



Assessment of genetic relationship among 15 accessions of bamboos of Kokrajhar district, BTAD, Assam, India based on RAPD and ISSR marker analysis

Anusriti Basumatary¹, Birendra Kumar Brahma², Amit Kumar Basumatary^{3*}

¹⁻³Department of Biotechnology, Bodoland University, Kokrajhar, BTAD, Assam, India

¹Department of Physiotherapy, Science College, Kokrajhar, BTAD, Assam, India

Abstract

Due to the unusual flowering behavior and long sexual cycle, the morphological exploration of bamboo solely cannot elucidate the taxonomic difficulties. So to validate and study the phyletic relation and the genetic diversity, molecular approaches were employed. In this study, different DNA fingerprinting techniques (RAPD and ISSR) were used to analyze the phylogenetic similarity among the 15 accessions of bamboos of district Kokrajhar, BTAD, Assam, India along with one out-group (*Oryza sativa*). 35 primers in total were used for RAPD analysis whereas ISSR analysis was carried out by using 25 primers. High polymorphism of DNA fragments were obtained from both ISSR and RAPD. Also the dendrogram was generated from the acquired data.

Keywords: bamboos; phylogenetic similarity; RAPD; ISSR

1. Introduction

Bamboos, the extraordinarily versatile, strong, valuable and woody perennial evergreen plants come as a 'boon' in various forms. With its massive ecological benefits alone, the 'green gold' is rapidly becoming more than just a poor man's timber. Moreover, in recent time, bamboo is perceived as the 'wonder plant' of the 21st century [2, 6]. It stands as a supreme species capable of attaining eco-restoration of degraded lands, conservation of soil, endowing with economic security as well [8]. For North-east region, the 'green gold' has not only being a traditional economic crop but also is established to have remarkable biogenomic resilience in combating the brunt of climate change such as the droughts faced by the people in 18th and 19th centuries [5].

Soil is eroded by wind, water and many more factors as a result of poor soil management. But with its widespread fibrous root system, bamboos like *Bambusa blumeana* and *Phyllostachys pubescens* prevents nutrient loss, binds the top soil, controls soil erosion and improves soil structure [3]. Its immeasurable environmental benefits involves restricting deforestation and mitigating climate change thereby liberating 35% more oxygen in the atmosphere as compared to the corresponding mass of trees and confiscates up to twelve tons of carbon dioxide from the air. These days bamboo is employed in creating corrugated sheets, hard boards, flooring, etc. aside from its wide- ranging use in the industries of paper and pulp [1]. Upliftment of bamboo as one of the prime renewable natural source of India presently is due to the influence of globalization. It has achieved the worldwide appreciation and attention because of its high growth pace, sustainability and demand [9, 4]. Therefore, ensuring protection and authentic determination of phylogenetic relationship and

genetic diversity of available plant germplasm is important for recognizing the potential germplasm groups as the vegetative characters and traditional method of classification are less dependable. Thus, molecular approaches have become an indispensable part in order to analyze the variation in the gene pool of the 'super material' of the 21st century. Furthermore, these techniques help to assess the relative diversity within and among the species and locate diverse accessions for breeding purposes [7]. RAPD is a cost effective simple and rapid technology and has been employed since its discovery fruitfully in the evaluation of genetic relationships in bamboos and other plant species. It does not require any information regarding the genome of the plant and requires very small amount of genomic DNA. ISSR markers on other hand determine intra-genomic and inter-genomic diversity compared to other arbitrary primers.

The present study was carried out to determine phylogenetic similarity and diversity of 15 accessions of bamboos of Kokrajhar district, BTAD, Assam, India using RAPD and ISSR markers.

2. Materials and methods

2.1 Collection of plant samples

The selected regions of Kokrajhar district such as Charaikhola, Titaguri, Debargaon, Pakhriguri, Dimalgaon, Balagaon, Karigaon, Chandrapara and Dotma were surveyed for sample collection. A total of 15 species of bamboos are known to exist in Kokrajhar district and the species were identified with the help of bamboo experts and literature sources. Healthy young tender leaves of bamboos were collected for genomic DNA extraction

Table 1: List of bamboo species and one outgroup (P)

| Sample | |
|--------|--------------------------------------------------------------------------------------------|
| Code | Scientific Name |
| B1 | <i>Bambusa assamica</i> Barooah <i>et</i> Borthakur |
| B2 | <i>Bambusa balcooa</i> Roxb. |
| B3 | <i>Bambusa bambos</i> Willd. |
| B4 | <i>Bambusa garuchokua</i> Barooah <i>et</i> Borthakur |
| B5 | <i>Bambusa multiplex</i> (Lour.) Raeusch. Ex Schult |
| B6 | <i>Bambusa pallida</i> Munro |
| B7 | <i>Bambusa tulda</i> Roxb. |
| B8 | <i>Bambusa vulgaris</i> Schard. ex J.C. Wendl. var. <i>Vittata</i> A. Riviere & C.Riviere |
| B9 | <i>Bambusa vulgaris</i> Schard. ex J.C. Wendl. var. <i>Vulgaris</i> A. Riviere & C.Riviere |
| B10 | <i>Bambusa vulgaris</i> Schard. ex J.C. Wendl. fo. <i>Waminii</i> (Brandis) Wen |
| B11 | <i>Dendrocalamus giganteus</i> Munro |
| B12 | <i>Dendrocalamus hamiltonii</i> Munro |
| B13 | <i>Dendrocalamus strictus</i> |
| B14 | <i>Melocanna baccifera</i> (Roxb.) Kurz |
| B15 | <i>Schizostachyum sp.</i> |
| P | <i>Oryza sativa</i> |

Collected from different regions of Kokrajhar district

2.2 Extraction of DNA

DNA of different species of bamboos was isolated by using Doyle and Doyle (1987) method with slight modification. Healthy and young leaves were taken (approx. 5g) and crushed with the help of liquid nitrogen into fine powder in mortar pestle. The powdered material was then mixed with 15 ml pre-warmed (65°C) CTAB buffer. Then the mixture was again warmed in hot water bath (EQUITRON) (65°C) for 1 hr. After 1 hr of incubation in water bath an equal volume of (24:1) chloroform (MERCK): isoamyl alcohol (MERCK) were added and mixed gently in the rotor spin (TARSONS) at 10 rpm for 20 minutes. The solution was centrifuged (GENETIX) at 6,500 rpm for 15 minutes at 24°C and then the upper aqueous was transferred carefully to a fresh tube. Then 0.6 ml of ice cold isopropanol (MERCK) was added and mixed upside down gently until the DNA-CTAB complex precipitates as a whitish network. Then the precipitated DNA was incubated at 4°C for 1 hr. After incubation it was then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the obtained pellet was washed with 70% ethyl alcohol (Omnis) and then air dried for 25-30 minutes. Then 500 µl of 1X TE buffer (pH 7.4) was added to dissolve the DNA pellet. The dissolved DNA was transferred to a fresh microcentrifuge tube and to it equal volume of Phenol (MERCK): Chloroform: Isoamyl alcohol (P: C: I) (25:24:1) was added. The solution was then mixed gently in the rotor spin for 20 minutes at 10 rpm. Then it was centrifuged at 10000 rpm at 24°C for 15 minutes and the supernatant was transferred carefully in a fresh microcentrifuge tube. Equal volume of C: I (24:1) was added in the tube containing supernatant and centrifuged at 10,000 rpm at 24°C for 15 minutes. The upper aqueous phase was transferred carefully in a fresh microcentrifuge tube and then 50µl of 3M sodium acetate (pH 5) (SIGMA) and 1 ml of absolute ethyl alcohol were added. It was mixed gently until the precipitated DNA appears and incubated at 4°C for 30 minutes then centrifuged for 30 minutes at 12,000 rpm at 4°C.

The upper aqueous phase was discarded and the pellet obtained was washed with 70% ethyl alcohol and air dried for 25-30 minutes. Then the DNA pellet was dissolved in 500 µl of 1X TE buffer (pH 7.4).

2.3 Purification of DNA and quantification

CTAB (cetyl trimethylammonium bromide) buffer was used while isolating DNA for purification. PVP (1%) (Polyvinylpyrrolidone) and 2-Mercaptoethanol (0.3%) was also added for cleaning polyphenols. The presence of RNA was removed from DNA by using RNase enzyme. To 500 µl of DNA dissolved 1X TE buffer (pH 7.4) RNase A (50 µl/ml) (SIGMA) was added and incubated in Dry bath (Bench Top) for 1 hour at 37°C. After incubation equal volume of Chloroform: Isoamyl alcohol (24:1) was mixed with it gently. Then the solution was centrifuged at 10,000 rpm for 15 minutes at 24°C. The upper aqueous phase was then transferred to a fresh microcentrifuge tube (Tarsons) and to it 3M sodium acetate (pH 5.2) and double volume of absolute ethyl alcohol was added. Then the solution was centrifuged at 12,000 rpm for 30 minutes at 4°C. The aqueous phase was removed carefully and the DNA pellet obtained was washed with 70% ethyl alcohol. The pellet was air dried for few minutes and then dissolved in 100 µl of 1X TE buffer (pH 7.4).

The DNA concentration was quantified with the help of spectrophotometer and agarose gel analysis technique. The ratio of absorbance at 260 nm and 280 nm was used to determine the purity and concentration of DNA in spectrophotometer. While on gel technique, DNA samples were run in 0.8 agarose gel) containing 0.5 µg/mL ethidium bromide. The concentration of the DNA was estimated by comparing the fluorescence intensities under UV- light on a Chemi Doc XRS gel documentation system (Bio Rad).

2.4 PCR analysis of RAPD and ISSR

For RAPD, 35 primers were used for the study of different species of bamboo. PCR conditions were standardized using varying concentrations of primers and template DNA. After standardization, the reactions were carried out in 50 µl volume which contain 5 µl of 10X reaction buffer, 1 µl of 10mM dNTP mix, 1 µl primer (0.2 µM), 0.5 µl of G9 *Taq* DNA polymerase (Cat No. G7115A; GCC Biotech, India) (1.25U), 5 µl MgCl₂ (2.5mM), 35.5 µl Nuclease free water, 2 µl template DNA. The thermal cycling program was carried out in a Veriti thermal cycle (Applied Biosystems). The PCR program had an initial denaturation step at 94°C for 5 min, followed by 44 cycles of 94°C for 1 min, 35°C for 1 min for annealing, and 72°C for 2 min for extension. A final extension step was given at 72°C for 7 min. The temperature was hold at 4 °C at the end. In ISSR the PCR program had an initial denaturation step at 94°C for 2 min, followed by 44 cycles of 94°C for 30s, 44°C for 45s for annealing, and 72°C for 1 min 30s for extension. A final extension step was given at 72°C for 20 min. The temperature was hold at 4 °C at the end. Then the amplified products of both RAPD and ISSR were resolved by electrophoresis at 50V for 3 hours in 2% agarose gel in 1× TAE buffer. The DNA bands were visualized by staining gel in 0.5 µg/ml ethidium bromide solution (10-15 min) and photographed under UV light using a Chemi Doc XRS gel

documentation system (Bio Rad). A 200 bp DNA ladder (Cat. No. G4668; GCC Biotech, India) and 1 kb ladder were used as a molecular weight marker for determining the molecular weight of the amplified products. NTC (No Template Control) was also used to monitor the contamination of PCR reaction.

2.5 Scoring and data analysis

Digitized gel photograph of RAPD and ISSR results were analyzed using NTSYS PC Ver.2.0 numerical software package. Data was recorded as 1 (presence) or 0 (absence), each of which were treated as an independent character. The bands which were very faint were not considered for scoring. For each primer, PCR reactions were repeated two times and only reproducible bands were considered for analysis. The primers which did not produce amplification were repeated thrice before discarding them. The pair wise similarity between isolates and polymorphic bands were calculated using Jaccard's coefficient (Jaccard, 1908), a common estimator of

genetic identity or estimates interspecific relationships. The similarity co-efficients were used to construct a dendrogram for determining relationship using unweighted pair group method with arithmetic average (UPGMA). Also 2D and 3D plot principle component analysis was done from the data to graphically show the diversity among the species along with the out group

3. Results and discussion

3.1 Molecular characterization through RAPD analysis

RAPD analysis was done by using 35 different primers each of 10-mer sequence for 15 different species of bamboo along with one outgroup. Amongst these, 20 primers showed distinct bands. The amplified DNA products with 20 primers from the 15 species of bamboo and one outgroup produced total 387 amplified DNA bands ranging from 178-2500 bp, out of which only 3 were monomorphic and remaining were polymorphic ("Table.2")

Table 2: Number of amplified bands, size of bands, monomorphic bands, polymorphic bands and percentage of polymorphism produced by selected 20 RAPD primers.

| Marker | Primers | Nucleotide sequence (5'-3') | Bands Amplified | Monomorphic Bands | Polymorphic bands | Polymorphism percentage | Range of band size (bp) |
|--------|---------|--------------------------------|-----------------|----------------------|----------------------|----------------------------|----------------------------|
| | OPA-01 | CAGGCCCTTC | 20 | 0 | 20 | 100% | 290-1469 |
| | OPA-03 | AGTCAGCCAC | 24 | 0 | 24 | 100% | 425-1773 |
| | OPA-04 | AATCGGGCTG | 23 | 0 | 23 | 100% | 371-2121 |
| | OPA-05 | AGGGGTCTTG | 16 | 0 | 16 | 100% | 305-1856 |
| | OPA-07 | GAAACGGGTG | 28 | 0 | 28 | 100% | 256-2162 |
| | OPA-08 | GTGACGTAGG | 18 | 0 | 18 | 100% | 307-1975 |
| | OPA-11 | CAATCGCCGT | 19 | 0 | 19 | 100% | 431-1817 |
| | OPA-17 | GACCGCTTGT | 10 | 0 | 10 | 100% | 346-2043 |
| RAPD | OPA-18 | AGGTGACCGT | 16 | 0 | 16 | 100% | 396-1948 |
| | OPA-20 | GTTGCGATCC | 15 | 0 | 15 | 100% | 499-2353 |
| | OPB-01 | GTTTCGCTCC | 19 | 0 | 19 | 100% | 178-2364 |
| | OPF-09 | CCAAGCTTCC | 21 | 0 | 21 | 100% | 355-2244 |
| | OPG-19 | GTCAGGGCAA | 20 | 1 | 19 | 95% | 409-2478 |
| | OPH-04 | GGAAGTCGCC | 22 | 0 | 22 | 100% | 397-2500 |
| | OPJ-04 | CCGAACACGG | 11 | 0 | 11 | 100% | 376-2362 |
| | OPN-04 | GACCGACCCA | 23 | 1 | 22 | 95.65% | 388-2035 |
| | OPN-13 | AGCGTCACTC | 26 | 0 | 26 | 100% | 427-2122 |
| | OPN-19 | GTCCGTA CTG | 21 | 0 | 21 | 100% | 226-2173 |
| | OPO-02 | ACGTAGCGTC | 18 | 0 | 18 | 100% | 392-2192 |
| | OPO-03 | CTGTTGCTAC | 17 | 1 | 16 | 94.11% | 472-1971 |
| | | Total | 387 | 3 | 384 | 99.23% | |

It displayed 99.23% of polymorphism. The total number of bands amplified with each primer OPA17 and OPA07 of 10 nucleotide length ranges from 10 to 28 respectively. A RAPD-PCR product profile of 15 species of bamboo and 1 outgroup using primer OPF-09 was delineated in ("Fig.1") which displayed that all the bands produced were polymorphic. In ("Fig.2") it showed monomorphic band with OPG-19 primer.

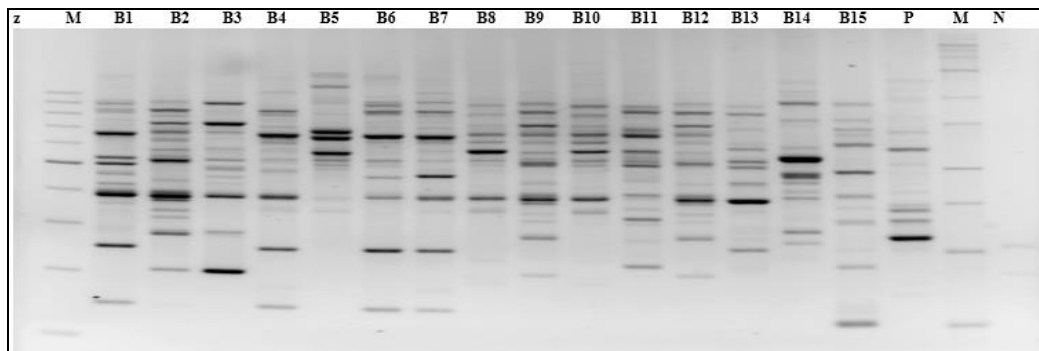


Fig 1: A representative RAPD profile of 15 species of bamboo and one outgroup P amplified with OPF-09 primer. Lane B1- *Bambusa assamica*; Lane B2- *B. balcooa*; Lane B3- *B. bambos*; Lane B4- *B. garuchokua*; Lane B5- *B. multiplex*; Lane B6- *B. pallida*; Lane B7- *B. tulda*; Lane B8- *B. vulgaris* var. Vittata; Lane B9- *B. vulgais* var. Vulgaris; Lane B10- *B. vulgaris* fo. Waminii; Lane B11- *Dendrocalamus giganteus*; Lane B12- *D. hamiltonii*; Lane B13- *D. strictus*; Lane B14- *Melocanna baccifera*; Lane B15- *Schizostachyum sp.*; Lane P- *Oryza sativa*

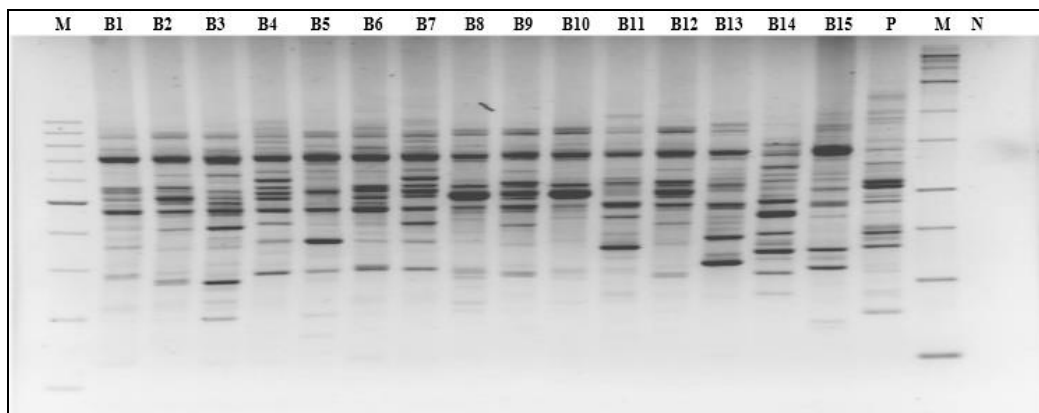


Fig 2: A representative RAPD profile of 15 species of bamboo and one outgroup P amplified with OPG-19 primer. Lane B1- *Bambusa assamica*; Lane B2- *B. balcooa*; Lane B3- *B. bambos*; Lane B4- *B. garuchokua*; Lane B5- *B. multiplex*; Lane B6- *B. pallida*; Lane B7- *B. tulda*; Lane B8- *B. vulgaris* var. Vittata; Lane B9- *B. vulgais* var. Vulgaris; Lane B10- *B. vulgaris* fo. Waminii; Lane B11- *Dendrocalamus giganteus*; Lane B12- *D. hamiltonii*; Lane B13- *D. strictus*; Lane B14- *Melocanna baccifera*; Lane B15- *Schizostachyum sp.*; Lane P- *Oryza sativa*. Lane M- 200bp DNA ladder; Lane M1- 1Kb ladder; Lane N- NTC (No Template Control).

The similarity matrix of 15 bamboo species and one outgroup acquired by using Jaccard’s coefficient from RAPD analysis is delineated in (“Table.3”). The highest similarity was observed between *B. vulgaris* var. Vittata and *B. vulgaris* fo. Waminii. Whereas, lowest similarity was found between *B. pallida* and *Melocanna baccifera*. The dendrogram generated from the

data acquired by RAPD analysis of bamboos and one outgroup showing genetic diversity is depicted in (“Fig.3”). “Table 3”) Similarity matrix of 15 species of bamboo and outgroup obtained using Jaccard’s coefficient from RAPD marker analysis

Table 3: Similarity matrix of 15 species of bamboo and outgroup obtained using Jaccard’s coefficient from RAPD marker analysis

| | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 | B13 | B14 | B15 | P |
|-----|-------|-------|-------|--------------|-------|-------|--------------|-------|-------|-------|-------|-------|-------|-------|-------|------|
| B1 | 1.00 | | | | | | | | | | | | | | | |
| B2 | 0.463 | 1.00 | | | | | | | | | | | | | | |
| B3 | 0.417 | 0.597 | 1.00 | | | | | | | | | | | | | |
| B4 | 0.472 | 0.478 | 0.481 | 1.00 | | | | | | | | | | | | |
| B5 | 0.396 | 0.450 | 0.447 | 0.476 | 1.00 | | | | | | | | | | | |
| B6 | 0.544 | 0.455 | 0.456 | 0.636 | 0.450 | 1.00 | | | | | | | | | | |
| B7 | 0.418 | 0.452 | 0.464 | 0.629 | 0.489 | 0.619 | 1.00 | | | | | | | | | |
| B8 | 0.429 | 0.501 | 0.486 | 0.444 | 0.465 | 0.489 | 0.511 | 1.00 | | | | | | | | |
| B9 | 0.452 | 0.524 | 0.429 | 0.433 | 0.479 | 0.442 | 0.490 | 0.571 | 1.00 | | | | | | | |
| B10 | 0.413 | 0.486 | 0.443 | 0.442 | 0.447 | 0.464 | 0.754 | 0.591 | 1.00 | | | | | | | |
| B11 | 0.391 | 0.457 | 0.408 | 0.462 | 0.375 | 0.442 | 0.429 | 0.456 | 0.505 | 0.535 | 1.00 | | | | | |
| B12 | 0.453 | 0.512 | 0.413 | 0.393 | 0.427 | 0.464 | 0.471 | 0.525 | 0.710 | 0.545 | 0.466 | 1.00 | | | | |
| B13 | 0.403 | 0.388 | 0.374 | 0.407 | 0.416 | 0.422 | 0.402 | 0.410 | 0.430 | 0.435 | 0.473 | 0.477 | 1.00 | | | |
| B14 | 0.405 | 0.385 | 0.371 | 0.359 | 0.417 | 0.339 | 0.394 | 0.422 | 0.457 | 0.399 | 0.416 | 0.431 | 0.415 | 1.00 | | |
| B15 | 0.408 | 0.433 | 0.396 | 0.407 | 0.393 | 0.437 | 0.424 | 0.404 | 0.419 | 0.418 | 0.446 | 0.457 | 0.407 | 0.387 | 1.00 | |
| P | 0.380 | 0.396 | 0.393 | 0.378 | 0.348 | 0.357 | 0.354 | 0.385 | 0.327 | 0.365 | 0.338 | 0.304 | 0.330 | 0.347 | 0.368 | 1.00 |

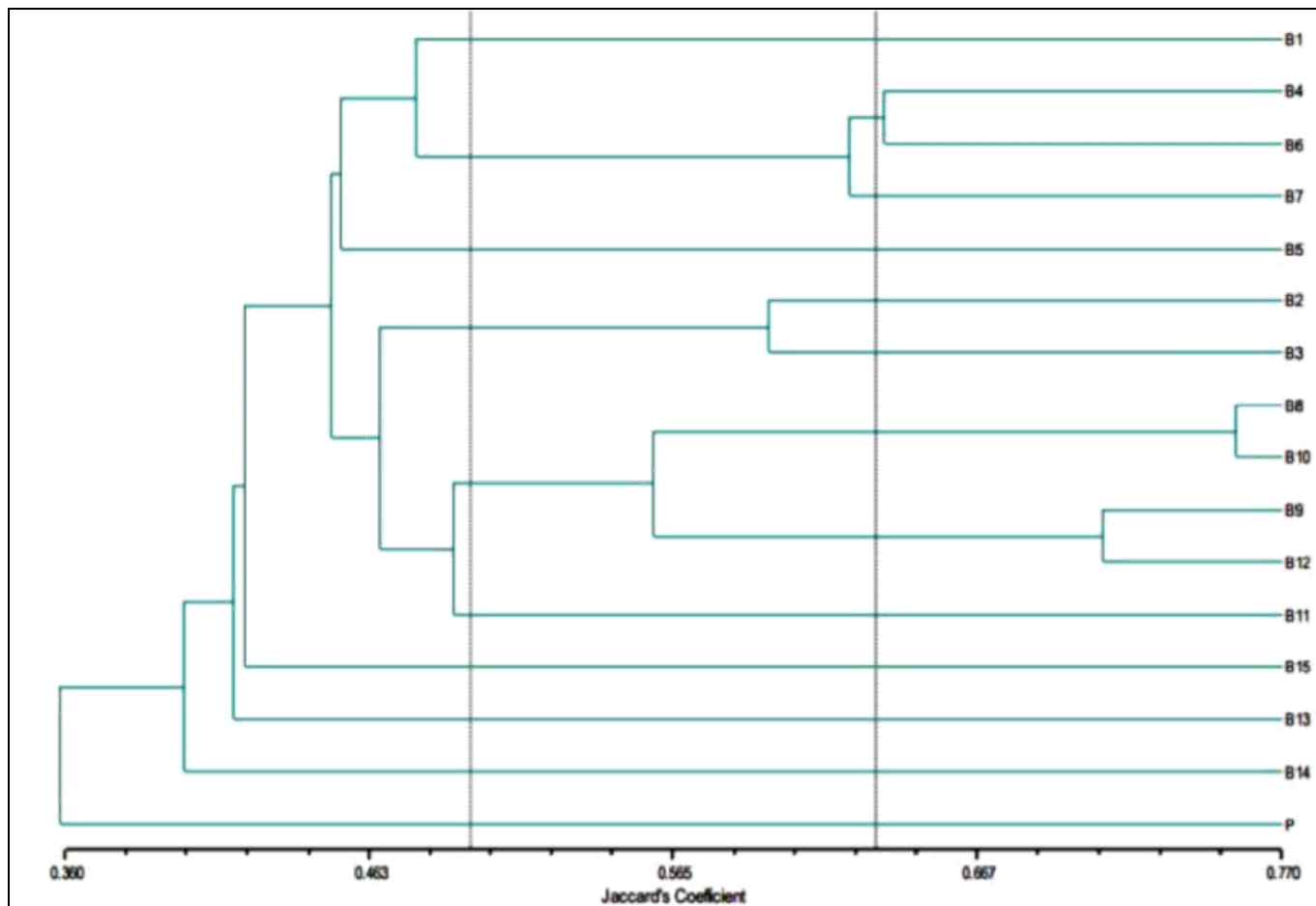


Fig 3: Dendrogram showing genetic diversity for RAPD markers in 15 bamboo genotype and one rice genotype.

To study the genetic diversity and the relationship among the 15 species of bamboo and outgroup, a comparison was done amongst the genus of bamboo and amongst the species and variety of bamboo. *B. vulgaris* var. *Vittata*. *vugaris* var. *Vulgaris*, the two varieties of the same species displayed in dendrogram showed 57.1% similarity coefficient. Moreover, the one forma of the same species i.e. *Bambusa vulgaris* fo. *Waminii* clustered closely with *B. vulgaris* var. *Vittata* sharing a node at 75.4% showing the highest similarity. Similarly Das *et al.*, 2007 recorded that *B. vulgaris* fo. *Waminii* and *B. vulgaris* var. *Vittata* (*B. striata*) clustered together in RAPD analysis and Mukherjee *et al.*, 2010 displayed that both the species showed highest similarity coefficient while using ISSR and EST based primers. *Bambusa pallida* and *Melocanna baccifera* showed 33.9% similarity. *B. garuchokua* and *B. pallida* were place together with 63.6% of similarity, while *B. balcooa* and *B. bamboos* were clustered together showing 59.7% of similarity. The dendrogram represented that

the genus *Bambusa* were close to each other. Whereas, *B. vulgaris* var. *Vulgaris* was found to be clustered closely with *Dendrocalamus hamiltonii* displaying 71% of similarity coefficient. The two species viz. *D. hamiltonii* and *D. giganteus* were grouped with genus *Bambusa*. *D. strictus* instead of clustering with its species remained in a different cluster. This segregation could be because of their difference in morphological features. The PCA analysis (Fig 4) based on the Jaccard similarity index displayed similar cluster as that of dendrogram. Hence, RAPD analysis showed that studying the phylogenetic relationship among the species solely through morphological analysis which is based on vegetative and reproductive organs is inadequate. Moreover, due to the unusual flowering of bamboo only the vegetative characters were taken into account for the study. So, there is a need for addition of the molecular corroboration to substantiate the phyletic relation among the taxa

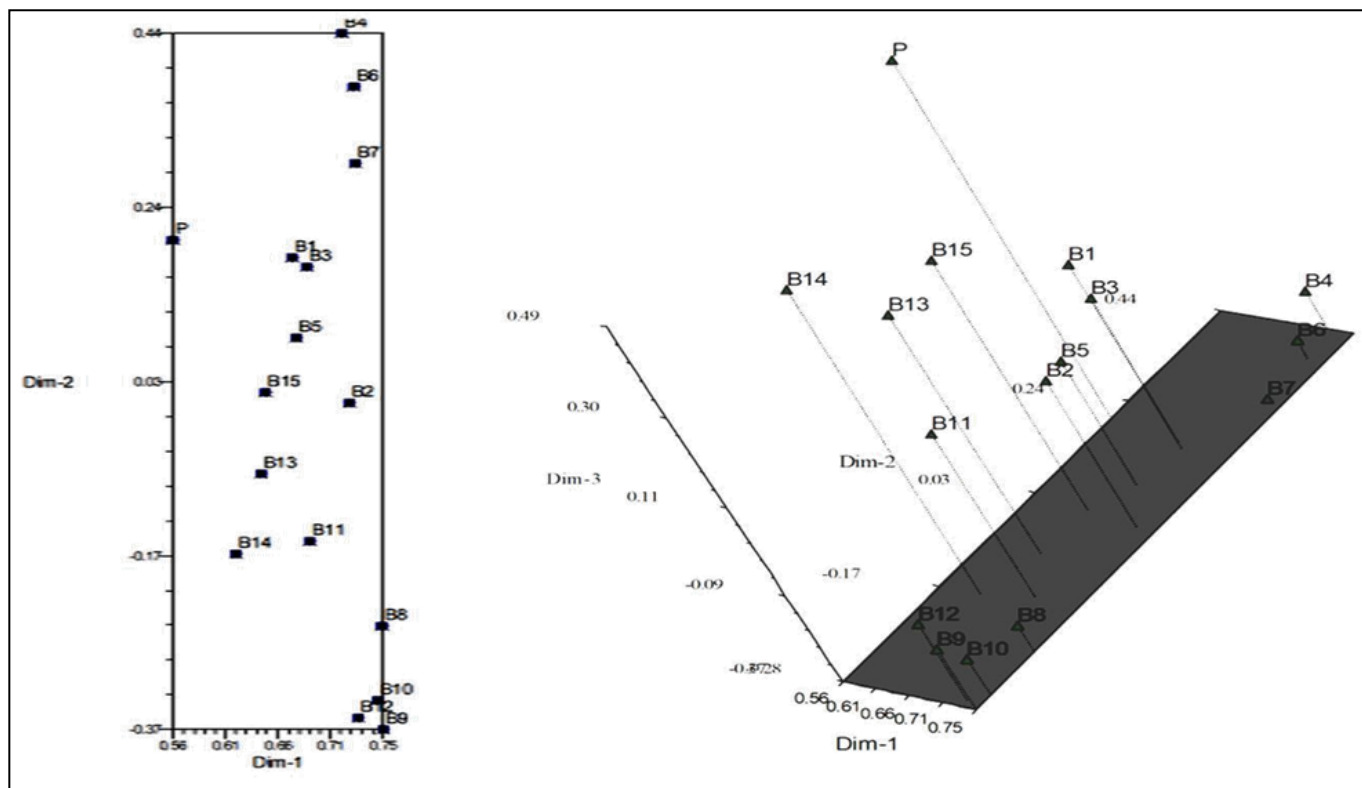


Fig 4: Principle component analysis showing genetic diversity for RAPD markers in 15 bamboo genotypes and one rice genotype. (A) 2-dimensional plot and (B) 3-dimensional plot.

3.2 Molecular characterization through ISSR analysis

For ISSR analysis of 15 species of bamboo DNA and one outgroup, 25 ISSR primers were used. Among these 25 primers, 18 ISSR markers showed distinct bands. Out of these primers, the primer BU-15 showed 21 bands while BU-815

primer produced 32 bands. The total numbers of amplified bands were 474 where all were polymorphic (100%). The amplified DNA bands size ranges from 170 - 3186 bp (“Table.4”)

Table 4: Number of amplified bands, size of bands, polymorphic bands and percentage of polymorphism produced by selected 18 ISSR primers.

| Marker | Primers | Nucleotide sequence (5'-3') | Bands Amplified | Polymorphic bands | Polymorphism percentage | Range of band size (bp) |
|--------|---------|--------------------------------|-----------------|----------------------|----------------------------|----------------------------|
| | BU14 | (AC)8TT | 30 | 30 | 100% | 235-2557 |
| | BU15 | (AC)8TA | 21 | 21 | 100% | 256-2315 |
| | BU 17 | (AG)8AA | 27 | 27 | 100% | 289-1763 |
| | BU19 | (AG)8TT | 24 | 24 | 100% | 271-2018 |
| | BU25 | (AC)8TG | 26 | 26 | 100% | 248-2553 |
| | BU41 | (GA)8YC | 23 | 23 | 100% | 170-2036 |
| | BU807 | (AG)8T | 29 | 29 | 100% | 276-3186 |
| | BU808 | (AG)8C | 31 | 31 | 100% | 283-2377 |
| ISSR | BU810 | (GA)8T | 30 | 30 | 100% | 405-1856 |
| | BU811 | (GA)8C | 23 | 23 | 100% | 411-2336 |
| | BU815 | (CT)8G | 32 | 32 | 100% | 358-2274 |
| | BU822 | (TC)8A | 31 | 31 | 100% | 319-2334 |
| | BU824 | (TC)8G | 27 | 27 | 100% | 378-2480 |
| | BU825 | (AC)8T | 22 | 22 | 100% | 364-2532 |
| | BU834 | (AG)8YT | 23 | 23 | 100% | 249-1976 |
| | BU856 | (AC)8YA | 25 | 25 | 100% | 392-2189 |
| | BU873 | (GACA)4 | 28 | 28 | 100% | 290-2238 |
| | SP2 | (GATA)5 | 22 | 22 | 100% | 302-2426 |
| | | Total | 474 | 474 | 100% | |

ISSR-PCR product profile of 15 bamboo species and one outgroup using primer BU-15 and BU-815 was delineated in (“Fig.5, 6”)

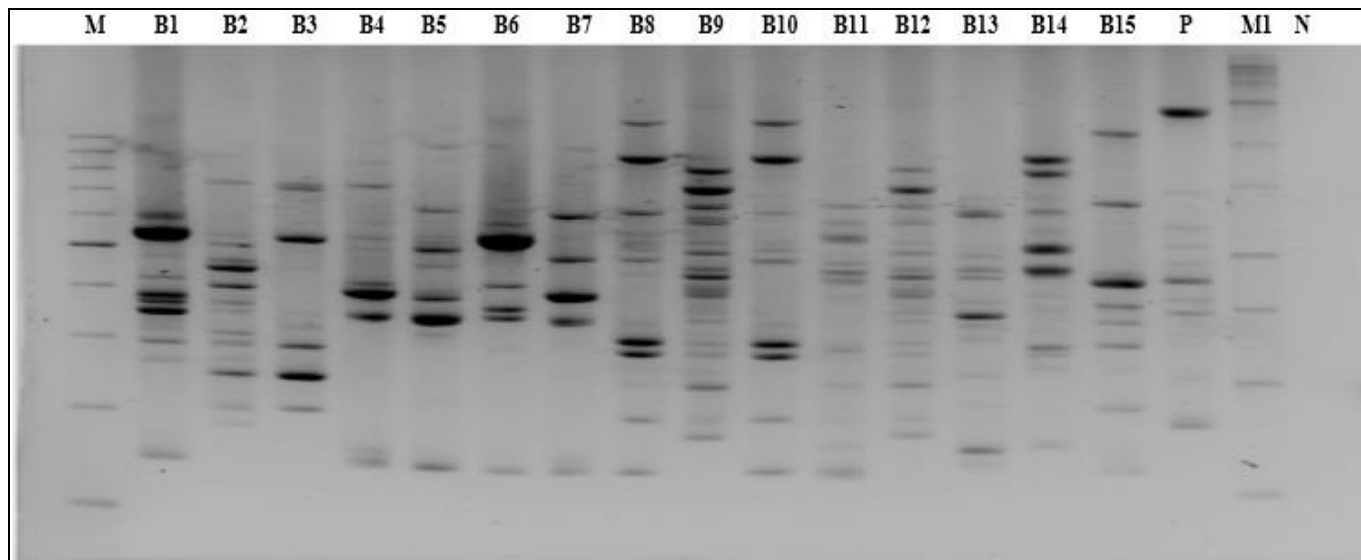


Fig 5: A representative ISSR profile of 15 species of bamboo and one outgroup P amplified with BU-15 primer. Lane B1- *Bambusa assamica*; Lane B2- *B. balcooa*; Lane B3- *B. bambos*; Lane B4- *B. garuchokua*; Lane B5- *B. multiplex*; Lane B6- *B. pallida*; Lane B7- *B. tulda*; Lane B8- *B. vulgaris* var. Vittata; Lane B9- *B. vulgais* var. Vulgaris; Lane B10- *B. vulgaris* fo. Waminii; Lane B11- *Dendrocalamus giganteus*; Lane B12- *D. hamiltonii*; Lane B13- *D. strictus*; Lane B14- *Melocanna baccifera*; Lane B15- *Schizostachyum sp.*; Lane P- *Oryza sativa*. Lane M- 200bp DNA ladder; Lane M1- 1Kb ladder; Lane N- NTC (No Template Control).

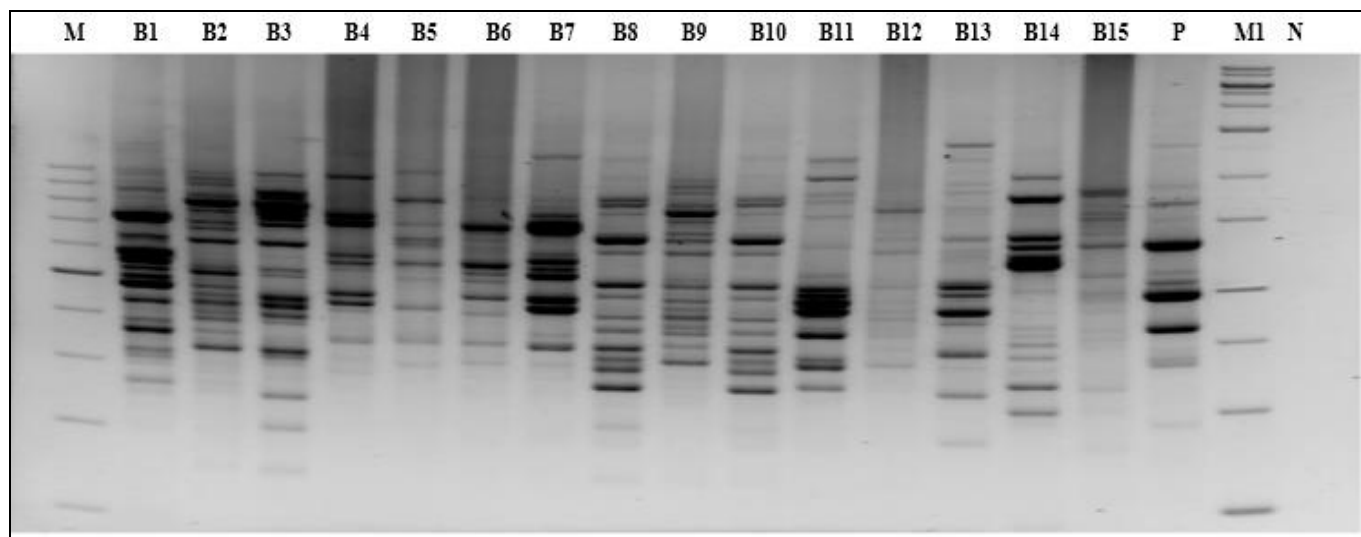


Fig 6: A representative ISSR profile of 15 species of bamboo and one outgroup P amplified with BU-815 primer. Lane B1- *Bambusa assamica*; Lane B2- *B. balcooa*; Lane B3- *B. bambos*; Lane B4- *B. garuchokua*; Lane B5- *B. multiplex*; Lane B6- *B. pallida*; Lane B7- *B. tulda*; Lane B8- *B. vulgaris* var. Vittata; Lane B9- *B. vulgais* var. Vulgaris; Lane B10- *B. vulgaris* fo. Waminii; Lane B11- *Dendrocalamus giganteus*; Lane B12- *D. hamiltonii*; Lane B13- *D. strictus*; Lane B14- *Melocanna baccifera*; Lane B15- *Schizostachyum sp.*; Lane P- *Oryza sativa*. Lane M- 200bp DNA ladder; Lane M1- 1Kb ladder; Lane N- NTC (No Template Control).

The dendrogram generated from the data obtained from ISSR analysis of bamboos and one outgroup showing genetic diversity is depicted in ("Fig.7"). The dendrogram was divided into two distinct clades. The 10 members of the genus *Bambusa* were split in different sub-clusters. Out of these 10 members of genus *Bambusa*, 5 members were grouped together viz. *Bambusa assamica*, *B.garuchokua*, *B. tulda*, *B. pallida* and *B. multiplex*. Whereas other 5 members viz.

Bambusa balcooa, *B. bambos*, *B. vulgaris* var. Vittata, *B. vulgaris* fo. Waminii and *Bambusa vulgaris* var. Vulgaris were grouped with *Dendrocalamus giganteus*, *D.hamiltonii*, *D. strictus*, *Schizostachyum sp.* and *Melocanna baccifera*. The variety of *Bambusa vulgaris* i.e. *B. vulgaris* var. Vittata and one forma *B. vulgaris* fo. Waminii were found to be clustered together with the highest similarity 64.4% (Similarity matrix "Table.5").

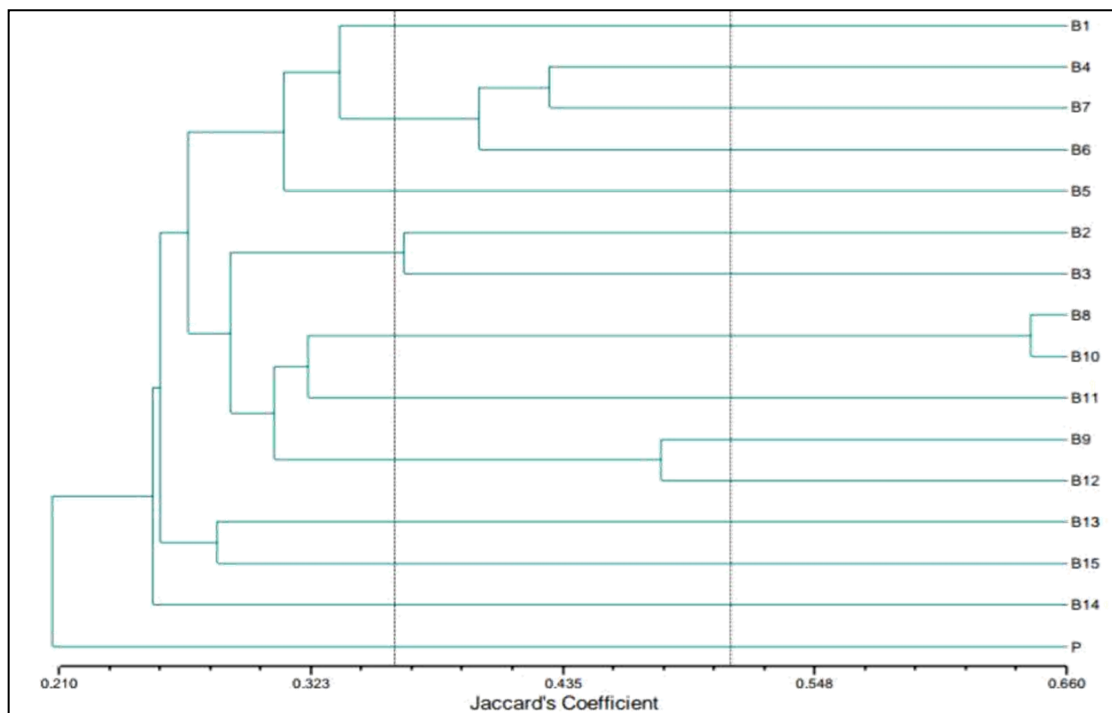


Fig 7: Dendrogram showing genetic diversity for ISSR markers in 15 bamboo genotypes and one rice genotype

The close relationship between these two species has been already recorded by *Nayak et al., 2003* [7], *Das et al., 2007* and *Mukherjee et al., 2010*). The distantly placed *Bambusa assamica* and *Melocanna baccifera* in dendrogram showed lowest similarity with 21.6%. *Bambusa garuchokua* and *B. tulda* clustered together with 42.9% of similarity coefficient. *Bambusa balcooa* and *B. bambos* clustered together while *B. vulgaris* var. *Vittata* clustered with *Dendrocalamus hamiltonii* showing 36.4% and 47.9% similarity respectively. *D. strictus* clustered with *Schizostachyum sp.* showing the node at 28% similarity instead of clustering with its same genus. The segregation of the same genus among the species in the dendrogram could be because of their dissimilar morphological features (*Nayak et al., 2003*) [7]. The PCA analysis (Fig 8) based on Jaccard's coefficient upheld the

dendrogram. Moreover, PCA analysis and dendrogram displayed similar cluster which has been also displayed while analyzing the genetic relationship among 22 species of bamboo by using IISR and EST (Expressed sequence tag) based random primers (*Mukherjee et al., 2010*). Hence, ISSR analysis showed that solely morphological analysis among species to demonstrate the genetic relationship is not satisfactory. The inadequacy could be due to the fact that the classical system of classification depends on both vegetative and reproductive organs. However, the present morphological study was based only on vegetative features due to the inaccessible of reproductive organs. So the involvement of molecular analysis is very important to reflect the genetic closeness among the species.

Table 5: Similarity matrix of 15 species of bamboo and outgroup obtained using Jaccard's coefficient from ISSR Marker analysis.

| | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 | B13 | B14 | B15 | P |
|-----|-------|-------|-------|-------|-------|-------|-------|-------------|-------|-------|-------|-------|-------|-------|-------|------|
| B1 | 1.00 | | | | | | | | | | | | | | | |
| B2 | 0.304 | 1.00 | | | | | | | | | | | | | | |
| B3 | 0.235 | 0.364 | 1.00 | | | | | | | | | | | | | |
| B4 | 0.340 | 0.275 | 0.251 | 1.00 | | | | | | | | | | | | |
| B5 | 0.317 | 0.277 | 0.258 | 0.287 | 1.00 | | | | | | | | | | | |
| B6 | 0.370 | 0.298 | 0.241 | 0.377 | 0.322 | 1.00 | | | | | | | | | | |
| B7 | 0.295 | 0.281 | 0.228 | 0.429 | 0.315 | 0.418 | 1.00 | | | | | | | | | |
| B8 | 0.291 | 0.335 | 0.294 | 0.318 | 0.285 | 0.312 | 0.278 | 1.00 | | | | | | | | |
| B9 | 0.253 | 0.328 | 0.257 | 0.247 | 0.265 | 0.250 | 0.262 | 0.300 | 1.00 | | | | | | | |
| B10 | 0.267 | 0.32 | 0.26 | 0.28 | 0.26 | 0.29 | 0.28 | 0.64 | 0.309 | 1.00 | | | | | | |
| 0 | | 7 | 9 | 2 | 6 | 1 | 0 | 4 | | | | | | | | |
| B11 | 0.256 | 0.301 | 0.244 | 0.250 | 0.295 | 0.256 | 0.271 | 0.318 | 0.310 | 0.324 | 1.00 | | | | | |
| B12 | 0.251 | 0.284 | 0.221 | 0.223 | 0.266 | 0.234 | 0.259 | 0.317 | 0.479 | 0.323 | 0.274 | 1.00 | | | | |
| B13 | 0.231 | 0.265 | 0.237 | 0.217 | 0.258 | 0.250 | 0.258 | 0.260 | 0.227 | 0.255 | 0.277 | 0.276 | 1.00 | | | |
| B14 | 0.216 | 0.289 | 0.268 | 0.230 | 0.258 | 0.218 | 0.248 | 0.273 | 0.257 | 0.271 | 0.263 | 0.244 | 0.227 | 1.00 | | |
| B15 | 0.255 | 0.270 | 0.249 | 0.231 | 0.234 | 0.286 | 0.249 | 0.272 | 0.255 | 0.263 | 0.289 | 0.249 | 0.280 | 0.258 | 1.00 | |
| P | 0.203 | 0.214 | 0.229 | 0.197 | 0.190 | 0.196 | 0.208 | 0.210 | 0.195 | 0.210 | 0.200 | 0.203 | 0.214 | 0.246 | 0.180 | 1.00 |

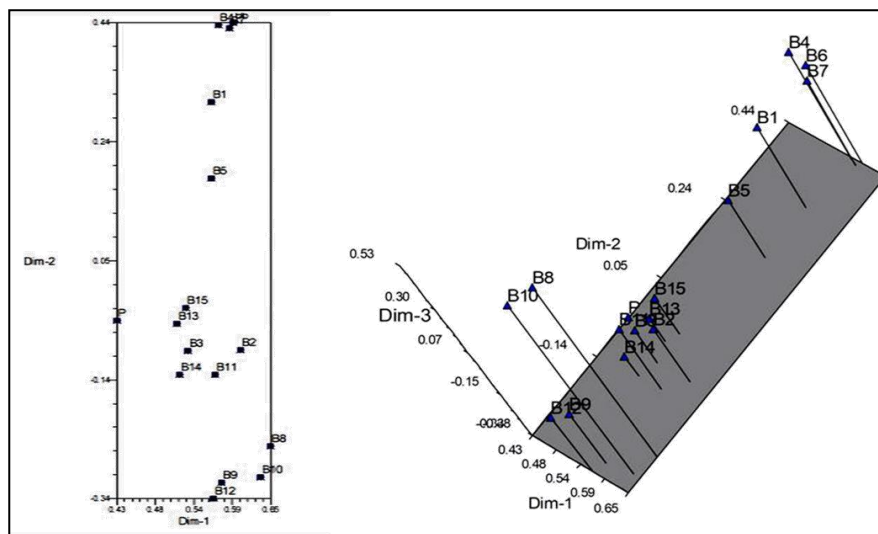


Fig 8: Principle component analysis showing genetic diversity for ISSR markers in 15 bamboo genotypes and one rice genotype. (A) 2-dimensional plot and (B) 3-dimensional plot.

4. Conclusion

The molecular techniques are therefore much more helpful and relied method to assess the relative diversity within and among the species and locate diverse accessions for breeding purposes (Nayak *et al.* 2003) [7]. Being a quick and sensitive method, RAPD analysis has proved its significance for diversity analysis through the sets of the data obtained from the molecular approach. (Qian *et al.*, 2001). RAPD and ISSR exposed enough polymorphism among the species of the bamboos of Kokrajhar. Based on the outcome of RAPD analysis, it is understandable that significant differences exist among those species of 'bio-steel' exhibiting 99.23% of polymorphism. The dendrogram prepared by the data acquired from RAPD analysis displayed the closeness of the genus '*Bambusa*' to each other. *Bambusa vulgaris* fo Waminii and *B. vulgaris* var. Vittata were clustered together sharing the highest similarity. While, *B. vulgaris* var. Vulgaris was found to be clustered closely with *Dendrocalamus hamiltonii* displaying similarity coefficient of 71% followed by *D. giganteus* which too was grouped with the genus '*Bambusa*'. *D. strictus* on other hand was singled out and remained in a different cluster instead of clustering with its own species. However, ISSR marker showed higher number of polymorphism (100%) than RAPD marker (Ge & Sun, 1998; Mattioni *et al.*, 2002) [1]. Due to their reproducible and specific amplifications of genomic regions, the ISSR markers can implement or perform effective genetic differentiation at varied levels. It is concluded from the result that RAPD and ISSR markers are strong, fast and reliable tools for characterizing and authenticating intrageneric classification and the genetic variation assessment for distinguishing and knowing the closely and distantly related species. The investigation and study among the different species of bamboo through molecular approaches is therefore indispensable as it reflects the true history of evolution thereby developing reliable keys for determination or identification.

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