Genetic diversity amongst landraces of a dioecious vegetatively propagated plant, betelvine (*Piper betle* L.)[§]

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Betelvine (*Piper betle* L., family Piperaceae) is an important, traditional and widely cultivated crop of India. The cultivators and consumers recognize more than 100 cultivars (landraces) based on regional and organoleptic considerations, while in terms of phytochemical constituents only five groups have been identified for all the landraces. Since betelvine is an obligate vegetatively propagated species, genomic changes, if any, may have become 'fixed' in the landraces. We carried out random amplified polymorphic DNA (RAPD) analysis in several landraces considered in four groups, namely, '*Kapoori*', '*Bangla*', '*Sanchi*' and '*Others*' in order to ascertain their genetic diversity. On the basis of the data from eleven RAPD primers, we distinguished genetic variation within and among the four groups of landraces. The results indicate the '*Kapoori*' group is the most diverse. The neighbour joining (NJ) tree after a bootstrap (500 replicate) test of robustness clearly shows the four groups to be well separated. Interestingly, all known male or female betelvine landraces have separated in the NJ tree indicating an apparent gender-based distinction among the betelvines.

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1. Introduction

Betelvine (*Piper betle* L., family Piperaceae) is an important, traditional and ancient crop of India. Leaves of betelvine have been used with condiments such as arecanut, kattha, cloves, cardamom, fennel and candied rose for chewing purposes. The leaves have also been used in Indian system of medicine and health (Rawat *et al* 1989a; Garg and Jain 1992; Sandhya *et al* 1995) and several attributes such as 'digestive', 'carminative', 'stimulant', 'antiseptic' and 'antifungal' activities have been described. 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).

Betelvine is widely cultivated in the states of Uttar Pradesh, Bihar, Madhya Pradesh, Northeastern India, Maharashtra, Karnataka, West Bengal, Orissa, Andhra Pradesh, Tamil Nadu, Kerala and Andamans in India. Betelvines are dioecious. 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 Shivashankara 1998). 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).

Cultivated betelvine is grown in traditional farming systems many of which are managed exclusively or communally. The betelvine growers invariably named their cultivars with local or vernacular names. These cultivated betelvines are therefore, nothing but landraces and it is this description that will be used consistently throughout the manuscript. A survey over several years indicated between 125 to 150 local cultivars (landraces) of betelvines in India. Many of these landraces differ from each other in several organoleptic properties. Scrutiny of the landrace names and their etymology, suggests that a given

Keywords. *Bangla*; *Kapoori*; NJ tree, RAPD, *Sanchi*, similarity index

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landrace may be named differently in different regions and more than one landrace may have the same name. Thus landraces with prefix *Desi* in their names invariably refer to the landrace '*Bangla*' in West Bengal, landrace '*Kapoori*' in Maharashtra and landrace '*Desavari*' in Madhya Pradesh (Balasubrahmanyam *et al* 1995). 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. On the basis of chemical constituent analysis of leaf essential oils, five prominent groups of betelvine landraces, namely, *Bangla*, *Kapoori*, *Meetha*, *Sanchii* and *Desawari* have been recognized (Rawat *et al* 1989b). The research work on genetic variation among the landraces using molecular or biochemical methods is, however, scanty.

The PCR based method for DNA profiling, random amplified polymorphic DNA or RAPD (Welsh and McClelland 1990; Williams *et al* 1990) was used to identify 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, at both intra- as well as inter-species levels, in *Amaranthus* spp., *Azadirachta indica* L. and *Prosopis* spp. (Ranade *et al* 1997; Farooqui *et al* 1998; Goswami and Ranade 1999). During our research on the molecular profiling of betelvine germplasm, we have earlier shown the utility of RAPD technique to distinguish between '*Kapoori*' and '*Bangla*' types of betelvine (Ranade *et al* 2002). In the present paper we now show the application of RAPD technique in assessing the diversity amongst the betelvine landraces collected from different centers under the All India Coordinated Research Project (AICRP) on betelvine.

2. Materials and methods

2.1 *Plant material*

Betelvine landraces were collected from some of the centres of the All India Coordinated Research Project (AICRP) on Betelvine and the details are given in table 1. Young leaf tissue was harvested from field-grown vines, washed free of dirt, mopped dry and quickly frozen and powdered 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. Leaf tissue was also collected from *Piper hamiltonii* and *Morus alba*, growing in the Botanical Garden, at NBRI, Lucknow. The latter two species were selected for comparison as outgroup in the RAPD analysis. Of these, the mulberry (*M. alba*) leaf tissue was already available in our laboratory, being the subject of a separate study (Bhattacharya and Ranade 2001).

2.2 *Isolation of DNA*

Total genomic DNA was isolated from the powdered and frozen young leaf tissue of the betelvine landraces and the outgroup plants using the procedure of de Kochko and Hamon (1990) with some modifications (Ranade *et al* 1997). At least three independent DNA preparations were made from leaf tissues collected from each landrace. The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel.

2.3 *RAPD reactions*

Sixty decamers from kits B, F and G (Operon Technologies Inc., Alameda, California, USA) were used as primers. DNA was amplified as described earlier (Ranade *et al* 2002). Initially a pilot experiment was conducted using various primer, template DNA and Mg^{2+} ion concentrations to determine the optimum concentrations. The final amplification reactions contained 10 mM TAPS (pH 8·8), 1·5 mM $MgCl_2$, 50 mM KCl, 0·01% gelatin, 0·2 mM each dNTP, 10 pmol primer, 0·5 U *Taq* DNA polymerase (Bangalore Genei, Bangalore) and 50 ng betelvine DNA template in a 25 µl reaction volume. The reaction mixes were overlaid with mineral oil prior to the amplification. The reaction was cycled 44 times at 94°C for 1 min, 35°C for 1·5 min and 72°C for 1·5 min in a thermo cycler (Robocycler 40, Stratagene GmbH, Germany). The final extension cycle allowed an additional incubation for 5 min at 72°C.

2.4 *Agarose gel electrophoresis*

Amplification products were separated by electrophoresis (at a constant current of 15 mA) through 1·0% agarose gels in 0·5 × TBE buffer according to Sambrook *et al* (1989), visualized and imaged using Nighthawk™ gel documentation system (pdi Inc., USA) after staining with ethidium bromide.

2.5 *Data analysis*

Data (fragment sizes of all the amplification products, estimated from the gel by comparison with standard molecular weight marker, 1 kbp DNA ladder) were scored as discrete variables, using '1' (one) to indicate presence and '0' (zero) to indicate absence of a band. A pair-wise matrix of distance between landraces was determined for the cumulative RAPD (eleven informative primers) data using Jaccard formula (Jaccard 1901) in the program FreeTree

Table 1. Plant material used in this study. Some of the landraces did not result in consistent profiles (landrace numbers, names and source are underscored) and for these landraces band data was not scored from the gels.

Number (DNA sample number)	Landrace	AICRP centre from where collected
K1 (422)	Karpuri	Sirugamani, Tamil Nadu
K2 (203)	Kapoori Chittikavata	Chinthalapudi, Andhra Pradesh
K3 (312)[a]	Kapoori Bolvad	Digraj, Maharashtra
K4 (302)	Kapoori Shirpurkata	Digraj, Maharashtra
K5 (513)	Kapoori Vellaikodi	NBRI, Uttar Pradesh
K6 (218)	Kapoori Doddipatla	Chinthalapudi, Andhra Pradesh
K7 (214)	Kapoori Pedachapelli	Chinthalapudi, Andhra Pradesh
K8 (212)	Kapoori Vasani	Chinthalapudi, Andhra Pradesh
K9 (211)	Kapoori Chillumuru	Chinthalapudi, Andhra Pradesh
K10 (213)	Kapoori Tuni	Chinthalapudi, Andhra Pradesh
K11 (228)	Kapoori Sangli	Chinthalapudi, Andhra Pradesh
K12 (301)	Kapoori Mhaisal	Digraj, Maharashtra
K13 (304)	Kapoori Bolvad	Digraj, Maharashtra
K14 (306)	Kapoori Karve	Digraj, Maharashtra
K15 (308)	Kapoori Veer	Digraj, Maharashtra
K16 (310)	Kapoori Arvi	Digraj, Maharashtra
K17 (323)	Kapoori Maharashtra	Digraj, Maharashtra
K18 (307)	Kapoori Indapur	Digraj, Maharashtra
K19 (414)	Kapoori Patchaikodi	Sirugamani, Tamil Nadu
K20 (408)	Tellaku	Sirugamani, Tamil Nadu
K21 (229)	Tellaku Chennur	Chinthalapudi, Andhra Pradesh
B1 (410)	Bangla Ramtek	Sirugamani, Tamil Nadu
B2 (426)[b]	Bangla Desi	Sirugamani, Tamil Nadu
B3 (1)	Bangla Kalkattiya	Mahoba, Uttar Pradesh
B4 (500)	Meetha-cum-Bangla	NBRI, Uttar Pradesh
B5 (115)	Bangla Bihar	Bangalore, Karnataka
B6 (512)	Bangla Ayurvedic	NBRI, Uttar Pradesh
B7 (201)	Bangla Mandsore	Chinthalapudi, Andhra Pradesh
B8 (423)	Bangla Jabalpur	Sirugamani, Tamil Nadu
B9 (103)	Meetha Bangla	Bangalore, Karnataka
B10 (5)	Bangla Calcutta	Mahoba, Uttar Pradesh
B11 (105)	Bangla Ramtek	Bangalore, Karnataka
B12 (7)	Bangla Desi	Mahoba, Uttar Pradesh
B13 (409)	Bangla Jal	Sirugamani, Tamil Nadu
B14 (415)	Bangla Godi	Sirugamani, Tamil Nadu
B15 (427)	Bangla Nova	Sirugamani, Tamil Nadu
B16 (502)	Ghanaghatte	NBRI, Uttar Pradesh
B17 (405)	Gachpan	Sirugamani, Tamil Nadu
S1 (413)	Halisahar Sanchi	Sirugamani, Tamil Nadu
S2 (204)	Kalipatti	Chinthalapudi, Andhra Pradesh
S3 (220)	Blackleaf	Chinthalapudi, Andhra Pradesh
S4 (403)	Kare	Sirugamani, Tamil Nadu
S5 (505)	Kakair	NBRI, Uttar Pradesh
S6 (424)	Kuljedu	Sirugamani, Tamil Nadu
O1 (411)	Sakkarai Kodi	Sirugamani, Tamil Nadu
O2 (406)	Dindugal	Sirugamani, Tamil Nadu
O3 (412)	Sreenivasa Naller	Sirugamani, Tamil Nadu
O4 (418)	Karapaku	Sirugamani, Tamil Nadu
O5 (504)	Mysore Chigaru	NBRI, Uttar Pradesh
O6 (506)	Desawari	NBRI, Uttar Pradesh
O7 (225)	Awanipan	Chinthalapudi, Andhra Pradesh
O8 (416)	SGM-1	Sirugamani, Tamil Nadu
O9 (309)	GN Hybrid	Digraj, Maharashtra
PH	*Piper hamiltonii*	NBRI, Uttar Pradesh
MO	*Morus alba*	NBRI, Uttar Pradesh

[a]Used as an outgroup (sample name = KO) in some gels as indicated in figure 1.
[b]Used as an outgroup (sample name = BO) in some gels as indicated in figure 1.

(Pavlicek *et al* 1999) available from the URL: http://www.natur.cuni.cz/~flegr/freetree.htm. The Neighbor Joining (NJ) tree was computed after a 500 replicate bootstrap analysis. This tree was saved as a text file used as input for the program TreeView (Page 2001, URL: http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The group analysis used the program POPGENE, ver. 1·32, 1997 (Yeh *et al* 1997) available from URL: http://www.ualberta.ca/~fcyeh/. For this program, the betelvine landraces were considered in four groups named as '*Kapoori*', '*Bangla*', '*Sanchi*' and '*Others*' while the two out group plants, *P. hamiltonii* and *M. alba* were included as a fifth group, 'Outgroups'.

3. Results

The betelvine DNAs were tested in RAPD 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 betelvine DNAs were tested with sixty decamer primers (20 each from the B, F and G kits, Operon Tech., CA, USA). Eleven primers (B-08, B-10, F-02, F-07, F-09, F-10, F-12, F-13, F-14, G-16 and G-19, table 2) resulted in consistent RAPD profiles in the landraces. 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 profiles obtained with two of the primers are shown in figure 1. Since some of the DNA samples did not result in discrete profiles (figure 1, profile with primer G-19, lanes marked as K9, K21, B3-4, B6-7, B9-12, S3 and O7) with some of the primers (though the same DNAs gave good profiles with the other primers), the band data for these DNAs was not scored from the agarose profiles and was not included in final calculations and analysis. The samples of landraces excluded from the final analysis are indicated in table 1. The data from the eleven primers was considered cumulatively and resulted in a data matrix of 208 bands. The Jacard distances among pairs of landraces were calculated using the program FreeTree and are given in table 3. The '*Bangla*' group landraces B13 and B14 were found to have the highest similarity (least distance, 0·03, table 3) while the landrace O9 and outgroup *M. alba*, exhibited the least similarity (highest distance, 0·97, table 3). The average similarities of the landraces within different groups as well as between groups are given in table 4. The mean diversity index (*H*) for each group calculated using the program POPGENE are given in table 4. This type of index of diversity, also known as the Shannon index is frequently used for RAPD data because it is insensitive to any bias in the data due to undetectable heterozygosity (Oiki *et al* 2001). On the basis of the mean similarity within groups and the number of polymorphic bands in that group, the probability that any landrace in the group would have the same polymorphic bands as any other randomly selected landrace from that group has been calculated according to Bruford *et al* (1992) and presented in table 4. Data given in table 5 indicate the trend for distribution of pairwise similarities within and between landrace groups and the outgroup plants. The NJ tree generated after a 500 replicate bootstrap analysis depicting the landrace clustering is shown in figure 2. The different betelvine landraces and the outgroup plants were clearly distinguished from each other in the NJ tree. Furthermore, the landraces have separated according to the respective groups and of these, the '*Kapoori*' landraces form one major cluster while the rest of the landraces form the second major cluster (cluster I and cluster II respectively, figure 2). Further, cluster II is made up of three smaller subclusters of the '*Bangla*', '*Sanchi*' and '*Others*' represented as sub-clusters IIA, IIB and IIC respectively (figure 2).

Table 2. The decamer sequences used as primers in RAPD reactions, which have resulted in consistent profiles.

Primer name	Operon kit	Sequence (5′–3′)
OP-B08	B	GTCCACACGG
OP-B10	B	CTGCTGGGAC
OP-F02	F	CAGGATCCCT
OP-F07	F	CCGATATCCC
OP-F09	F	CCAAGCTTCC
OP-F10	F	GGAAGCTTGG
OP-F12	F	ACGGTACCAG
OP-F13	F	GGCTGCAGAA
OP-F14	F	TGCTGCAGGT
OP-G16	G	AGCGTCCTCC
OP-G19	G	GTCAGGGCAA

4. Discussion

Betelvine is one of the heritage crops of India. At present, several landraces are grown throughout India. Despite the importance of this crop, very little research has been done on the genetic variation of several characters. The fact that the plant is propagated vegetatively may also have resulted in this situation. The different landraces 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. We earlier showed a clear distinction between the '*Kapoori*' and '*Bangla*' landraces on the basis of RAPD profiles (Ranade *et al* 2002). In this study these two groups of landraces were clearly distinguishable from each other as well as from other groups. The landraces belonging to '*Bangla*' group were however; more

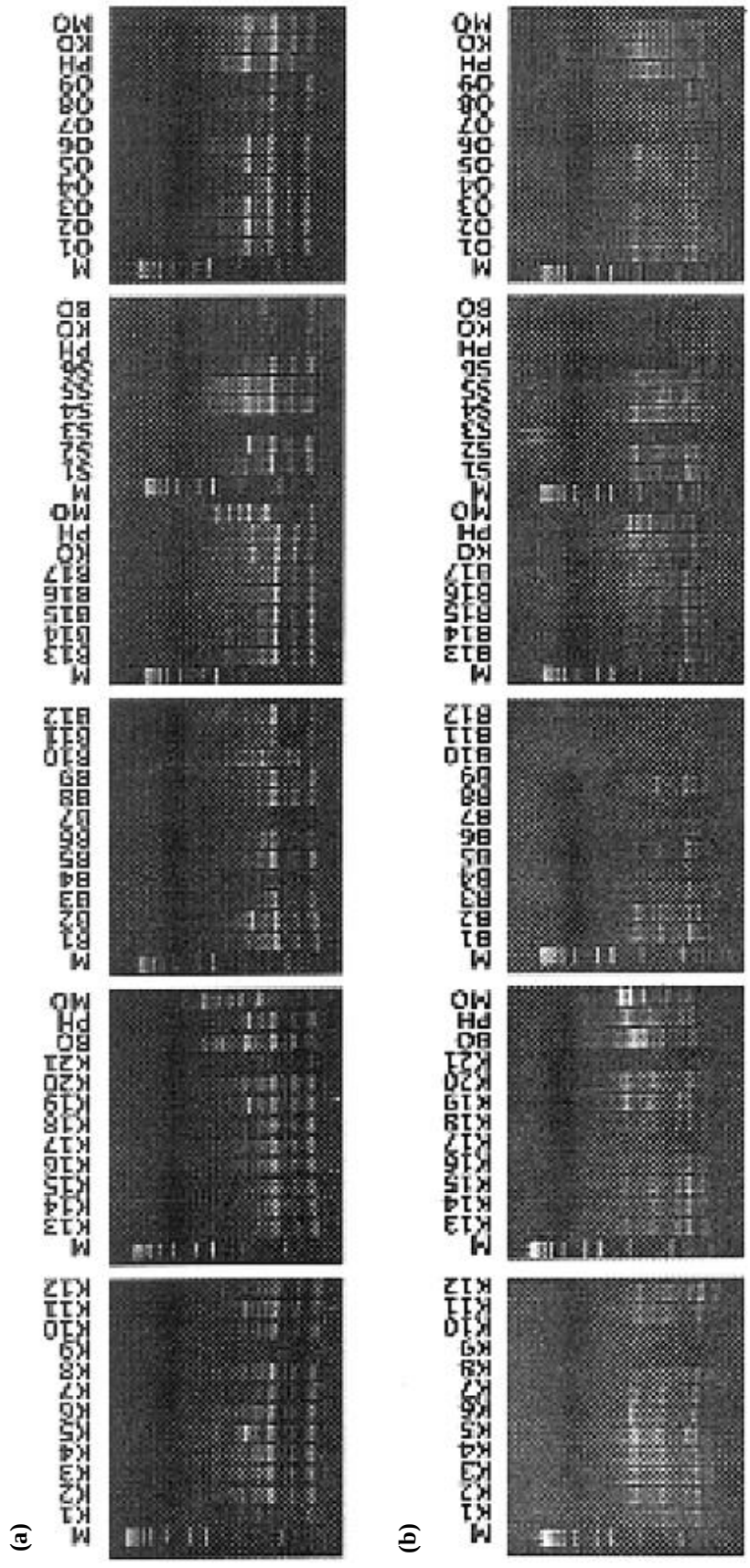

Figure 1. RAPD agarose gel electrophoresis profiles obtained in case of the betelvine landraces using primers OP -F07 (**a**) and OP -G19 (**b**). Lanes indicated by M co n-tain 1 kbp DNA ladder as the molecular weight marker. The landrace numbers are according to table 1 and indicate the template DNA from the appropriate lan drace.

Table 3. The pair-wise distances for the RAPD data, calculated by Jaccard's algorithm, NJ method using FREETREE ver. 1·0 from final 11 primer data.

	K1	K2	K3	K4	K5	K6	K7	K8	K10	K11	K12	K13	K14	K15	K16	K17	K18	K19	K20	B1	B2
K1																					
K2	0·64																				
K3	0·70	0·25																			
K4	0·66	0·07	0·27																		
K5	0·69	0·37	0·38	0·35																	
K6	0·67	0·48	0·52	0·49	0·57																
K7	0·65	0·44	0·52	0·47	0·58	0·26															
K8	0·65	0·31	0·39	0·35	0·46	0·33	0·33														
K10	0·71	0·63	0·70	0·64	0·70	0·51	0·53	0·49													
K11	0·65	0·54	0·60	0·56	0·61	0·48	0·50	0·46	0·45												
K12	0·62	0·31	0·46	0·35	0·51	0·40	0·38	0·32	0·53	0·47											
K13	0·66	0·51	0·53	0·54	0·51	0·57	0·57	0·46	0·72	0·60	0·51										
K14	0·75	0·60	0·49	0·63	0·62	0·67	0·67	0·54	0·73	0·60	0·62	0·32									
K15	0·77	0·57	0·46	0·60	0·60	0·67	0·67	0·53	0·74	0·62	0·64	0·34	0·06								
K16	0·78	0·56	0·47	0·59	0·60	0·66	0·66	0·52	0·72	0·61	0·64	0·39	0·12	0·09							
K17	0·77	0·63	0·54	0·65	0·64	0·68	0·69	0·56	0·71	0·63	0·71	0·47	0·26	0·23	0·15						
K18	0·73	0·60	0·51	0·62	0·65	0·65	0·65	0·55	0·75	0·57	0·61	0·35	0·14	0·16	0·19	0·26					
K19	0·76	0·62	0·53	0·63	0·57	0·69	0·67	0·59	0·75	0·60	0·65	0·51	0·28	0·30	0·28	0·29	0·30				
K20	0·73	0·58	0·49	0·57	0·60	0·61	0·68	0·54	0·69	0·54	0·64	0·49	0·31	0·32	0·31	0·35	0·33	0·32			
B1	0·77	0·68	0·65	0·71	0·70	0·73	0·69	0·66	0·77	0·68	0·69	0·70	0·68	0·68	0·68	0·70	0·72	0·65	0·71		
B2	0·72	0·68	0·62	0·70	0·68	0·70	0·67	0·64	0·75	0·72	0·68	0·71	0·69	0·69	0·67	0·67	0·70	0·66	0·70	0·32	
B5	0·77	0·76	0·69	0·78	0·74	0·78	0·75	0·74	0·81	0·76	0·76	0·70	0·66	0·68	0·70	0·73	0·71	0·71	0·76	0·37	0·41
B8	0·76	0·76	0·73	0·78	0·75	0·75	0·73	0·71	0·77	0·76	0·74	0·69	0·70	0·71	0·71	0·74	0·73	0·72	0·77	0·40	0·40
B13	0·82	0·74	0·70	0·76	0·70	0·75	0·72	0·69	0·77	0·74	0·75	0·71	0·71	0·71	0·69	0·69	0·75	0·68	0·72	0·36	0·52
B14	0·82	0·74	0·70	0·76	0·71	0·75	0·72	0·69	0·77	0·76	0·75	0·71	0·71	0·71	0·69	0·69	0·75	0·70	0·72	0·36	0·52
B15	0·81	0·75	0·70	0·77	0·72	0·75	0·72	0·69	0·76	0·76	0·75	0·69	0·68	0·69	0·67	0·67	0·72	0·70	0·72	0·41	0·49
B16	0·87	0·78	0·72	0·80	0·74	0·80	0·78	0·72	0·75	0·75	0·80	0·73	0·70	0·70	0·67	0·67	0·74	0·70	0·72	0·44	0·55
B17	0·78	0·72	0·67	0·74	0·68	0·74	0·72	0·66	0·74	0·73	0·73	0·67	0·66	0·66	0·64	0·64	0·70	0·65	0·69	0·36	0·49
S1	0·81	0·76	0·73	0·76	0·73	0·76	0·79	0·74	0·87	0·86	0·79	0·74	0·80	0·78	0·79	0·79	0·79	0·75	0·80	0·65	0·64
S2	0·74	0·74	0·68	0·75	0·66	0·75	0·78	0·70	0·83	0·77	0·76	0·64	0·68	0·68	0·71	0·69	0·70	0·68	0·73	0·69	0·67
S4	0·80	0·73	0·75	0·74	0·72	0·79	0·80	0·74	0·82	0·78	0·77	0·78	0·81	0·79	0·80	0·81	0·80	0·76	0·79	0·70	0·68
S5	0·80	0·71	0·66	0·72	0·65	0·76	0·77	0·70	0·84	0·81	0·76	0·69	0·70	0·68	0·69	0·68	0·70	0·64	0·69	0·67	0·67
S6	0·83	0·71	0·70	0·72	0·72	0·76	0·76	0·70	0·83	0·81	0·76	0·71	0·74	0·73	0·72	0·74	0·73	0·73	0·74	0·69	0·70
O1	0·81	0·72	0·67	0·71	0·67	0·75	0·75	0·72	0·80	0·75	0·73	0·76	0·69	0·68	0·67	0·67	0·71	0·59	0·67	0·62	0·59
O2	0·84	0·78	0·78	0·77	0·74	0·80	0·78	0·75	0·78	0·72	0·79	0·82	0·79	0·77	0·75	0·74	0·78	0·68	0·76	0·67	0·68
O3	0·81	0·75	0·72	0·74	0·71	0·80	0·78	0·76	0·85	0·76	0·77	0·77	0·72	0·70	0·71	0·72	0·72	0·66	0·73	0·65	0·62
O4	0·88	0·80	0·76	0·79	0·79	0·84	0·84	0·82	0·84	0·79	0·82	0·83	0·77	0·75	0·74	0·75	0·77	0·74	0·78	0·74	0·70
O5	0·83	0·73	0·69	0·72	0·73	0·83	0·81	0·78	0·85	0·76	0·77	0·77	0·71	0·69	0·70	0·70	0·70	0·66	0·72	0·68	0·64
O6	0·84	0·73	0·69	0·73	0·72	0·81	0·79	0·77	0·84	0·78	0·75	0·79	0·74	0·72	0·73	0·73	0·74	0·68	0·75	0·68	0·66
O8	0·89	0·87	0·80	0·87	0·88	0·88	0·89	0·88	0·91	0·91	0·86	0·86	0·80	0·80	0·81	0·82	0·81	0·79	0·80	0·82	0·76
O9	0·89	0·81	0·81	0·82	0·85	0·84	0·82	0·83	0·84	0·83	0·80	0·79	0·78	0·79	0·79	0·81	0·76	0·80	0·78	0·77	0·75
PH	0·85	0·71	0·60	0·72	0·72	0·73	0·73	0·70	0·81	0·77	0·74	0·66	0·66	0·65	0·63	0·64	0·68	0·64	0·65	0·72	0·66
MO	0·93	0·89	0·84	0·90	0·87	0·88	0·85	0·85	0·90	0·87	0·88	0·87	0·87	0·86	0·86	0·85	0·86	0·84	0·89	0·84	0·84

B5	B8	B13	B14	B15	B16	B17	S1	S2	S4	S5	S6	O1	O2	O3	O4	O5	O6	O8	O9	PH	MO
0·44																					
0·53	0·55																				
0·53	0·55	0·03																			
0·53	0·52	0·13	0·13																		
0·58	0·58	0·27	0·27	0·20																	
0·50	0·49	0·17	0·20	0·15	0·18																
0·69	0·63	0·65	0·65	0·65	0·71	0·63															
0·69	0·70	0·66	0·68	0·64	0·69	0·62	0·53														
0·78	0·71	0·73	0·75	0·73	0·78	0·72	0·53	0·50													
0·69	0·66	0·68	0·68	0·68	0·71	0·66	0·46	0·52	0·52												
0·74	0·70	0·69	0·69	0·71	0·76	0·71	0·48	0·55	0·42	0·43											
0·70	0·71	0·62	0·62	0·62	0·64	0·58	0·54	0·57	0·59	0·55	0·61										
0·73	0·77	0·68	0·68	0·68	0·66	0·63	0·60	0·66	0·56	0·66	0·63	0·28									
0·73	0·69	0·67	0·67	0·67	0·68	0·63	0·55	0·63	0·56	0·60	0·56	0·20	0·27								
0·73	0·74	0·73	0·73	0·73	0·72	0·71	0·60	0·74	0·69	0·66	0·66	0·45	0·49	0·40							
0·75	0·70	0·68	0·70	0·68	0·70	0·64	0·57	0·67	0·56	0·62	0·58	0·29	0·40	0·19	0·37						
0·73	0·71	0·69	0·70	0·69	0·71	0·65	0·56	0·65	0·57	0·58	0·59	0·31	0·41	0·24	0·38	0·14					
0·82	0·78	0·80	0·80	0·80	0·83	0·80	0·67	0·78	0·71	0·76	0·67	0·60	0·70	0·53	0·55	0·50	0·48				
0·78	0·75	0·74	0·74	0·74	0·78	0·74	0·68	0·75	0·70	0·75	0·63	0·61	0·66	0·57	0·53	0·54	0·58	0·50			
0·71	0·74	0·73	0·73	0·75	0·78	0·73	0·80	0·79	0·79	0·74	0·78	0·78	0·83	0·80	0·82	0·79	0·81	0·84	0·85		
0·83	0·85	0·84	0·84	0·86	0·86	0·86	0·90	0·88	0·87	0·83	0·88	0·88	0·89	0·91	0·93	0·92	0·90	0·94	0·97	0·78	

closely related to those of the other two groups, namely, '*Sanchi*' and '*Others*' than to the '*Kapoori*' group. Furthermore, the groups '*Kapoori*', '*Bangla*', '*Sanchi*' and '*Others*' were separated as clusters thereby validating the earlier distinction of the betelvines into five groups (Rawat *et al* 1989b) on the basis of the leaf essential oils.

On the basis of diversity analysis the '*Kapoori*' group is the most diverse ($H = 0{\cdot}247$, table 4), while the landraces in the group '*Others*' were the least diverse ($H = 0{\cdot}129$, table 4) of the four groups studied. The table further indicates that the probabilities of the polymorphic bands in one landrace in a group to be present in another randomly selected landrace in that group are very low ($1{\cdot}6 \times 10^{-4}$ to $2{\cdot}0 \times 10^{-2}$, table 4). The average similarity values for the RAPD profiles differed from 0·48 to 0·61 within the betelvine landrace groups, while between the groups the plants had average similarity values in the range 0·26 to 0·29 (table 5). Similarly, the average similarity of the landraces in one group to those of the outgroup plants was only 0·25 for *P. hamiltonii* and 0·12 for mulberry (table 5). Surprisingly, the betelvines, despite being vegetatively propagated showed considerably less similarity than expected or as reported for other vegetatively propagated plants (Breto *et al* 2001; Vega *et al* 2001). In fact, Vega *et al* (2001) reported one of the lowest levels of polymorphism (0·8%) detected for a plant species by RAPD analysis, for

Table 4. The proportion of polymorphic bands, average of pair-wise similarities, index of diversity and probability of random match for the RAPD band data.

Group (number of landraces)	Number of bands (cumulative data for all primers)			Mean index of diversity[a] (*H*)	Mean probability[b] (within group) *P*
	Total	Polymorphic	Polymorphic per landrace in the group		
Kapoori (19)	118	113	5·95	0·247	$1{\cdot}3 \times 10^{-2}$
Bangla (9)	95	71	7·89	0·187	$2{\cdot}0 \times 10^{-2}$
Sanchi (5)	80	63	12·60	0·161	$1{\cdot}6 \times 10^{-4}$
Others (8)	70	58	7·25	0·129	$1{\cdot}7 \times 10^{-2}$

[a]Mean index of diversity *H* was calculated according to Shannon using the program POPGENE ver. 1·31.
[b]Mean probability $P = (SI)^n$ for all *n* polymorphic bands in any individual landrace in a group are present in another random landrace in that group where average similarity index is *SI* (based on the equation given in Bruford *et al* (1992).

Table 5. The distribution of pair-wise similarity index within and between landrace groups and the outgroup plants.

Parameter	Within *Kapoori*	Within *Bangla*	Within *Sanchi*	Within *Others*	Between *Kapoori* and rest of the landrace groups	Between *Bangla* and rest of the landrace groups	Between *Sanchi* and rest of the landrace groups	Between *Others* and rest of the landrace groups	Between *Piper hamiltonii* and rest of the landrace groups	Between *Morus* spp· and rest of the landrace groups
Similarity index range[a]	Proportion (%) of genotypes pairs in the similarity range									
0·00–0·10					0·48			0·76		24·39
0·11–0·20					19·082	5·21	10·56	22·35	21·95	75·61
0·21–0·30	11·11				60·87	56·60	47·22	46·21	53·66	
0·31–0·40	31·58			3·57	19·32	37·50	33·89	23·86	24·39	
0·41–0·50	21·053	36·11	60·00	10·71	0·24	0·69	8·33	6·82		
0·51–0·60	12·28	22·22	40·00	28·57						
0·61–0·70	14·035	13·89		21·43						
0·71–0·80	5·26	11·11		10·71						
0·81–0·90	2·92	13·89		17·86						
0·91–1·00	1·75	2·78		7·14						
Number of pairs	171	36	10	28	418	288	180	264	41	41
Average similarity	0·48	0·61	0·51	0·57	0·26	0·29	0·29	0·27	0·25	0·12

[a]Similarity index *SI* was calculated from the distance values given in table 3, by using the relation $SI = \sum(1 - d)$, where *d* is the distance between landrace pairs.

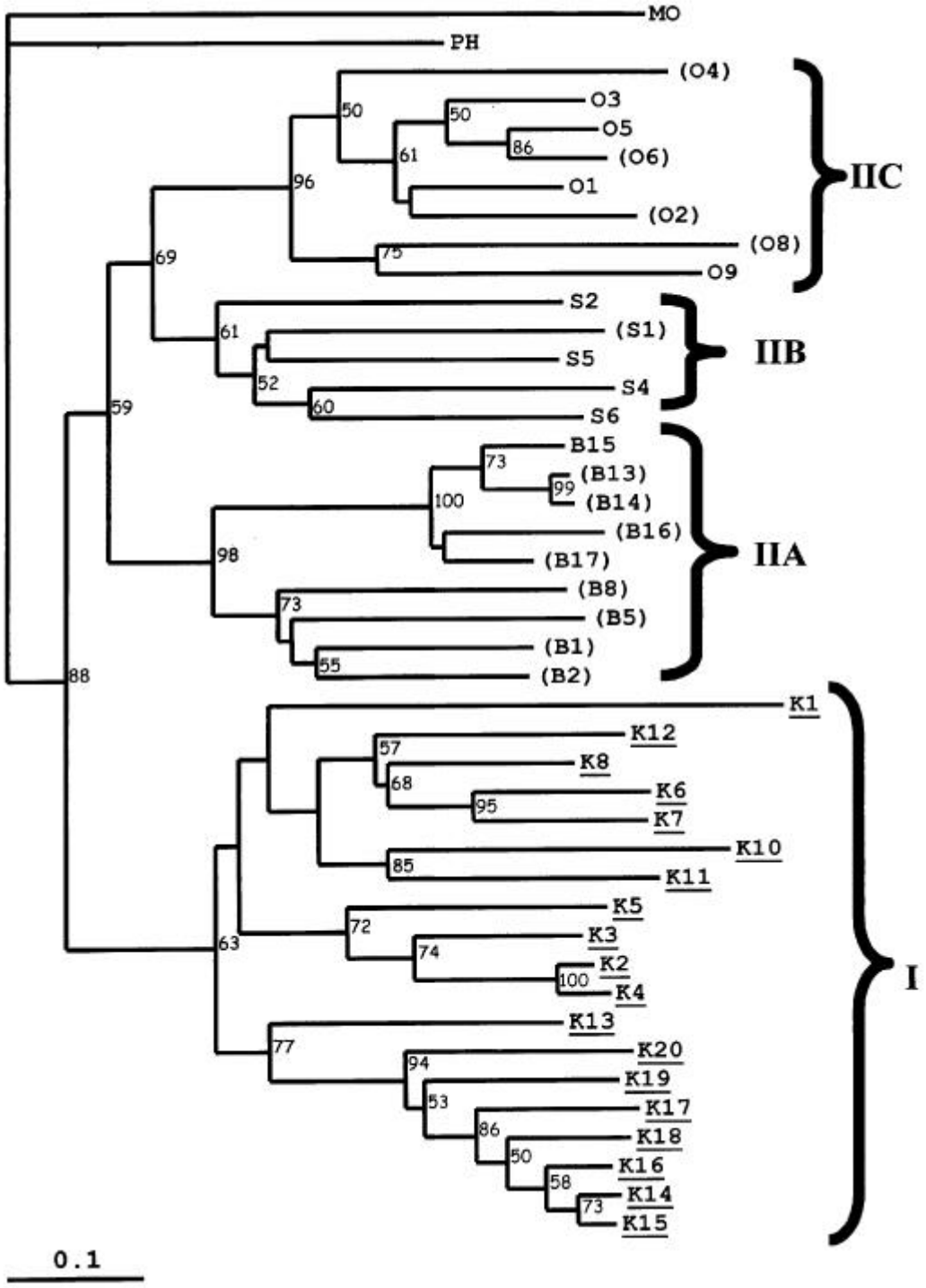

Figure 2. Cluster analysis of cumulative RAPD data for betelvine landraces. The phenogram was generated by the NJ method and a 500 replicate bootstrap analysis was used to assess the robustness of the tree. The tree is rooted at the outgroup MO. The scale represents the distance scale. The numbers at the nodes are bootstrap percentages for the branches to the right of the nodes. For minimizing density of numbers, only the values greater than or equal to 50% bootstrap percentage are shown. The landraces are numbered as given in table 1 and are indicated to the right of the phenogram. The underscored numbers are for known male betelvines while those included in parenthesis are for the known female betelvines. The thick brackets to the right of the landrace numbers indicate the major clusters.

Agave tequilana var. *azul* plants. They attributed such low levels of polymorphism to selective cultivation of a single conserved genotype over many years by exclusive vegetative propagation, a situation resulting from Federal legislations for Tequila production in Mexico. 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. This could be another reason for the greater than expected diversity among the landraces and groups.

RAPD technique has been employed to screen germplasm for several higher plant species including obligate, facultative cross-pollinated plants and clonally propagated plants (Virk *et al* 1995; Al-Zahim *et al* 1997; Degani *et al* 1998; Nair *et al* 1999). For tissue culture or micropropagated plants, RAPD technique has enabled the testing of fidelity of micro-propagation methods (Rani *et al* 1995). For garlic, a seed sterile crop, RAPD analysis along with isozymes allowed the infraspecific differentiation of plants (Maass and Klaas 1995). Betelvine types are similar to garlic plants as both lack propagation through seed. However, the betelvines exhibit less variability in morphological characters compared to garlic. The RAPD profiles, however, revealed relative variability both within as well as between the groups of betelvine landraces. Clearly there is scope for large-scale application of RAPD for analysis of obligate vegetatively propagated plants.

One interesting result from the present study suggests the primary splitting of the groups in the NJ tree was based on gender since all the landraces in the group '*Kapoori*' clustered together and are known to be male vines. On the other hand, all known female vines amongst the groups '*Bangla*', '*Sanchi*' and '*Others*' were clustered separately along with vines of unknown gender. It is tempting to speculate that gender-based distinction exists among the betelvines. If this were so, it would also suggest the male vines were more diverse than the others since '*Kapoori*' group of vines had the highest diversity index amongst all the betelvines. This is also evident from the distribution of pairwise similarity within and between landrace groups and outgroups. The '*Kapoori*' group exhibited a wide range with some 11% pairs exhibiting similarities in the low range 0·21–0·30 and some 2% pairs showing similarities in the high range 0·91–1·00 (table 5). These data suggest that this group of landraces, namely, '*Kapoori*' or the male betelvines are more heterogeneous relative to the female vines. The gender distinction and determination has not been well studied in betelvines. Thus it is not known whether the betelvines have the similar chromosomal basis of sex determination as the other dioecious plants have. While plants of *Sliene latifolia* have a X-Y chromosomal system for sex determination, in the plants of *Rumex acetosa* sex determination is due to the X-to-autosome ratio (Lebel-Hardenack and Grant 1997). Since in betelvines, it is the leaves of both sexes and not just the fruits that are commercially valued, sex determination does not seem to have much importance. The absence of seed propagation and the fact that the cultivators generally never have mixed plantations of landrace clones also indicates that the genetic diversity in the male and female betelvines has probably been fixed in populations that are probably as ancient as the start of the betelvine cultivation itself. Our results are the first systematic evidence that gross molecular profiling also reflects gender distinction in plants such as the betelvines.

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