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Assessment of Genetic Diversity Among Six Species of Calamus (Arecaceae) in Eastern Ghats of India using Molecular Markers

Priyajeet Sinha, Pankajini Bal and Pratap Chandra Panda
DNA Fingerprinting Laboratory, Taxonomy and Conservation Division,
Regional Plant Resource Centre, Nayapalli, Bhubaneswar 751015, Odisha, India

Abstract:- Cane and rattans (Calamus spp.) are spiny climbing palms belonging to the subfamily Calamoideae of the family Arecaceae. They are principal non-timber forest products in international trade and have social and economic importance because of their unique characteristics such as strength, durability, looks and bending ability. Canes play a significant role in the livelihood of rural and tribal people in terms of income generation and employment. In Eastern Ghats of India, the genus Calamus is represented by six species; C. guruba, C. viminalis and C.latifolius being the widespread species. In the present paper, the genetic relationship and phylogeny of 6 species (30 accessions) of Calamus namely, C. guruba, C. viminalis, C. latispinus, C. latifolius, C. caesius and C. rotang have been assessed using RAPD and ISSR markers. RAPD analysis was performed using 20 decamer oligonucleotide primers. Out of a total of 272 bands amplified 5 were found to be monomorphic, while 267 bands were polymorphic including one unique band. In ISSR analysis, 20 primers amplified a total of 224 bands, of which, 210 were polymorphic in nature. By combining the data of both markers, a total of 496 bands were produced including 477 polymorphic loci. The dendrogram obtained from the UPGMA analysis revealed the extent of intra-species diversity and phylogeny among species. C. viminalis was found to be genetically more diverse; and C. viminalis and C. rotang were found to be phylogenetically closer to each other.

***Keywords*:-** *Genetic variation, Phylogenetic relationship, RAPD, ISSR, Calamus, Dendrogram*.

I. INTRODUCTION

Cane and rattans are spiny climbing palms belonging to the subfamily Calamoideae of the family Arecaceae or Palmae (Uhl and Dransfield, 1987). They comprise about 600 species under 14 genera, which are distributed in equatorial Africa, South Asia, Southern China, the Malay Archipelago, Australia and the Western Pacific. Rattans, prickly climbing palms or canes with solid stem are principal non-timber forest products in international trading. Rattans have properties that make them very popular as raw materials for furniture, construction, handicrafts and other industries. They play a significant role in the livelihood of rural and tribal people in terms of income generation and employment. More than a million people in India belonging to tribal communities and backward classes, depend upon canes and cane crafts for their sustenance (Sarmah *et al*., 2007). The largest rattan genus is *Calamus* with ca. 370 species (Mabberley, 1997). *Calamus* is predominantly an Asian genus and ranges from the Indian subcontinent and south China southwards and east through the Malaysian region to Fiji, Vanuatu and tropical and subtropical parts of eastern Australia. In India, the genus is represented by 32 species (Karthikeyan *et al.,* 1989) and 22 species are reported to occur in Peninsular India alone.

Eastern Ghats (between 9^{o} 95' to 20^{o} 74' N latitudes and 77^{o} 22' to 85^{o} 20' E longitudes) are a long chain of isolated, broken hills ranges and elevated plateus, running along the Indian east coast and passes through the states of Odisha, Andhra Pradesh and Tamila Nadu. Six species of *Calamus* are reported to occur in Eastern Ghat region (Fischer, 1932; Saxena and Brahmam, 1994; Mahapatra *et al*., 2012).

The species of *Calamus* are wind-pollinated and their phenological behaviour is 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). Due to rapid urbanization and overexploitation for trade purposes, the natural reserves of rattan are fast declining, causing genetic erosion of the existing resources. All these necessitate study of genetic diversity among and within species and populations of genus *Calamus*.

Genetic variation at the intra-species level is a prerequisite for future adaptive change or evolution, and has profound implications for species conservation (Schaal *et al*., 1991). Understanding genetic variation within and between populations is essential for the establishment of effective and efficient conservation practices for rare species (Hamrick and Godt, 1996). DNA fingerprinting is an effective method for palm identification, examining genetic diversity and phylogenetic analysis. Different markers used in DNA fingerprinting and phylogeny analysis of canes and rattans

include RAPD, RFLP, AFLP, ISSR and SSR but each of them have some limitations related to their cost, ease of use, robustness, dominance/co- dominance and polymorphism level and are playing increasingly important roles in investigating genetic diversity within and among populations (Nebauer *et al*., 2000).

The present study aims at establishing of inter-population phylogenetic relationship among the six species of *Calamus* (viz. *C. guruba*, *C. rotang, C. latifolius, C. viminalis, C. latispinus* and *C. caesius*) in Eastern Ghats of India using molecular markers (RAPD and ISSR) and their combination, which will assist in identifying closely related species for breeding and other improvement programmes.

II. MATERIALS AND METHODS

A. Plant materials

Thirty (30) individuals belonging to six different populations of each species of *Calamus* (viz. *C. guruba*, *C. rotang, C. latifolius, C. viminalis, C. latispinus* and *C. caesius*) were studied (Fig. 1). Leaves from each individual plant were collected at random and samples of each species were pooled together for genomic DNA isolation. The botanical names and details of collection localities of the species studied are provided in Table-1.

B. Genomic DNA isolation

DNA was isolated from freshly collected young leaves by the 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 (Eppendorf-5430), the upper aqueous phase was separated, 1/10 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(hydroxy methyl) 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/ ISSR analysis.

C. Randomly Amplified Polymorphic DNA (RAPD) analysis

For RAPD analysis, PCR amplification of 25 ng of genomic DNA was carried out using 25 standard decamer oligonucleotide primers out of which 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 produced satisfactory amplification. The RAPD analysis was performed by the methods of Williams *et al.* (1990). Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5 μl of 10X assay buffer (100 mm Tris- HCl, pH 8.3, 0.5 m KCl and 0.01% gelatin), 1.5 mm $MgCl_2$, 200 μm each of dNTPs, 20 ng of primer and 0.5 U Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler, Gene Amp$^@$ PCR System 9700(Applied Biosystems). The first cycle consisted of denaturation of template DNA at 94 ^{0}C for 5 min, primer annealing at 37 ^{0}C 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 were maintained as in the case of the first cycle. The last cycle consisted of only primer extension at 72 ^{0}C for 7 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide solution (0.5 μg/ml of gel solution). The size of the amplicons was determined using standards (GeNei Medium Range DNA Ruler, Merk Millipore, Merk Specialities Private Limited, Mumbai). The DNA fragments were observed under UV light and photographed.

D. Inter simple sequence repeat (ISSR) analysis

Inter Simple sequence repeats were used for PCR amplification. Thirty numbers of 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). Among those primers twenty 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)) were produced reproducible bands. The ISSR analysis was performed as per the methodology given by (Zietkiewicz *et al*., 1994). Each amplification reaction mixture of 25 µl contained 20ng of template DNA, 2.5µl of 10X assay buffer (100mM Tris-HCl pH 8.3, 0.5M KCl and 0.01%gelatin), 1.5mM $MgCl_2$, 200µm each of dNTPs, 44ng of primer and 0.5U Taq DNA polymerase. 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 2% 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 55°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.

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E. Data analysis

The presence/absence of bands in RAPD/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): Rp = ΣIB, where IB (band informativeness) = 1 D [2 \ (0.5 D *P*)], *P* being the proportion of the 5 species containing the band.

III. RESULTS

A. 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} with good quality, which was evident from the Agarose gel analysis.

B. Analysis of genetic diversity and molecular phylogeny of the genus Calamus

Two PCR-based molecular markers i.e. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) were employed to assess the genetic diversity and establish phylogenetic relationship among 30 accessions of six species of *Calamus* belonging to the palm family Arecaceae. The results of molecular characterization of each of these groups are presented separately and discussed and interpreted at the end.

C. Genomic relationship as inferred from RAPD analysis

Out of 25 primers screened, 20 RAPD primers yielded reproducible bands in all the six species of *Calamus*. The DNA profiles obtained from RAPD analysis are represented in Table-2. A total of 272 amplified loci were generated including 267 polymorphic bands. Only five bands were monomorphic in nature and one was unique. Twenty (20) primers resulted in the amplification products of various sizes ranging from 250 bp to 3700 bp. The resolving power (Rp) of primers ranged from 6.6 (OPA 05) to 13.73 (OPC 02) where as the primer index (Pi) varied from 3.28 (OPD-08) to 6.3 (OPN-05). The PIC value of the primers varied from 0.863 (OPD-08) to 0.928 (OPN-05).The RAPD banding pattern is represented in Fig. 2. The primers OPN04 and OPN05 produced highest number of amplicons (16), whereas OPD 08 and OPD 18 produced least number of loci (10). Fifteen primers such as OPA 02, OPA 04, OPA 05, OPA 1O, OPA 16, OPC 05, OPD 02, OPD 05, OPD 18, OPD 20, OPN 03, OPN 04, OPN05, OPN 06, OPN08 showed 100% polymorphism and OPD 08 had the least polymorphism (90%) with all the primers used. The average percentage of polymorphism was 98.1%. The average number of bands and polymorphic bands per primer was 13.6% and 13.35%. Primer OPA 03, OPA20, OPAF14, OPC 02 and OPD 08 each produced single monomorphic bands and primer OPN03 produced one unique band.

The similarity matrix was calculated using Jaccard's similarity coefficient (Jaccard, 1908) for the pooled data of all RAPD primers. The accessions CLA1 and CLA2, CLA1 and CLA3, CLA2 and CLA3 of *Calamus latifolius* had highest similarity (100%) between them. Similarity of as low as 0.16% was observed between *Calamus guruba* and *Calamus rotang* accessions (CG1 and CR1, CG1and CR2, CG1 and CR4, CG1 and CR5). All the accessions of *Calamus* had an average similarity of 0.48.

The dendrogram constructed using RAPD data from 30 accessions of six species of Calamus was primarily formed of two major clusters- 25 accessions of five Calamus species in one cluster and five accessions of Calamus rotang in the other, sharing a similarity of 22%. The major cluster was further divided into two sub-clusters, one with C. guruba-C. viminalis accessions and the other with C. latifolius- C. caesius- C. latispinus accessions; both groups sharing similarity of 23%. The five accessions of Calamus guruba collected from Khandalabandha (Berhampur Forest Division) and five accessions of Calamus viminalis collected from Sisupalgarh (Bhubaneswar of Khurda Forest Division) formed separate clades and had genetic similarity of 30%. In the C. latifolius- C. caesius- C. latispinus clade, the accessions of C. latispinus collected from Rajin RF (Balugaon range) of Khurda Forest Division were the first to segregate from the rest at a similarity level of 26%. Subsequently, accessions of C. latifolius and C. caesius formed two distinct groups and got separated from each other sharing a node at 33% similarity level.

In general, all accessions of a particular species formed compact cluster in the dendrogram validating its species affiliation. Five (5) accessions of *Calamus guruba* were further segregated into two sub-groups, one with the lone accession (CG1) and the other with four accessions (CG2, CG3, CG4 and CG5), both sharing a genetic similarity of 92%. The genetic similarity among these four accessions varied in the range of 95-99%. Among the five accessions of *Calamus viminalis*, CV1 got clearly segregated from the rest (CV2, CV3, CV5 and CV4) with an average genetic similarity of 96%. Further, genetic relatedness between the accessions CV4 & CV2 and CV3 & CV5 was observed to be 97%.

Out of the five accessions of Calamus latifolius collected from Rajin RF (Balugaon range) of Khurda Forest Division, four accessions (CLA1, CLA2, CLA3 and CLA4) formed a cluster and got segregated from CLA5 at the level of similarity of 97%, as shown in the cladogram constructed using RAPD data. The genetic similarity among the four accessions varied in the range of 98-99%. In Calamus caesius, the five accessions collected from Bhatapada (Balugaon range) of Khurda forest division was separated in to two clades in the dendrogram; one with three accessions CC1, CC2 and CC3

and the other with CC4 and CC5 sharing a node at a similarity level of 90%. The range of variation in genetic similarities among individual accessions was found to be 95-97%. Five accessions of Calamus latispinus were collected from Dhatangarh (Jagatsinghpur range) of Cuttack Forest Division. Of the five accessions, two distinct groups could be observed; one with three accessions CLP1, CLP2 and CLP3 and the second with two accessions CLP4 and CLP5. Both the clusters had a genetic similarity of 86%. The genetic closeness among the accessions varied from 95% to 99%. Similarly, out of the five accessions of Calamus rotang, which belong to one population from Bhatapada (Balugaon range) of Khurda Forest Division, two accessions (CR1 and CR2) got separated from rest three (CR3, CR4 and CR5) with similarity of 85% between the clades. Further, the genetic relatedness between CR1 and CR2; and CR3 and CR4 were 96% and 90%, respectively.

D. Genomic relationship as inferred from ISSR analysis

The data obtained by molecular fingerprinting of 30 accessions belonging to six species of *Calamus* using ISSR primers are represented in Table-3. Out of the 30 ISSR primers screened, 20 primers produced good and reproducible amplified bands. A total 224 bands were amplified which include 210 polymorphic and 14 monomorphic bands. In this case, no private band could be detected. The size of amplicons ranged from 170 bp to 4300bp. The resolving power (Rp) of primers ranged from 7.26 (Oligo 5b) to 13.2 (PCP 1) and the primer index (Pi) varied in the range of 2.44 (Oligo9a) to 6.04 (Oligo 8a). The PIC value of the primers varied from 0.825 (PCP-12) to 0.917 (Oligo 8a).The ISSR banding pattern is shown in Fig. 3. The primer Oligo 8(a) produced highest number of amplified loci (16) whereas, Oligo9a produced least number (8) of bands. Ten primers (PCP3, PCP8, PCP9, Oligo1b, Oligo2b, Oligo4a, Oligo5b, Oligo8a, Oligo11a and Oligo11b) yielded 100% polymorphic bands and only the primer PCP6 gave least polymorphism of 66.66%. The average percentage of polymorphism was 93.18. The average number of amplified bands and polymorphic bands per primer was 11.2 and 10.5 respectively. PCP06 amplified maximum number of monomorphic loci (3).

The similarity matrix was calculated using Jaccard's similarity coefficient (Jaccard, 1908) for the pooled data of all ISSR primers. Calamus guruba (CG3 and CG4), Calamus viminalis (CV3 and CV4) and (CV4 and CV5) showed maximum similarity of 1.0 (100%) and the lowest (0.50) was observed between Calamus guruba and Calamus caesius (CG3 and CC4, CG4 and CC2, CG5 and CC2) and between Calamus viminalis and Calamus caesius (CV1 and CC5, CV2 and CC2, CV2 and CC3, CV2 and CC4, CV2 and CC5, CV3 and CC3). All the accessions of six species of Calamus had an average genetic similarity of 0.71.

The dendrogram constructed using ISSR data of 30 accessions of six species of Calamus formed two primary clusters. The minor clade contained 10 accessions; five of Calamus guruba collected from Khandalabandha of Brahmapur Forest Division and five of Calamus viminalis collected from Sisupalgarh (Bhubaneswar) of Khurda Forest Division. The major group had 20 accessions: five accessions of Calamus latifolius collected from Rajin RF of Balugaon range (Khurda Forest Division), five accessions of Calamus caesius collected from Bhatapada of Balugaon range (Khurda Forest Division), five accessions of Calamus latispinus from Dhatangarh of Jagatsinghpur range (Cuttack Forest Division) and five Calamus rotang accessions from Bhatapada of Balugaon range (Khurda Forest Division). These two main clusters showed a genetic similarity of 55%.

The smaller cluster of 10 accessions of C. guruba-C. viminalis was further divided into two distinct clades of 5 accessions each of Calamus guruba and Calamus viminalis having 59% genetic relatedness. The intra-population genetic diversity and similarity of C. guruba and C. viminalis varied considerably among individuals but accessions of each species formed distinct clusters justifying their taxonomic affiliation as species. In the major cluster of 20 accessions comprising of four species of Calamus, there was further division into two clades (C. latifolius-C. caesius and C. latispinus-C. rotang) at 57% level of similarity. Again, the accessions of Calamus latifolius and Calamus caesius got segregated to distinct clades showing 71% similarity between the two species. The intra-population genetic relatedness of Calamus latifolius varied in the range of 94% to 98%. Similarly, among the accessions of Calamus caesius, the genetic similarity ranged from 97% to 99%. In Calamus latispinus-Calamus rotang clade, the accessions of both the species formed clear clusters and shared a node at the level of similarity 67%. The genetic similarity within the population of Calamus latispinus was between 95-98% and for Calamus rotang the range was between 95-99%.

The results of the study as derived from the phylogenetic tree indicate that all the six species of Calamus maintain their genetic identity as biological species with varying levels of genetic similarities. Phylogenetically, Calamus guruba and Calamus rotang were found to be distantly related, where close relationship between Calamus latifolius and Calamus caesius could be noticed.

D. Assessment of genomic relationship with the application of RAPD and ISSR marker combination

A total of 496 bands were produced with the use of both ISSR and RAPD markers in all the six species and accessions of *Calamus*, out of which 477 bands were polymorphic, 19 were monomorphic and only one band was unique in nature. *Calamus viminalis* (CV1) produced the highest number of fragments (243) with all the primers, whereas 165 bands, the lowest, were amplified in case of *Calamus rotang* (CR1).

Jaccard's similarity derived from the data obtained from both combined RAPD and ISSR marker analysis revealed that the accessions CG3 and CG4 of Calamus guruba had highest similarity (0.99) and the lowest (0.23) was observed between Calamus guruba and Calamus latispinus accessions, between Calamus guruba and Calamus rotang (CG1 and CR5) and between Calamus latifolius and Calamus rotang (CLA1 and CR1, CLA2 and CR1, CLA4 and CR2, CLA5 and CR2). All

the 30 accessions of six species of Calamus had an average similarity of 0.51 (Table-4).

The dendrogram constructed using combined RAPD and ISSR data revealed that all the six species of Calamus maintain their taxonomic identity and all accessions of a particular species form compact cluster. The cladogram, now constructed, divided the 30 accessions of six species of Calamus into two major clusters. The smaller clade was comprised of 5 accessions each of C. latispinus and C. rotang and the larger one with 20 accessions of four other species namely, C. guruba, C. viminalis, C. latifolius and C. caesius. Both the major groups shared a common node at 27% level of genetic similarity (Fig. 4). The members of Calamus guruba and Calamus viminalis, which comprised the larger cluster as described above, got separated from each other in the dendrogram sharing a similarity of 35%. The infra-species variability in Calamus guruba ranged from 95% to 99% and that of Calamus viminalis from 97% to 99%. Similarly, C. latifolius and C. caesius segregated from each other at a genetic similarity level of 40%. The within population genetic variability was found to be between 91-98% in Calamus caesius and in Calamus latifolius from 95% to 97%.

In the minor cluster, the accessions of Calamus latispinus and Calamus rotang were separated from each other at 31% similarity level in the dendrogram. The five accessions of Calamus latispinus had genetic relatedness ranging from 88% to 98% and similarly, the range was between 88-97% in Calamus rotang accessions.

IV. DISCUSSION

Twenty RAPD primers were used for amplification of bands for genetic diversity analysis. A total of 272 bands were scored in all the six species (30 accessions) of Calamus, out of which 98.16% (267 bands) were polymorphic. With 20 ISSR primers, as many as 210 (93.18%) polymorphic loci were generated. The high level of polymorphism detected in the present investigation could be due to dioecious nature of the species, which facilitated cross-pollination and increased genetic diversity. Sarmah et al. (2007) found similar high polymorphism in 15 rattan genotypes of Northeast India using RAPD markers. In the above study, 13 accessions of 8 species of Calamus and one species of each of Daemonorops and Plectocomia, were included. RAPD technique was also found suitable for genetic diversity assessment among 10 Malaysian rattan species and ten primers of OPA series were used to obtain species-specific amplification patters (Changtragoon et al., 1997). In an attempt to validate the taxonomic status of two closely related Calamus species of Western Ghats namely, C. rivalis and C. metzianus, Sreekumar et al. (2006) conducted molecular analysis using RAPD marker and detected pronounced polymorphism (95%) across all primers. Witono & Rondo (2006) made a genetic analysis of 13 species of Pinanga (Palmae) using ISSR markers and found exceptionally high (98.99%) genetic polymorphism. Out of 9 ISSR primers, 8 primers produced 100% polymorphic loci. Bacon & Bailey (2006) also obtained higher polymorphism in Chamaedorea tepejilote and C. alternans. It can be derived that the genetic polymorphism detected in calamoid palms is comparable with that found in other allogamous and perennial species (Forapani et al., 2001; Kapteyn and Simon, 2002; Li and Luukkanen, 2001; Wachira et al., 1995). The average number of loci amplified per accession from both RAPD and ISSR markers was highest (238.8) in C. viminalis followed by C. guruba (207.2), C. caesius (196.0) and least in C. rotang (169.6). It can be presumed that with the standard set of primers, C. viminalis is genetically more diverse in comparison to other studied species.

Pair-wise genetic similarities generated by Jaccard's coefficient ranged from 0.99 between two accessions of Calamus guruba (CG3 and CG4) from Khalikote, Ganjam to 0.23 between C. guruba and Calamus latispinus accessions (CG1-CLP1, CG2-CLP2, CG3-CLP3). The accessions collected from a particular geographical location exhibited close genetic similarity. Sarmah et al. (2007) also observed similar genetic closeness between two accessions of Calamus tenuis from Golaghat, Assam. Among the genotypes of Calamus now analysed, the most divergent pairs (0.23 similarity value) were several accessions of C. guruba collected from Berhampur and C. latispinus from Jagatsinghpur of Odisha. In other allogamous species, similar results have also been reported (Forapani et al., 2001; Kapteyn and Simon 2002; Nebauer et al., 1999).

The dendrogram based on the cluster analysis revealed the distinct identities of all the six species of Calamus. All the five accessions of a species came together in the cladograms constructed using RAPD and ISSR data separately and in combination justifying their status as biological species. Witono & Rondo (2006) estimated the genetic similarity among the 13 species of Pinanga using Inter Simple Sequence Repeat (ISSR) markers and observed alignment of species based on their geographical distribution. They opined that possibly this genetic similarity was caused by their geographical history and the natural barriers between them. Hahn & Sytsma (1999) also made similar observations on phylogeny of Southeast Asian species of Caryota. C. latispinus and C. rotang came together in a smaller cluster and another cluster was formed with C. guruba, C. viminalis, C. caesius and C. latifolius. Though no formal infra-generic classification of Calamus is yet available, Beccari & Hook. f. (1894) placed the then known 160-170 species of Calamus in 13 groups based on morphology. Of these, C. viminalis and C. rotang come under group-III, C. guruba under group-IV; C. latifolius and C. caesius under the group-X. In the present molecular phylogenetic study, C. latifolius and C. caesius were found to be genetically closer to each other, which is in agreement with the classification put forth by Beccari & Hooker (1894). However, genetic closeness between C. guruba and C. viminalis could not be explained. Besides, the coming together of C. rotang and C. latispinus, a taxonomically less-known species, in a common clade in the phylogenetic tree needs some further study.

V. CONCLUSION

The present research involves species, population and intra-population level genetic diversity assessment of six species of *Calamus* (*C. guruba, C. latifolius, C. viminalis, C. latispinus, C. rotang* and *C. caesius*) with the application of PCR-based molecular markers. This is the first of its kind as none of the species included in the work has been subjected to molecular studies in India and elsewhere. In the current investigation, 20 RAPD and 20 ISSR markers were used. With both the markers, very high level of polymorphism (93.18%-98.16%) was detected, which may be attributed to the dioecious nature of the species, which facilitated cross-pollination and increased genetic variation. Of the six species, *C. viminalis* was found to possess more genomic diversity compared to other species. In the phylogenetic tree constructed using molecular data, all accessions of a particular species formed distinct justifying their status as biological species. *Calamus viminalis* and *C. rotang* come under one cluster and *C. latifolius* and *C. caesius* under the other in the cladogram, which is in agreement with the intra-generic classification proposed by Beccari & Hooker (1894) based on morphological characters. However, genetic closeness between *C. guruba* and *C. viminalis* could not be explained from molecular data interpretation.

VI. ABBREVIATIONS

CTAB, Cetly trimethyl ammonium bromide; AMOVA, Analysis of molecular variance; UPGMA, Unweighted pair group method using arithmetic averages.

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Fig 1:- Natural habitats of canes (a) Calamus guruba (b) Calamus viminalis (c) Calamus latifolius (d) Calamus latispinus (e) Calamus rotang and (f) Calamus caesius

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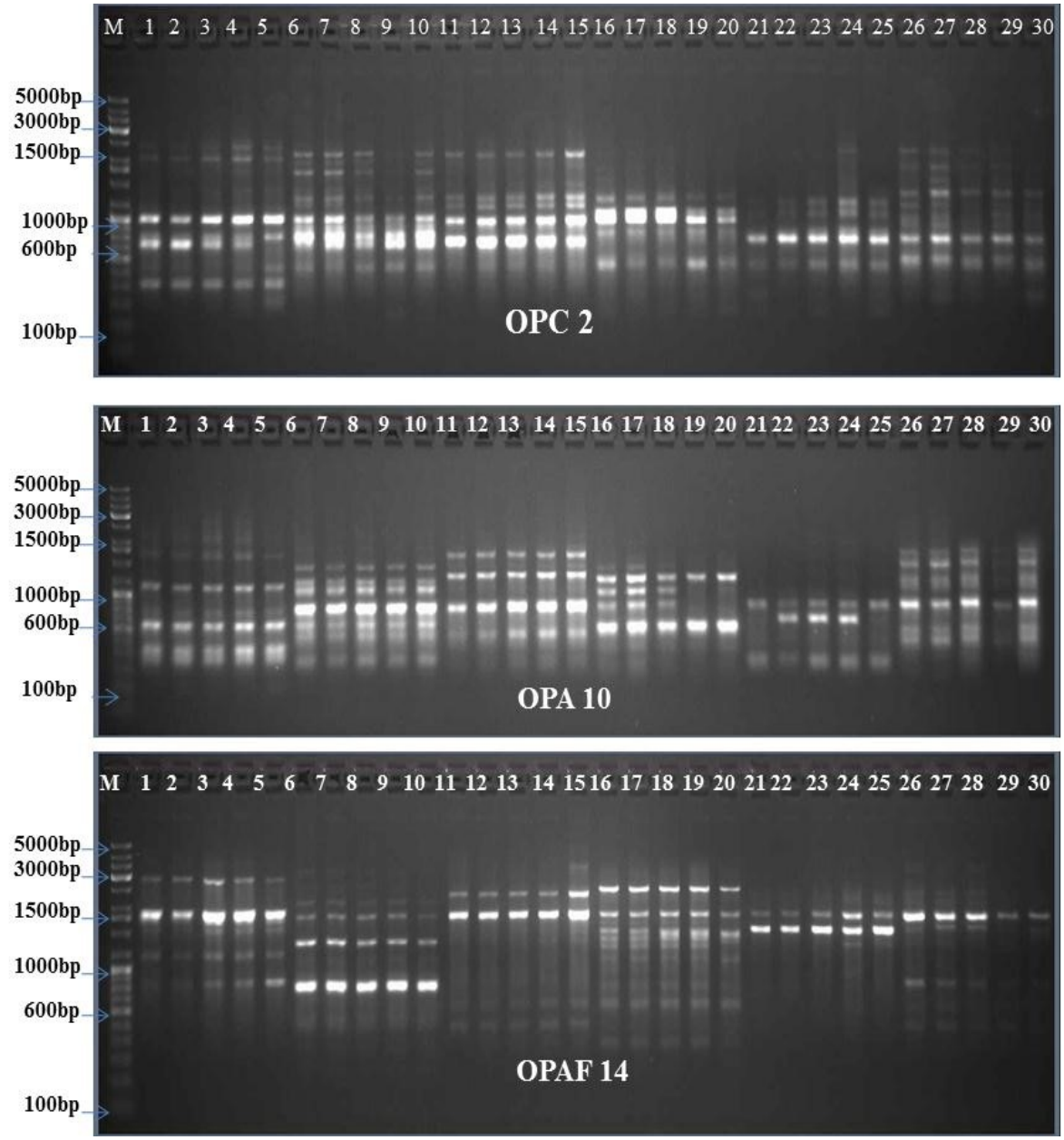

Fig 2. RAPD banding patterns of 30 accessions of six species of *Calamus* as revealed by the primer OPC2, OPA10 and OPAF 14

[Gene ruler (Medium range) 5kb: lanes 1 to 5; C.guruba: lanes 6 to 10; C. viminalis: lanes 11-15;C.latifolius: lanes 16-20; C. latispinus: lanes 21-25; C.rotang and lanes 26-30; C.caesius]

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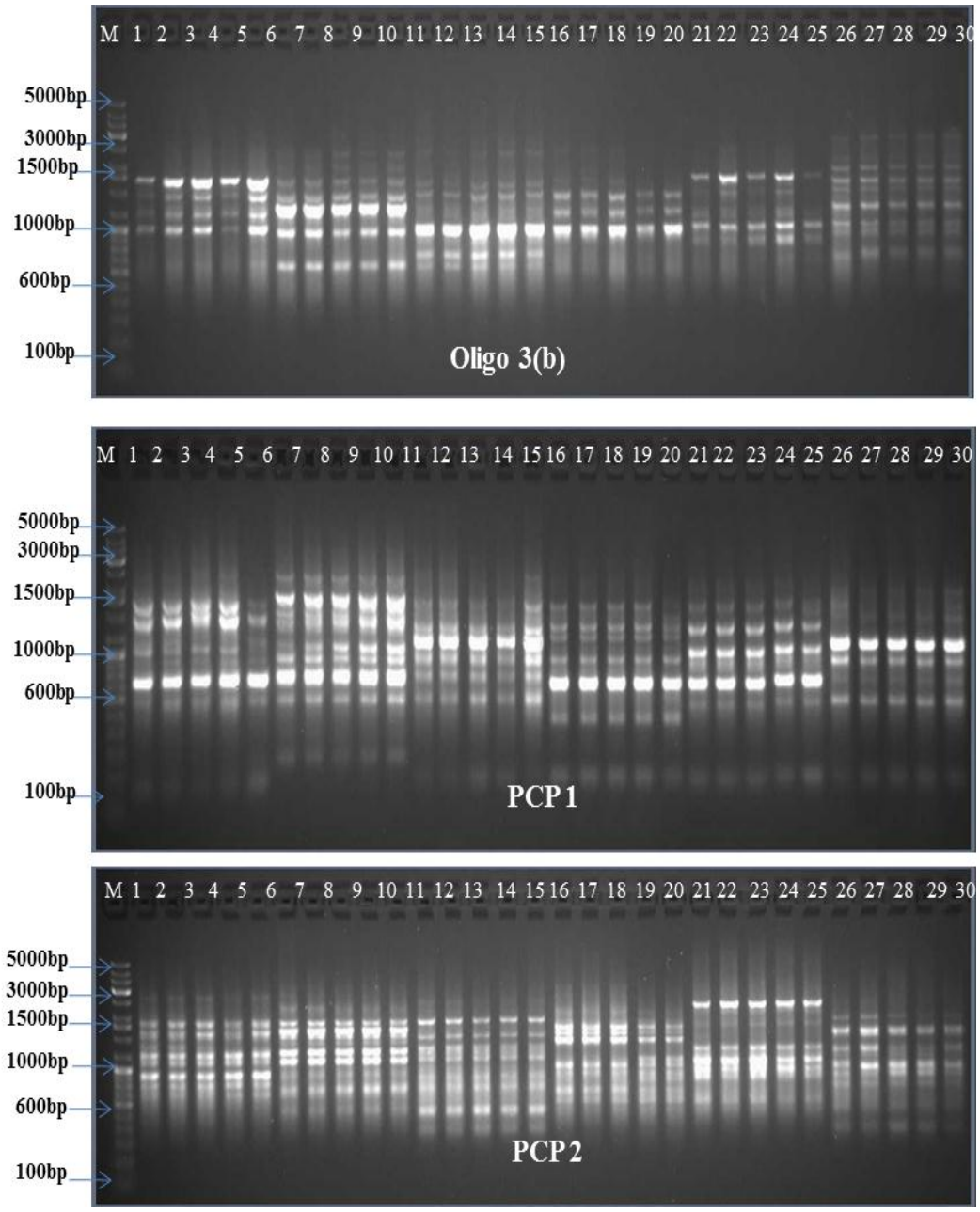

Fig 3:- ISSR banding patterns of 30 accessions of six species of *Calamus* as revealed by the primer Oligo 3(b), PCP1 and PCP2

[Gene ruler (Medium range) 5kb: lanes 1 to 5; C.guruba: lanes 6 to 10; C. viminalis: lanes 11-15; C. latifolius: lanes 16-20; C. latispinus: lanes 21-25; C.rotang and lanes 26-30; C. caesius]

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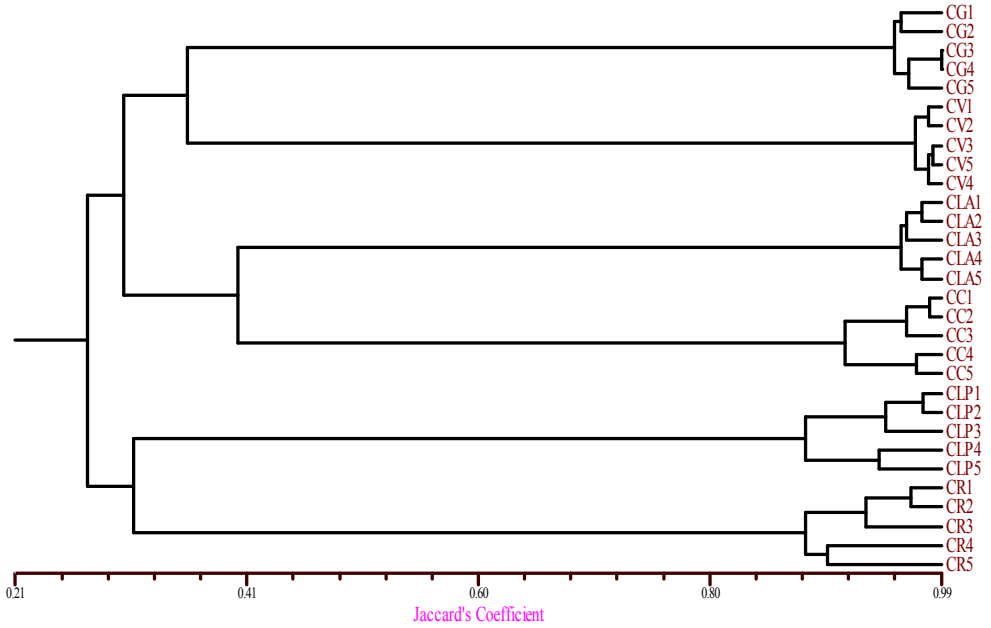

Fig 4:- Dendrogram showing genetic relationship among 30 accessions of six species of *Calamus* as revealed from combined RAPD and ISSR analysis

Species	Code	Forest Division	Forest Range	Location	Latitude	Longitude	Altitude
Calamus guruba	CG1-CG5	Brahmapur	Brahmapur	Khandalabandha	N 19^0 13.581'	E 084^0 39.262'	398 ft
Calamus viminalis	CV1-CV5	Khurda	Bhubaneswar	Sisupalgarh	N 20^0 14'.008"	E 085^0 50'.903"	220 ft
Calamus latifolius	CLA1-CLA5	Khurda	Balugaon	Rajin	N 19^0 53' 01.2"	E 084^0 59' 32.7"	1741 ft
Calamus latispinus	CLP1CLP5	Cuttack	Jagatsinghpur	Dhatangarh	N 20^0 18' 34.6"	E 086^0 14' 45.00"	621 ft
Calamus rotang	CR1-CR5	Khurda	Balugaon	Bhatapada	N 19^0 49' 53.9"	E 085^0 01' 24.2"	301ft
Calamus caesius	CC1-CC5	Khurda	Balugaon	Bhatapada	N 19^0 49' 54.2"	E 085^0 01' 23.7"	290 ft

Table 1. Details of collection sites of six species of *Calamus* from Eastern Ghats of India and codes used for each of them in the text.

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SL. NO	Primer Name	Primer Sequences	GC (%)	Annealing temperature $T_A(^0C)$	Range of amplification (bp)	Total number of bands (TNB)	Number of polymorphic bands (NPB)	Number of monomorphic bands (NMB)	Number of unique bands (NUB)	Percentage of polymorphic band PPB (%)	Resolving Power (Rp)	Primer Index (Pi)	Polymorphism Information Content (PIC)
11	OPA-02	5'-TGC CGA GCT G-3'	70	37	450-2100	13	13	0	0	100	7.33	4.88	0.909
2	OPA-03	5'-AGT CAG CCA C-3'	60	37	380-2100	15	14	1	0	93.33	10.8	5.57	0.910
3	OPA-04	5'-AAT CGG GCT G-3'	60	37	300-2400	14	14	0	0	100	11.4	5.35	0.906
4	OPA-05	5'-AGG GGT CTT G-3'	60	37	360-2400	13	13	0	0	100	6.6	4.63	0.909
5	OPA-10	5'-GTG ATC GCA G-3'	60	37	370-1750	13	13	0	0	100	9.46	4.85	0.897
6	OPA-16	5'-AGCCAGCG AA-3'	60	37	250-2900	15	15	0	0	100	9.86	5.81	0.916
7	OPA-20	5'-GTT GCG ATC C-3'	60	37	400-2900	13	12	1	0	92.30	11.4	4.38	0.891
8	OPA F-14	5'-GGT GCG CAC T-3'	70	37	380-3600	14	13	1	0	92.85	12.33	5.41	0.909
9	OPC-02	5'-GTG AGG CGT C-3'	70	37	260-2200	15	14	1	0	93.33	1373	5.31	0.910
10	OPC-05	5'-GAT GAC CGC C-3'	70	37	280-3000	14	14	0	0	100	11.73	6.09	0.918
11	OPD-02	5'-GGA CCC AAC C-3'	70	37	360-2900	12	12	0	0	100	11.7	4.63	0.896
12	OPD-05	5'-TGA GCG GAC A-3'	60	37	310-2500	12	12	0	0	100	6.8	4.51	0.901
13	OPD-08	5'-GTG TGC CCC A-3'	70	37	570-3000	10	9	1	0	90	9.66	3.28	0.863
14	OPD-18	5'-GAG AGC CAA C-3'	60	37	500-2500	10	10	0	0	100	7.53	3.67	0.864
15	OPD-20	5'-ACC CGG TCA C-3'	70	37	360-2200	14	14	0	0	100	9.73	5.68	0.914
16	OPN-03	5'-GGT ACT CCC C-3'	70	37	420-3100	15	15	0	1	100	8.2	4.7	0.897
17	OPN-04	5'-GAC CGA CCC A-3'	80	37	310-3700	16	16	0	0	100	11.6	6.13	0.918
18	OPN-05	5'-ACT GAA CGC C-3'	60	37	500-3600	16	16	0	0	100	9.53	6.3	0.928
19	OPN-06	5'-GAG ACG CAC A-3'	60	37	400-2600	14	14	0	0	100	8.93	5.33	0.909
20	OPN-08	5'-ACC TCA GCT C-3'	60	37	280-2500	14	14	0	0	100	10.6	5.55	0.910
TOTAL						272	267	5	1	1962	198.92	102.06	18.07
AVERAGE						13.6	13.35	0.25	0.05	98.1	9.94	5.10	1.721

Table 2. Details of RAPD banding pattern of 30 accessions of six species of *Calamus* using 20 primers

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SL NO	Primer Name	Primer Sequences	GC (%)	Annealing temperature $T_A(^0C)$	Range of amplification (bp)	Total number of bands (TNB)	Number of polymorphic bands (NPB)	Number of monomorp-hic bands (NMB)	Number of unique bands (NUB)	Percentage of polymorphic band PPB (%)	Resolving Power (Rp)	Primer Index (Pi)	Polymorphism Information Content(PIC)
1	PCP-1	5’- GAC GACGACGAC GAC-3’	66.66	51.7	170-2450	14	13	1	0	92.85	13.2	5.10	0.906
2	PCP-2	5’-AGG AGG AGGAGGAGG AGG-3’	66.66	57.4	480-2500	14	13	1	0	92.85	15	5.27	0.913
3	PCP-3	5’-GTCG GTGC GTGC GTGC-3’	75	66.1	300-1600	11	11	0	0	100	9.26	4.08	0.879
4	PCP-5	5’-GA GA GAGAGAGAGAGAGA T-3’	47.36	43	380-2520	9	8	1	0	88.85	8.66	3.55	0.863
5	PCP-6	5’-GACA GACA GACAGACA -3’	50	40	600-2520	9	6	3	0	66.66	11.8	2.77	0.869
6	PCP-7	5’-GGA GGA GGA GGA-3’	66.66	38.9	420-2500	12	11	1	0	91.66	11	3.94	0.883
7	PCP-8	5’-GTG GTG GTGGTG GTG-3’	66.66	52.2	580-2400	10	10	0	0	100	11.66	3.57	0.881
8	PCP-9	5’ GACAC GACACGACAC GACAC-3’	60	60.7	300-1780	13	13	0	0	100	8.6	5.12	0.904
9	PCP-12	5’ GACA GACAGACAGACAGA CAGACAGACAGACA G-3’	51.5	68.5	300-1740	9	8	1	0	88.85	7.46	2.60	0.825
10	Oligo 1(b)	5’-AGA GAG AGA GAG AGA GG-3’	52.94	47	480-2100	9	9	0	0	100	10.2	3.40	0.869
11	Oligo 2(b)	5’-GAG AGA GAG AGA GAG AG-3’	52.94	47	500-1580	9	9	0	0	100	7.66	4.05	0.877
12	Oligo 3(b)	5’-GAC AGA CAG ACA GAC A-3’	50	43	610-3000	14	13	1	0	92.85	11.13	4.5	0.892
13	Oligo 4(a)	5’- GAC AGA CAG ACA GAC AT-3’	47.05	45	720-2300	10	10	0	0	100	7.33	4.17	0.882
14	Oligo 4(b)	5’- TGA CAG ACA GAC AGA CA-3’	47.05	45	700-3000	13	12	1	0	92.30	9.46	4.4	0.886
15	Oligo 5(a)	5’- GGA CAG ACA GAC AGA CA-3’	52.94	47	560-2500	11	9	2	0	81.81	11.06	2.87	0.866
16	Oligo 5(b)	5’- GAC AGA CAG ACA GAC AG-3’	52.94	47	600-3000	12	12	0	0	100	7.26	4.45	0.893
17	Oligo 8(a)	5’- CTC TCT CTC TCT CTC TG-3’	52.94	47	280-2200	16	16	0	0	100	12.66	6.04	0.917
18	Oligo 9(a)	5’- GCT CTC TCT CTC TCT CT-3’	52.94	47	570-1400	8	6	2	0	75	8.66	2.44	0.834
19	Oligo 11(a)	5’- GCT GTC TGT CTG TCT GT-3’	52.94	47	700-3500	11	11	0	0	100	8.73	3.97	0.875
20	Oligo 11(b)	5’- CTG TCT GTC TGT CTG TC-3’	52.94	47	700-4300	10	10	0	0	100	7.46	3.73	0.866
TOTAL						224	210	14	0	1863.68	198.25	80.02	17.58
AVERAGE						11.2	10.5	0.7	0	93.18	9.91	4.00	1.674

Table 3. Details of ISSR banding pattern of 30 accessions of six species of *Calamus* using 20 primers