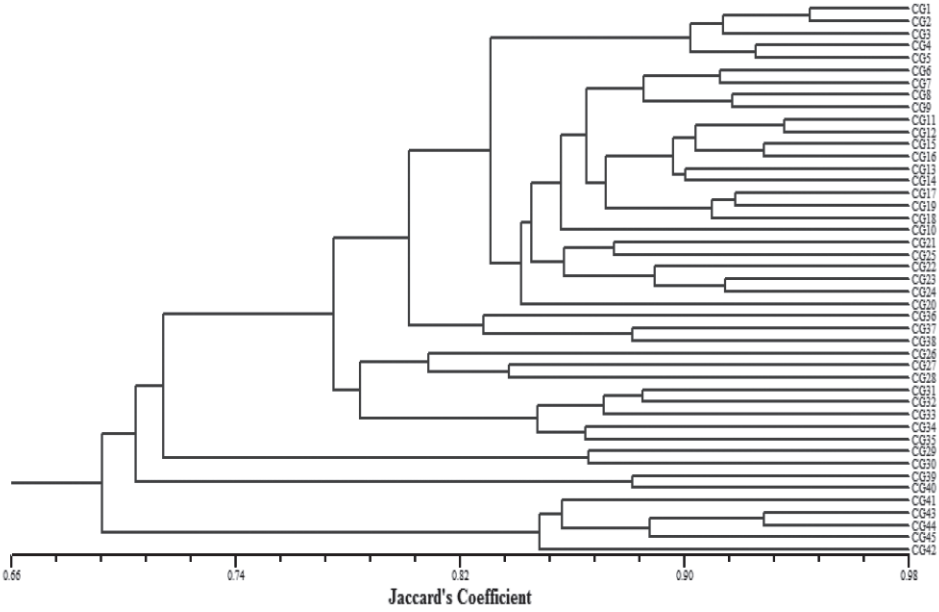

Fig. 6. Dendrogram showing genetic relationship among accessions of *Calamus guruba* (CG1-CG45) using combination of RAPD and ISSR markers

A total 121 bands were amplified using RAPD primers which include 82 polymorphic bands and the average percentage of polymorphism was 66%. Similar range of polymorphism has been obtained in different Indian and Sri Lankan populations of *Calamus rivalis* and *C. metzianus* using RAPD markers (Shreekumar *et al*., 2006). High level of polymorphism was also detected in India rattan genotypes by Sarmah *et al*. (2006) as in case of the present study. The genetic diversity of *Calamus thwaitesii* occurring in Western Ghats of India and Sri Lanka was estimated by using RAPD markers (Shreekumar and Renuka, 2006) and high percentage of polymorphism (40.00 to 60.83) was detected. They observed that majority of genetic diversity resides within population (70.79%) and only 29.21% among populations.

With ISSR primers, a total number of 138 bands got amplified. Out of these, 87 loci were polymorphic, 51 monomorphic and no unique band could be found. Highest level of polymorphism (88.88%) was observed with primers PCP1 and PCP9; the average percentage of polymorphism being 60.62%. Karim *et al*. (2015) reported as high as 90% polymorphism in *Phoenix dactylifera* in South Tunisia with the application of ISSR molecular markers. The finding of the present work corroborates the above data. Similar observations were also made by Zehdi *et al*. (2004) in Tunisian date palm.

Using RAPD and ISSR markers in combination, a total of 259 bands were produced in the present investigation, of which 169 bands (65.25%) were polymorphic. Adawy (2002) reported high polymorphism among and within the populations of Egyptian date palm (*Phoenix dactylifera*) applying RAPD and ISSR marker combination. Similar observations were made by Khierallah *et al*. (2014) in 17 date palm cultivars of Iraq. Our results are in conformity with the above observations on genetic diversity of palms.

The 45 accessions selected for genetic diversity assessment using molecular markers belonged to nine wild populations of Odisha with 5 individuals per population. Out of these, the population from Chandaka Wildlife Sanctuary, Bhubaneswar was found to possess maximum genetic diversity in terms of percent polymorphism (37.5%) and minimum (18.98%) in case of Khallikote, Ganjam population. In view of this, it is suggested that *Calamus guruba* population from Chandaka possessing higher genetic variability be used as source of seed and other planting materials for enrichment of genetic stock and raising quality planting materials for raising plantations in suitable forest habitats.

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