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Comparative Study of RAPD and ISSR Markers to Assess the Genetic Diversity of Betel Vine (*Piper betle* L.) in Orissa, India

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ABSTRACT
In Orissa Betel vine (*Piper betle* L., family Piperaceae) is an important asexually propagated cash crop comprising of several cultivars. There are many cultivares but they are not well demarcated due to similarities in the morphological characters and in certain places same cultivars are cultivated under different local name. Therefore, in the present study DNA fingerprinting technique has been used to differentiate cultivars of betel vine for crop improvement programme. So Comparative study of both RAPD and ISSR markers analysis were used to establish genetic identities and evaluate genetic diversity among fifteen cultivars of betel vine grown in different parts of Orissa. Thirty RAPD and 25 ISSR primers were tested to resolve the genetic diversity among the cultivars. Twenty RAPD and 18 ISSR primers resulted in 523 amplicons. Out of these 504 were polymorphic loci and 54 were found to be unique. The extent of genetic diversity and relatedness among 15 cultivars were computed through Jaccard's similarity coefficient. Maximum similarity (0.68) was observed between Balipana and Birkoli and minimum (0.114) for Banglamandesore chitalpudi and Halisahar Sanchi. All the cultivars were related with each other with an average similarity of 0.2913. Dendrogram showed Godibangala was separated from rest of the species into isolated clade in both the analysis. Correlation between RAPD and ISSR marker was very low (r = 0.17). RAPD showed high correlation with all the primers.

Key words: Genetic diversity, betelvine, RAPD, ISSR, molecular markers, cluster analysis

INTRODUCTION
Betel vine (*Piper betle* L., family Piperaceae) is an important, traditional and ancient crop of India. Leaves of betel vine have been used with condiments such as areca nut, kattha, cloves, cardamom, fennel and candid rose for chewing purpose. It has also been used in the Indian system of medicine (Rawat *et al*., 1989a; Garg and Rajshree, 1992; Sandhya *et al*., 1995) for digestive, caraminative, stimulant, antiseptic and antifungal purposes. A phenolic compound, hydroxy-chavicol, with anticarcinogenic property has also been identified in betel leaves (Bhide *et al*., 1991). Fresh juice of betel leaves is also used in many ayurvedic preparations (Sharma, 1991).

Betel vine is widely cultivated in the states of Uttarpradesh, Bihar, Madhyapradesh, Northeastern India, Maharashtra, Karnataka, West Bengal, Orissa andhra Pradesh, Tamilnadu,

Kerala and Andamans in India. Betel vines are dioecious in nature. Under controlled hybridization, attempts have been made to cross different landraces and in some of these experiments, viable seed set has been reported (Maiti and Shivashankar, 2002). However, as a crop, propagation is only through vegetative means. Its cultivation in northern India under sub-tropical conditions has been shown to be a unique case of plant establishment under anthropogenically regulated microclimatic conditions (Kumar, 1999).

The betel vine growers invariably named their cultivars with local or vernacular names. Therefore, these cultivated betel vines are nothing but landraces. A survey over several years indicated that there are many local cultivars (landraces) of betel vines in Orissa, India. Many of these land races differ from each other in organoleptic properties (Verma *et al.*, 2004). Scrutiny over the landrace names and their etymology, suggests that a given landrace may be named differently in different regions and more than one land race may have same name. On the basis of chemical constituents and essential oils, five prominent groups of betel vine landraces, namely, Bangla, Kapoori, Metha, Sanchii and Desavari have been recognized (Rawat *et al.*, 1989b). The research work on genetic variation among the landraces using molecular or biochemical methods is however, scanty. In the absence of any systematic attempts to resolve this nomenclature problem and since betelvines are vegetatively propagated, most of these names are as ancient as the cultivation of betelvine itself. A few isolated efforts have been made to rationalize the different landraces and to identify similar or dissimilar types among them. No studies have been reported on the phylogeny of betelvine in Orissa. So this report will be the first to disclose the phylogenetic relationship among the betelvine cultivars. The evaluation of genetic diversity and phylogenetic relationship among the cultivars would promote the efficient use of genetic variations in the breeding programme (Paterson *et al.*, 1991). As it is a cash crop of coastal Orissa, many people depend on it for their livelihood. This study will give a better idea about the distribution and cropping pattern of Betel vine.

The PCR based method for DNA profiling, Random Amplified Polymorphic DNA (RAPD) (Welsh and Mc Clelland, 1990; Williams *et al.*, 1990) and inter simple sequence repeats (ISSR) (Bornet and Branchard, 2001; Zietkiewicz *et al.*, 1994) were used to identify the duplicates or sort the germplasm and to estimate genetic diversity among the plants (Virk *et al.*, 1995). This technique was used in our laboratory to determine genetic variation in intra specific levels (Ranade *et al.*, 1997; Farooqui *et al.*, 1998; Goswami and Ranade, 1999). In the present study we show the application of RAPD (Lynch and Milligan, 1994; Tembe and Deodhar, 2010; Tertivanidis *et al.*, 2008) and ISSR (Gantait *et al.*, 2010; Fares *et al.*, 2009) technique in assessing the diversity amongst the betel vine landraces collected from different parts of Orissa and maintained under All India Coordinated Research Project (AICRP) on betel vine, Bhubaneswar, Orissa, India. Although, RAPD (Colagar *et al.*, 2010) and ISSR profiling differ mainly in the pattern of fragment they amplify, the data interpretation is identical and can be combined for statistical analysis (Souframanien and Gopalakrishna, 2004). ISSRs are semiarbitrary markers amplified by PCR in the presence of one primer complementary to a target microsatellite. Like RAPDs, ISSRs markers are also quick and easy to handle and have been successfully utilized for the phylogenetic analysis.

MATERIALS AND METHODS

Sample collection: Betel vine landraces were collected and maintained under All India Coordinated Research Project (AICRP), OUAT, Bhubaneswar on Betel vine (Table 1). The study was conducted during the period of 2005-06. Young leaf tissue was harvested from field grown

Table 1: Name, Acc No. and source of collection of germ plasm of betel vine

Name of cultivars	Acc No.	Source of collection
Godi bangala	Opb-1	Kakatapur (Orissa)
Nua bangala	Opb-2	Chandanpur (Orissa)
Balipana	Opb-3	Puri (Orissa)
Birkoli	Opb-7	Balangir (Orissa)
Kali mahata	Opb-8	Puri (Orissa)
SGM-1	Opb-9	Sirugamani (Tamilnadu)
Kali bangala	Kpb-4	Howrah (West bengal)
Halisahar sanchi	Kpb-10	24- pragana (West bengal)
Awani	Apb-2	Jorhat (Assam)
Gandhi pana	Apb-5	Jorhat (Assam)
Ramtek bangala	Rpb-1	Ramtek (Madhya pradesh)
Maghai	Rpb-3	Jutalpur (Madhya pradesh)
Bangala mandasore chinthalpudi	Cpb-10	Chinthalpudi (Andhra pradesh)
Karapaku bangala	Cpb-12	Baputal (Andhra pradesh)
Andaman local	Cpb-13	Andaman Nicober

vines, washed properly to remove dirt, mopped dry and quickly frozen. Then the frozen leaves were powered using liquid nitrogen. The powders were either used for isolation of DNA immediately or were stored in a deep freezer (-80°C) for long term storage.

Isolation of genomic DNA: The total genomic DNA was isolated from powdered and young leaf tissue of betel vine landraces by using modified CTAB method (Doyle and Doyle, 1990). Three independent DNA preparations were made from leaf tissue collected from each land races. The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel (Ranade *et al*., 2002).

RAPD marker analysis: RAPD amplification was performed with random decamer primers obtained from *Operon Technologies* (Alameda, CA, USA). Thirty arbitrary RAPD primers were tested for PCR amplification. Twenty of them were chosen for the analysis because they produced highly readable and reproducible bands (Table 2). RAPD analysis was done by using primers from A, B, D, H and N series (Table 2) were produced highly reproducible bands. The experiment was standardized using various primers, template DNA and Mg^{2+} concentration to determine the optimum result. The final amplification reactions contained Each reaction mixture of 25 μL contained 20 ng template DNA, 2.5 μL of 10 X assay buffer (100 mM Tris HCl pH 8.3, 500 mM KCl and 0.1% gelatin), 1.5 mM $MgCl_2$, 200 mM each dNTPs, 15 ng primer and 0.5 U Taq DNA polymerase (Banglore genei, Banglore). The reaction was cycled 42 times at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min in a thermo cycler (Applied Biosystem, Model 9700). The final extension cycle allowed an additional incubation for 7 min at 72°C.

ISSR marker analysis: For ISSR amplification some anchored and non anchored microsatellite primers designed in our laboratory were randomly selected and used. Twenty-five arbitrary ISSR primers were used for PCR amplification. Eighteen of them were chosen for the analysis because they produced highly readable and reproducible bands (Table 3). Each reaction mixture of 25 μL contained 20 ng template DNA, 2.5 μL of 10 X assay buffer (100 mM Tris HCl pH 8.3, 500 mM KCL and 0.1% gelatin), 1.5 mM $MgCl_2$, 200 mM each dNTPs, 15 ng primer and 0.5 U Taq DNA

Table 2: Details of RAPD primers analysis

[a]Primers	Sequences	Range of amplicons (bp)	Resolving power	Primer index
OPA18	5'GAACGGACTC3'	>3000-650	10.000	6.257
OPA19	5'CAAACGTCGG3'	2800-670	11.230	5.340
OPA12	5'TCGGCGATGA3'	3000-450	8.390	4.560
OPA06	5'GGTCCCTGAC3'	2500-750	5.540	3.850
OPA17	5'GACCGCTTGT3'	2300-300	12.540	7.850
OPA07	5'GATGACCGCC3'	3000-600	9.570	4.960
OPA 18	5'GAACGGACTC3'	2500-400	8.670	5.670
OPB01	5'GTTTCGCTCC3'	2250-760	10.560	6.780
OPB11	5'GTAGACCCGT3'	2300-950	6.780	3.240
OPB 12	5'GGCACTGAGG3'	2550-900	10.640	6.780
OPD 01	5'GTCAGGGCAA3'	2800-700	8.480	3.920
OPD 02	5'GGTCGGAGAA3'	2600-1000	5.380	3.680
OPD 03	5'GTCGCCGTCA3'	3000-650	7.730	3.467
OPD 06	5'GGGAATTCGG3'	2750-550	8.160	4.480
OPD 04	5'GGTGAGGTCA3'	2500-400	5.840	3.460
OPD 08	5'GTGTGCCCCA3'	3000-550	6.933	5.013
OPD 18	5'AGGTGACCGT3'	3000-450	10.133	2.824
OPD 20	5'ACCCGGTCAC3'	3000-500	12.667	6.009
OPH04	5'GGAAGTCGCC3'	2000-700	5.780	3.560
OPN 16	5'AAGCGACCTG3'	2900-275	12.667	5.404

[a]Primers are from operon technology

Table 3: Details of ISSR primers analysis

[a]Primers	Sequences	Range of amplicons (bp)	Resolving power	Primer index
1	(GACA)4	2600-350	6.4	4.75
2	(AGG)6	1350-375	8.933	4.356
3	(GTG)5	1175-400	3.867	2.75
4	(AC)8T	2260-500	6.56	5.34
5	(AG)8T	2300-700	5.69	3.24
6	(CA)8AT	1975-625	7.36	4.36
7	(AG)8G	2350-550	6.25	4.78
8	(GA)9T	2530-650	9.32	5.86
9	T(AG)9	1890-370	5.46	3.58
10	(AC)9G	1500-650	4.58	2.88
11	(AC)9T	1960-480	6.46	3.74
12	(TC)8G	1450-400	5.92	3.96
13	(CCG)6	2450-540	8.12	5.46
14	(CTC)6	2150-450	7.84	4.38
15	(CA)6GG	1650-500	5.92	3.78
16	(ATG)6	1750-650	4.76	3.14
17	(GA)9T	2100-760	5.48	3.28
18	(AGC)5CG	2350-460	7.56	4.84

[a]Primers are from operon technology

polymerase (Bangalore genei, Bangalore). The amplification was carried out in a thermal cycler (Applied Biosystem, Model 9700). The reaction was cycled 42 times at 94°C for 1 min, 45-55°C for 1 min for annealing and 72°C for 2 min in a thermo cycler. The final extension cycle allowed an additional incubation for 7 min at 72°C for complete polymerization.

Agarose gel electrophoresis: The amplified products were separated by electrophoresis through 1.5% agarose gel in 1X TAE buffer pH 8 (Sambrook *et al.*, 1989) visualized and photographed using gel documentation system (Bio rad USA) after staining with ethidium bromide.

Band profile reproducibility: Three replicate DNA extractions from leaves of *Piper betel* were used to assess the consistency of the band profiles. RAPD and ISSR amplifications were repeated at least three times and only the reproducible PCR products were scored.

Data analysis: Data (fragment size of all the amplification products estimated from gel by comparison with standard molecular weight marker, 1 kbp DNA ladder) were scored as discrete variables using 1 to indicate presence and 0 to indicate absence of a band. A pair wise matrix of distance between landraces was determined for the cumulative RAPD and ISSR data.

Jaccard's coefficient of similarity (Jaccard, 1901) was measured and a phylogram based on similarity coefficients generated by Unweighted Pair Group Method using Arithmetic average (UPGMA) (Sneath and Sokal, 1973) and sequential agglomerative hierarical nested clustering (SHAN) was obtained. Most informative primers were obtained by comparing all primers with that of pooled data using Mantel Z statistics (Mantel, 1967). The entire analysis was performed using the statistical package NYTSY 2.0 e. Principal Co ordinate Analysis (PCA) was used to retrieve information about the clustering pattern of analyzed the primers (Semagan *et al.*, 2000).

RESULTS AND DISCUSSION

The betelvine DNAs were tested in RAPD and ISSR reactions in triplicate. The initial pilot reactions were carried out to determine the optimum primer, template and Mg^{2+} concentrations (data not shown). Subsequently, the entire set of betel vine DNAs were tested with thirty decamer primers for RAPD. Out of these twenty RAPD primers produced highly reproducible and scorable bands (Table 2) were chosen for analysis. The profiles were considered consistent if at least two of the three DNA preparations revealed identically sized prominent bands after amplification with a given primer.

The highest number of fragments (16) was amplified by the primer OPA18 (Fig. 1), OPD20 and OPN16 and that were lowest (10) by primer OPD03. The primers OPA18 (Fig. 1), OPD20 and OPN16 (Fig. 2) produced maximum number of polymorphic bands (16) and minimum (9) in case of OPD03. The OPD03 and OPD18 produced single monomorphic band. Resolving power was highest for primer OPD20 and OPN16 (12.667) and lowest for OPD02 (5.38) (Table 2). Primer

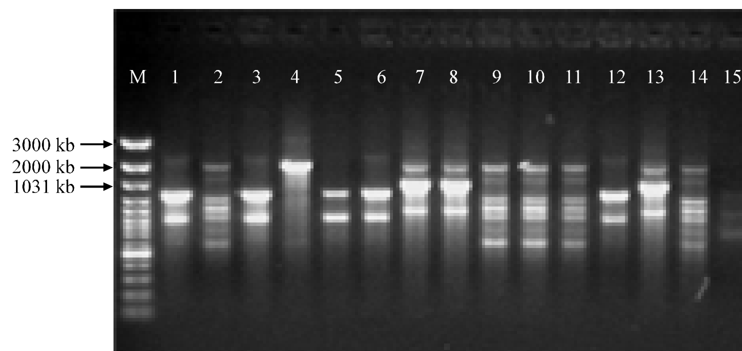

Fig. 1: Banding pattern of OPA18 on 1.5% agarose gel

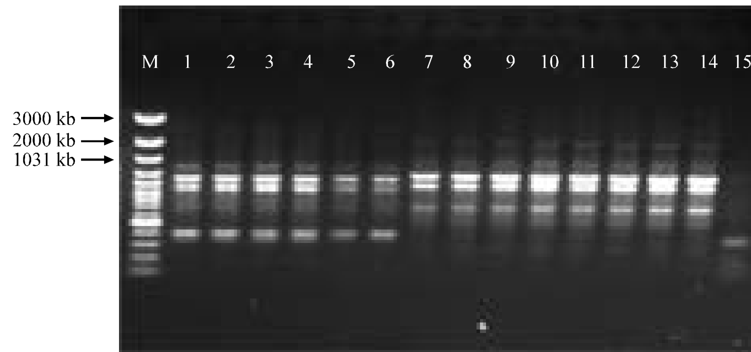

Fig. 2: Banding pattern of OPN16 on 1.5% agarose gel

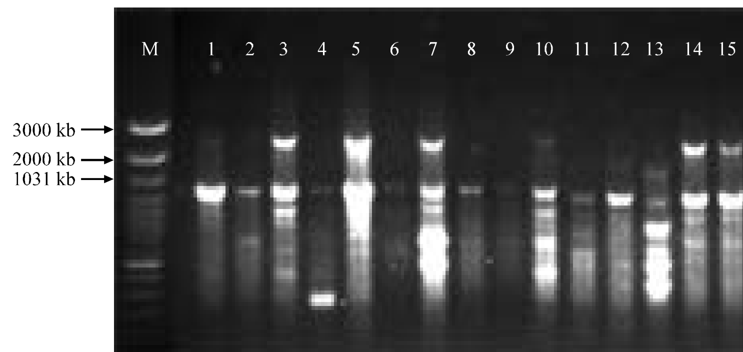

Fig. 3: Banding pattern of (GACA)4 on 1.5% agarose gel

index was highest in OPA17 (7.85) and lowest in case of OPD18 (2.824) (Table 2). Jaccard's similarity coefficient showed landrace Balipana and Birkoli were closely related having similarity value 0.779 and landrace Andaman Local and Birkoli were widely apart with similarity of 0.119.

Twenty-five random oligonucleotide primers were tested for ISSR. Out of these eighteen ISSR primers produced highly reproducible and scorable bands (Table 3) were chosen for analysis. Resolving power and primer index for ISSR were nearly same as comparison to RAPD marker. Maximum numbers of bands (16) were resolved for the primer (GACA)4 and lowest numbers of bands (10) were resolved for (GTG)5. The highest number of polymorphic loci were detected by primer (GACA)4 (Fig. 3) and lowest by (GTG)5. Resolving power of primer (AGG)6 was maximum 8.933 and in case (GTG)5 it was lowest 3.867 (Table 3). Maximum Primer index was 4.75 in case of (GA)9T and minimum in case of (GTG)5 2.75 (Table 3).

When both markers were combined together it was observed that the average similarity between two cultivars was 0.2913. Highest similarity observed between the cultivars Birkoli and Balipana (0.68). Lowest similarity observed between Halisahar Sanchi and Bagla Mandasore Chitalpudi (0.114). From the above analysis RAPD maker shows Godibangala landrace was clustered into separate clade (Fig. 4). In ISSR analysis Godibangla andaman Local and Bagla Mandasore Chitalpudi are the subclade of one main clade (Fig. 5). Both RAPD and ISSR (Fig. 6) showed similar results as RAPD showing Godibangala as separate clade. The clustering of 15 cultivars was confirmed using Principal Co ordinate Analysis (PRINCORDA) which revealed the similar result obtained from SHAN clustering (Fig. 7). The correlation between RAPD and ISSR markers obtained using Mentel Z correlation analysis was very low (Fig. 8).

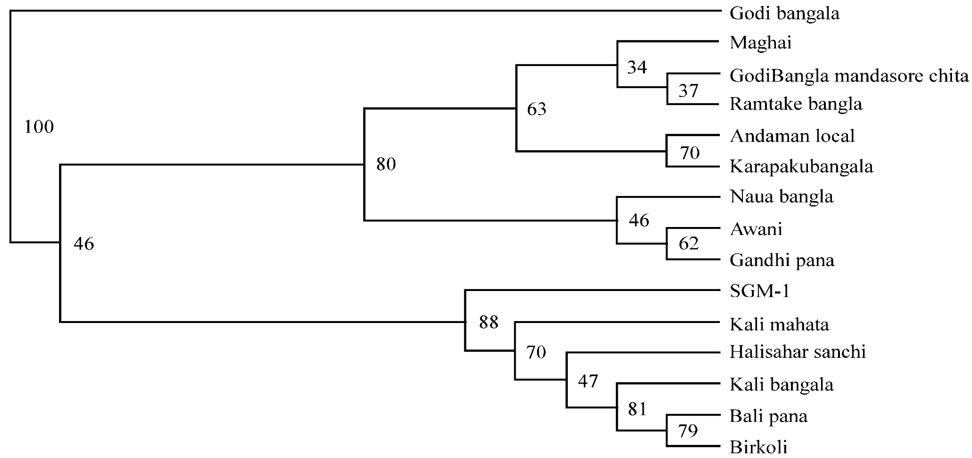

Fig. 4: Cluster analysis of cumulative RAPD data for betel vine land race

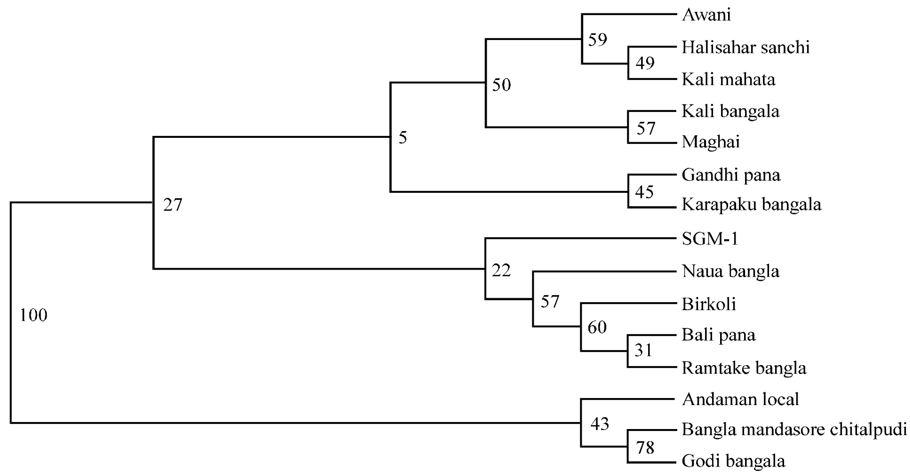

Fig. 5: Cluster analysis of cumulative ISSR data for betel vine land race

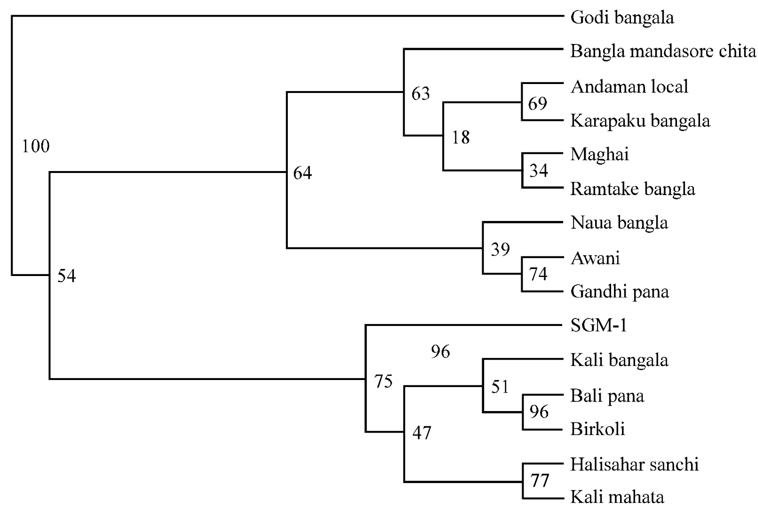

Fig. 6: Cluster analysis of cumulative RAPD and ISSR data for betel vine land race

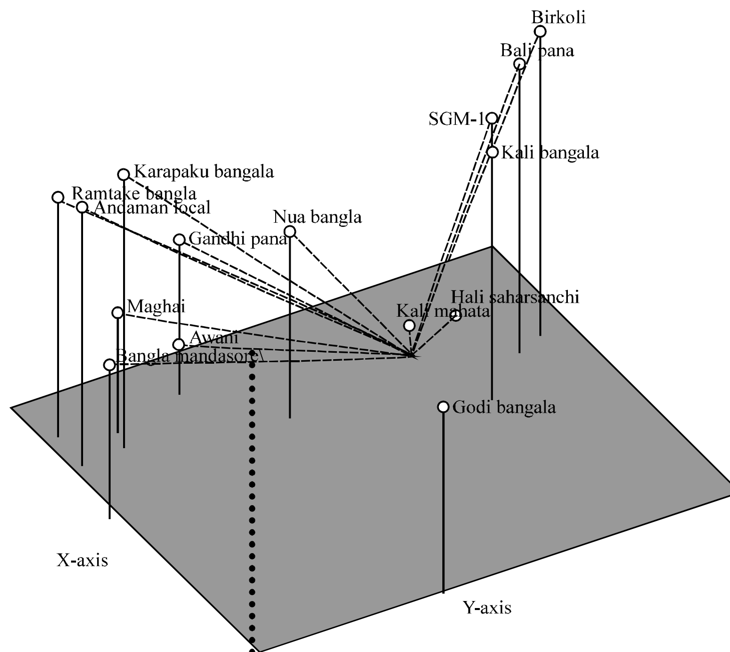

Fig. 7: Principal Co ordinate analysis of betel vine varieties

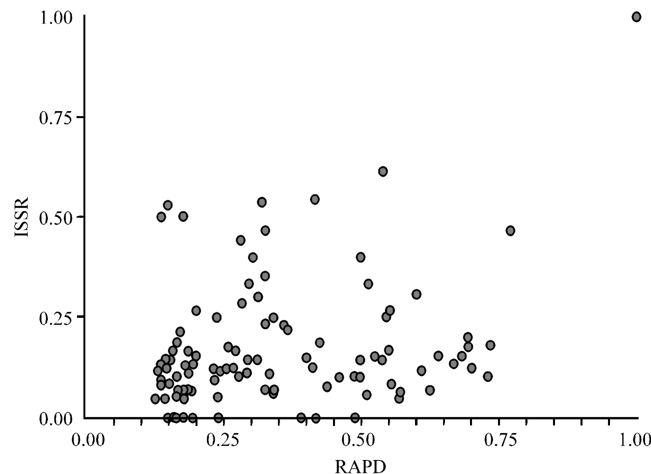

Fig. 8: Correlation analysis of combined RAPD and ISSR markers (R = 0.17)

Betel vine is a very important medicinal and cash crop in Orissa as well as in India. It is one of the heritage crops of India. Improvement of this crop is primarily based on recombination breeding programme that utilizes superior landraces possessing resistance to biotic and abiotic stress, taken as parents. The different land races were distinguished earlier on the basis of the leaf essential oils (Rawat *et al.*, 1989b). However, the extent of variation among and between them is not easily analysed due to its vegetative propagation attributes. Under these conditions, RAPD technique could reveal within-landrace type variation more efficiently (Verma *et al.*, 2004). Since, success of the improvement programme is primarily dependent on choice of genetically diverse parents. So an attempt has been taken in this study to assess the genetic diversity of 15 cultivars at molecular level for identification of superior parents for the purpose of recombination breeding.

RAPD and ISSR markers were used to characterize and compare the genetic diversity among the Betel vine cultivars. Both RAPD and ISSR showed similar result. But the ISSR technique generated more bands per primer and revealed higher levels of polymorphism, so we recommend ISSR for future studies. RAPD and ISSR are PCR based arbitrary oligonucleotides primers which generally used to amplify the complementary sequence in the genome. So it can be easily used without prior knowledge of the genome sequence. On an average 13.78 bands have been amplified by each primer which is reasonably good (Ranade *et al.*, 2002).

Similarity value showed that Bali pana and Birkoli both are from Orissa are the closest cultivars. Similarly Bangla Mandasore Chitalpudi from Andhra Pradesh and Halisahar Sanchi from West Bengal are most distantly related. Other varieties like Awanipan and Gandhi pan shared a same node interestingly both of them were collected from Assam (Table 1). Maghai and Ramtake Bangala shared same node and both of them were collected from Madhya Pradesh (Table 1) which indicates that there are certain relationship between geographical distribution and genetic diversity.

However, there is no relationship among the cultivars if response to different diseases were considered. In fact it was expected that DNA fragments from the entire genomic DNA amplified, only one or few genes control the disease reactions. The high boot strap value of different cultivars in the present investigation indicated that the major clustering pattern won't change even if some other markers are added (Nybom, 2004). The different groups like Bangla, Sanchi and others could not be separated out which contradicted the earlier report of Rawat *et al.* (1989a), Ranade *et al.* (2002) and Verma *et al.* (2004). When both the markers are compared, it was observed that RAPD shows high correlation with that of combined markers. Surprisingly, the betel vines, despite being vegetatively propagated crop showed considerably less similarity than expected or as reported for other vegetatively propagated crop (Breto *et al.*, 2001; Vega *et al.*, 2001). Lowest levels of polymorphism (0.8%) detected for a plant species by RAPD analysis, for *Agave tequilana* var *azul* plants (Vega *et al.*, 2001). However, the betelvines exhibit less variability in morphological characters compared to other vegetatively propagated plant. The betelvines, though vegetatively propagated, differ considerably from the agaves by exhibiting greater diversity amongst the landraces. It is thus possible that centuries of cultivation by vegetative means have fixed the differences among the groups of landraces. Alternatively, the different landraces may have been derived from several ancestral and diverged founders or seed derived plants before intensive vegetative cultivation practices fixed the variations by eliminating selection of the plants on the basis of sexual reproduction (Verma *et al.*, 2004). This could be another reason for the greater than expected diversity among the landraces and groups. The RAPD and ISSR profiles, however, revealed relative variability among the betelvine landraces. Clearly there is scope for large-scale application of RAPD (Bussell, 1999) for analysis of obligate vegetatively propagated plants.

One interesting result from the present study suggests the landraces collected from different geographical region and climatic condition were clustered into one clade. Godibangla found in a separate clade with all the primers it may genetically different from other lanraces. So further indepth study required revealing this difference.

CONCLUSIONS

In the betelvine, DNA methodologies have become a clear and powerful impact on understanding the origin, evolution and genome relationships among the plant species. However the routine use of molecular DNA marker for identification of plant collection might be very important to get more and better understanding about the genome relationship of the related plant

species. Therefore, more studies are planned on chromosome complements and genomes to clarify and justify the species taxonomical relationships and evolution of the species. We are also planning to extend present studies by using RFLP, AFLP and microsatellite marker on the other congneric species in betelvine distributed in India also to use those molecular markers for studies of genetic differentiation among the wild species to identify areas of maximum diversity and to estimate genetic variability in natural population.

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