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# SPAR Profiles for the Assessment of Genetic Diversity Between Male and Female Landraces of the Dioecious Betelvine Plant (*Piper betle* L.)

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## 1. Introduction

Betelvine (*Piper betle* L., family Piperaceae) is an important, traditional and an ancient crop of India and is a shade loving, perennial evergreen climber of tropical origin. It is generally known as "Paan" in Hindi in the Indian subcontinent and by different names in the Asiatic region and is a plant of considerable antiquity. It is distributed in several countries in including India as well as other countries of Indochina region - Indonesia, Malaysia, Vietnam, Laos, Kampuchea, Thailand, Myanmar, Singapore and the Far-East (Figure 1), where its cultivation or ethnomedicinal properties are well known. In India, 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 and almost the entire production of betel leaves is consumed fresh as a masticatory.

Betelvines are dioecious and therefore, under controlled hybridization, attempts have been made to cross different landraces and in some of these experiments, viable seed set has also been reported. However, as a crop, propagation is obligately only through vegetative means. Its cultivation in northern India under sub-tropical conditions (Figure 2) 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 by families or communities. 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.

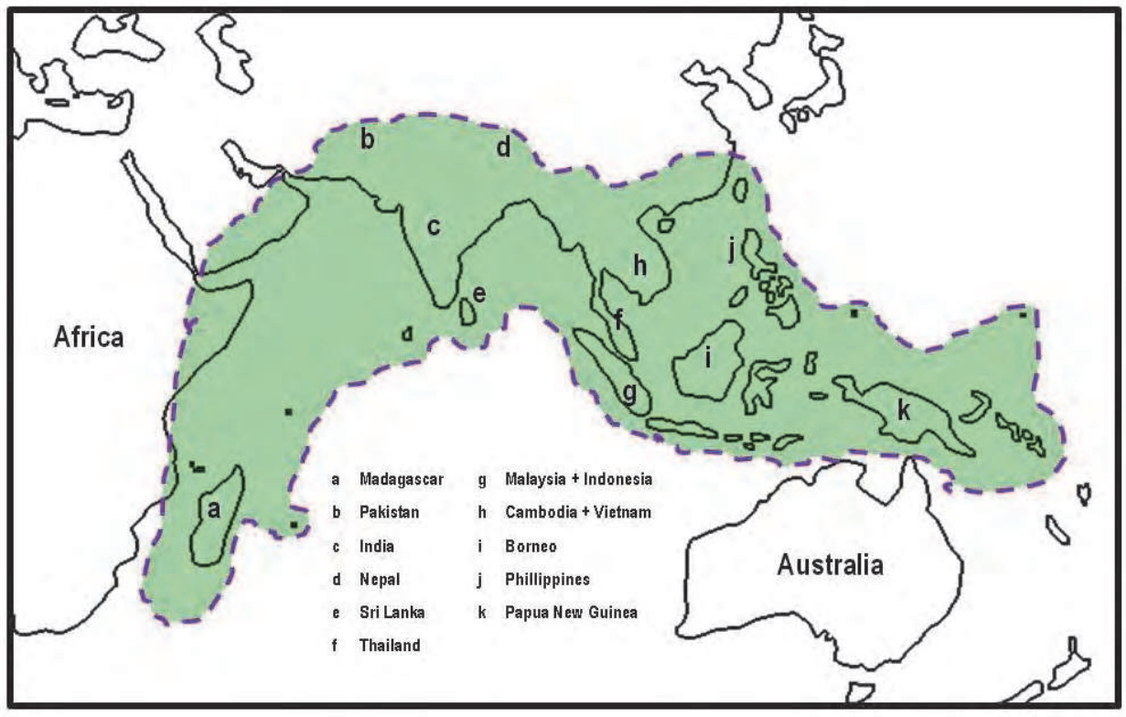


Fig. 1. Schematic map showing the regions of the world where betelvine is cultivated or consumed.

Currently more than 200 landraces are cultivated in India and are often named after the localities, villages or towns where they are grown. As a result of this, though the geographical distribution of the betelvines under cultivation is vast, it is possible that the genetic variation may not be so well distributed. However, no systematic study of betelvine genetic diversity had been carried out to our knowledge. Thus the assessment of genetic variation amongst the different landraces became an important objective for which, PCR-based parallel approaches were used. We have shown, in an earlier study with different landraces using RAPD method, a clear distinction between male and female betelvines or between types of landrace groups (Ranade et al. 2002; Verma et al. 2004). A further substantiation of those results as well as an in-depth analysis was carried out using four different primer sets including RAPD primers on a specific subset of landraces clearly distinguished from each other by flowering behavior as either male or female betelvines (Figure 3). On such a set of known male and female betelvines, hereafter referred to as the "gender set" we have assessed genetic diversity using four primer types revealing polymorphic profiles from discrete but widely distributed genomic regions. These primers include (i) arbitrary sequence decamer primers amplifying from several anonymous regions; (ii) SSR primers amplifying several inter-SSR regions; (iii) minisatellite core sequence primers amplifying from miniatellite rich regions and (iv) primers derived from X and Y chromosomes of a known dioecious plant with heteromorphic sex chromosomes, *Silene latifolia* that were expected to specifically amplify from genomic regions homologous to the X or Y chromosomes in case of the male and female betelvines. Such a PCR-based study was

planned because there were no prior reports about chromosomal basis of gender distinction in case of betelvines. A better understanding of how gender discrimination takes place in those plants where chromosomal basis of inheritance of sex is undocumented or negligible or non-existent too is important as the study also assumes significance in understanding the resource allocation and metabolism costs attributed to gender discrimination in those plants where the economic importance of plant is defined by a metabolite or a phytochemical that is recovered from a somatic tissue. Such a study in case of a dioecious plant is expected to throw light on whether or not there is a difference in diversity of the two genders and if so whether such differences are significant in terms of the economic values of the plant.

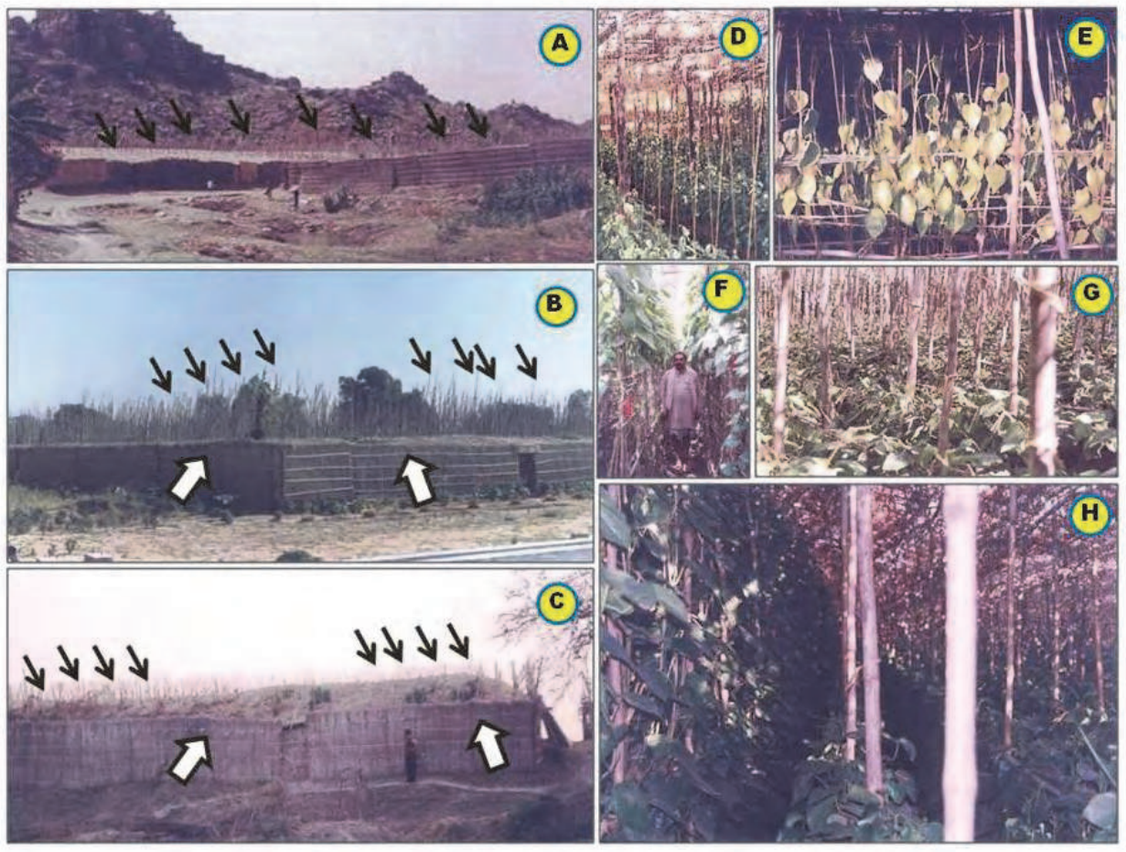


Fig. 2. The stages during the construction of the unique man-made structures created to regulate the micro-climate for cultivation of the betelvines in the sub-Tropical parts of India. These structures are called as "bareja" and are almost exclusively made out of natural raw materials such as palm thatch and bamboo. The panels labeled A through H depict the various stages. The small black arrows in panels A through C point to the bamboo sticks that are used to support the betelvines. The white larger white arrows in panels B and C point to the thatch walls of the bareja sides.

Fig. 3. Photos of the female (top panel) and male (bottom panel) betelvines. The solid white arrows (top panel) and the solid black arrow (bottom panel) point to the female and male inflorescences respectively.

## 2. Materials and methods

*Plant material*: Betelvine landraces were collected from three of the centers of the All India Co-coordinated Research Project (AICRP) on Betelvine at Chinthalapudi (Bapatla) in Andhra Pradesh, Sirugamani (Tiruchirappalli) in Tamil Nadu and Digraj (Sangli) in Maharastra. The landraces of betelvine for which leaf tissue were collected are listed in Table 1. Young leaf tissue was harvested from the vines, washed free of dirt and dust and then quickly mopped dry on blotting sheets. The leaves were de-ribbed and powdered rapidly in liquid nitrogen, and then either the DNA isolation procedures were immediately followed or the powdered tissue was stored at -70°C till further use. The leaves of, the *Piper* species outgroup (*Piper hamiltonii*) as well as non-*Piper* outgroup, Mulberry variety 'MI-0129' were collected from NBRI, Lucknow. As in the case of betelvines the young leaf tissue was harvested, washed free of dirt and dust and then quickly mopped dry on blotting sheets. Then the leaves were de-ribbed and powdered rapidly in liquid nitrogen, and then either the DNA isolation procedures were immediately followed or the powdered tissue was stored at -70°C till further use.

*Isolation of DNA*: Total plant DNA was isolated from the frozen tissue powder according to the method of de Kochko and Hamon (1990) with some modifications as described earlier (Bhattacharya and Ranade, 2001). At least three to five independent DNA preparations were made from leaf tissues collected from each plant. The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel as well as by fluorometry (DyNA Quant 200, Pharmacia) using Hoechst 33258 as the fluorochrome.

*Minisatellite, SSR, SLXY and RAPD Primers*: Four minisatellite core sequence primers and five SSR primers and three SLXY primers were custom synthesized from Bangalore Genei, Bangalore, India. The fifteen RAPD primers were procured from Qiagen Operon Technology Inc., Alameda, CA, USA. The sequences of all these primers as well as the annealing temperatures used in PCR with these primers are given in Table 2.

| Sample # | Landrace | Collection | |
|---|---|---|---|
| | | Source | Time |
| 207F | Bangla Nagaram | Chinthalapudi (A.P.)[a] | Jan. 2000 |
| 211M | Kapoori Chillumurru | -- Ditto -- | Jan. 2000 |
| 213M | Kapoori Tuni | -- Ditto -- | Jan. 2000 |
| 214M | Kapoori Peddachapelli | -- Ditto -- | Jan. 2000 |
| 218M | Kapoori Doddipatla | -- Ditto -- | Jan. 2000 |
| 219M | Kapoori Chinnachapelli | -- Ditto -- | Jan. 2000 |
| 223F | Bangla (U.P.) | -- Ditto -- | Jan. 2000 |
| 226M | Kapoori Vuyyur (A.P.) | -- Ditto -- | Jan. 2000 |
| 234F | Godi Bangla (Orrisa) | -- Ditto -- | Jan. 2000 |
| 235F | Bangla (M.P.) | -- Ditto -- | Jan. 2000 |
| 239F | Bangla Ramtek | -- Ditto -- | Jan. 2000 |
| 244F | Kali Bangla (Assam) | -- Ditto -- | Jan. 2000 |
| 301M | Kapoori Mhaisal | Digraj (Maharashtra)[b] | Jul. 2000 |
| 304M | Kapoori Bolvad | -- Ditto -- | Jul. 2000 |
| 305M | Kapoori Viddi | -- Ditto -- | Jul. 2000 |
| 306M | Kapoori Karve | -- Ditto -- | Jul. 2000 |

| | | | |
|---|---|---|---|
| 307M | Kapoori Indapur | -- Ditto -- | Jul. 2000 |
| 310M | Kapoori Arvi | -- Ditto -- | Jul. 2000 |
| 319F | Calcutta Bangla | -- Ditto -- | Jul. 2000 |
| 323M | Kapoori Maharashtra | -- Ditto -- | Jul. 2000 |
| 326M | Kapoori Solapur | -- Ditto -- | Jul. 2000 |
| 409F | Bangla Jal | Sirigamani (T.N.)[c] | Aug. 2000 |
| 410F | Bangla Ramtek | -- Ditto -- | Aug. 2000 |
| 423F | Bangla Jabalpur | -- Ditto -- | Aug. 2000 |
| 426F | Bangla Desi | -- Ditto -- | Aug. 2000 |
| 427F | Nav Bangla | -- Ditto -- | Aug. 2000 |
| 601F | Kakair | Sirugamani (T.N.)[c] | Aug. 2003 |
| 602F | Banchi Kodi | -- Ditto -- | Aug. 2003 |
| 603F | Bangla Jal | -- Ditto -- | Aug. 2003 |
| 604F | Kuljedu | -- Ditto -- | Aug. 2003 |
| 605M | Kapoori Vasani | -- Ditto -- | Aug. 2003 |
| 606M | Kapoori Bihar | -- Ditto -- | Aug. 2003 |
| 607F | Deshawari | -- Ditto -- | Aug. 2003 |
| 608F | SGM-1 | -- Ditto -- | Aug. 2003 |
| 609F | Sreenivasa Nallur | -- Ditto -- | Aug. 2003 |
| 610F | Bangla Ramtek | -- Ditto -- | Aug. 2003 |
| 612F | Bangla | -- Ditto -- | Aug. 2003 |
| 613M | Kapoori Chittikavata | -- Ditto -- | Aug. 2003 |
| 614M | Tellaku Ponnur | -- Ditto -- | Aug. 2003 |
| 615F | Shirpurkata | -- Ditto -- | Aug. 2003 |
| 616M | Tellaku | -- Ditto -- | Aug. 2003 |
| 617M | Tellaku Uttukuru | -- Ditto -- | Aug. 2003 |
| 618M | Karpuri (T.N.) | -- Ditto -- | Aug. 2003 |
| 619M | Sangli Kapoori | -- Ditto -- | Aug. 2003 |
| 620F | Kalipatti | -- Ditto -- | Aug. 2003 |
| 621F | Gach Pan | -- Ditto -- | Aug. 2003 |
| PH | Piper hamiltonii | NBRI (Lucknow)[d] | Aug. 2000 |
| MO | Mulberry | NBRI (Lucknow)[e] | Sep. 1999 |

[a] AICRP on Betelvine Center at Acharya NG Ranga Agricultural University, Bapatla, A. P. State.
[b] AICRP on Betelvine Center at MPKV ARS at Kasbe Digraj, Sangli, Maharashtra State.
[c] AICRP on Betelvine Center at TNAU Sugarcane Research Station at Sirugamani, Tiruchirapalli, T. N. State.
[d] Betelvine Conservatory, at Betelvine Biotechnology Laboratory, NBRI, Lucknow, U. P. State.
[e] Mulberry accession number MI-0129 from an earlier NBRI collection of tissue for R&D work on mulberry.
Samples in shaded cells were not included in data analysis since their PCR profiles were not always consistent amongst replicate experiments.

Table 1. Betelvine landraces (gender set), *Piper hamiltonii* and mulberry included for the studies on PCR-based profiling are given below. In all cases, leaf tissue was harvested, washed and stored frozen as described. The source locations have been detailed as footnotes to the table. The sample number refers to the numbers assigned to the tissue samples while **M** or **F** suffixes to sample numbers indicate the appropriate gender as Male or Female respectively.

| Primer Name | Primer Sequence (5′- 3′) / (Length in numbers of bases) | Annealing Temperature (°C) |
|---|---|---|
| **RAPD Method primers** | | |
| OPG-02 | GGCACTGAGG / (10) | **35** |
| OPG-03 | GAGCCCTCCA / (10) | **35** |
| OPG-08 | TCACGTCCAC / (10) | **35** |
| OPG-10 | AGGGCCGTCT / (10) | **35** |
| OPG-11 | TGCCCGTCGT / (10) | **35** |
| OPG-13 | CTCTCCGCCA / (10) | **35** |
| OPG-16 | AGCGTCCTCC / (10) | **35** |
| OPG-17 | ACGACCGACA / (10) | **35** |
| OPG-18 | GGCTCATGTG / (10) | **35** |
| OPG-19 | GTCAGGGCAA / (10) | **35** |
| OPH-04 | GGAAGTCGCC / (10) | **35** |
| OPH-06 | ACGCATCGCA / (10) | **35** |
| OPH-12 | ACGCGCATGT / (10) | **35** |
| OPH-18 | GAATCGGCCA / (10) | **35** |
| OPH-19 | CTGACCAGCC / (10) | **35** |
| **ISSR method primers** | | |
| $(GACA)_4$ | GACAGACAGACAGACA / (16) | 37, **40**, 45 |
| $(GATA)_4$ | GATAGATAGATAGATA / (16) | **30**, 33, 37 |
| $(GAA)_6G$ | GAAGAAGAAGAAGAAGAAG / (19) | 41, 45, **48** |
| $(CA)_8GC$ | CACACACACACACACAGC / (18) | **45**,48,53 |
| $(ACTG)_4$ | ACTGACTGACTGACTG / (16) | **38**, 41, 45 |
| **DAMD method primers** | | |
| M-13 | GAGGGTGGCGGTTCT / (15) | 41, 45, **48** |
| HVR(-) | GCTCCTCCCTCCT / (13) | **50**, 55 |
| HBV | GGTGTAGAGAGGGGT / (15) | 41, 45, **48** |
| 33.6 | GGAGGTGGGCA / (11) | **50**, 55 |
| **SLXY primers** | | |
| SLXY6 | TGGACTTCCACTGGAATTCGAT / (21) | 45, **50**, 55 |
| SLXY7 | ACTTGCAACGACTTCACTTTGAG / (25) | 45, **50**, 55 |
| SLXY8 | ATCGAATTCCAGTGGAAGTCC / (22) | 45, **50**, 55 |

Table 2. Various primers used for PCRs in present study. Annealing temperatures given in boldface are the optimum for that primer.

*SPAR with arbitrary sequence decamers (RAPD)*: Decamers from kits G and H (Operon Technologies Inc., Alameda, California, USA) were used as primers. DNA was amplified essentially following Williams *et al.* (1990). Initially a pilot experiment was carried out varying primer, template DNA and $Mg^{++}$ ion concentrations. The final amplification reactions contained 1x buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmoles primer, 0.6 U *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) and 50 ng betelvine DNA template in a 10µl reaction volume. The reaction was predenatured at 94°C for 1 min and thereafter cycled 44 times at 94°C for 15 s, 35°C for 20 s and 72°C for 45 s in the Air Thermal Cycler. Additionally a final extension cycle allowed incubation for 240 s at 72°C. The PCR products were separated by electrophoresis (at constant current of 5A) through 1% agarose gels in 0.5X TBE buffer, visualized and imaged using Nighthawk™ gel documentation system (pdi Inc., USA) after staining with ethidium bromide. Only distinct and well-separated bands repeated in replicated experiments were included in the analysis.

*SPAR with SSR primers (ISSR-PCR)*: The PCR conditions were according to Gupta *et al.* (1994). A pilot experiment was carried out to determine the optimum annealing temperature for each primer in the range 3-10°C lower than the denaturation temperature. The denaturation temperature was calculated according to Berger and Kimmel (1987), by adding 2°C for each A or T and 4°C for each G or C in the oligomer. The final reaction was carried out in 10 µl volumes and contained 50 ng of template DNA, 10 pmoles of SSR primer, 200 µM each dNTP, 1.5 mM $Mg^{2+}$ ion concentration in suitable 1X assay buffer supplied along with the enzyme and 0.6 Units of the thermostable *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). The tubes were placed in the Air Thermal Cycler (Idaho Technology, USA) for the PCR. The Air Thermal Cycler was programmed to include pre-denaturation at 94°C for 60 s. This was followed by 30 cycles of denaturation at 94°C for 20 s, annealing at the optimized temperature for 30 s, and extension at 72°C for 45 s. The final cycle allowed an additional 240 s period of extension at 72°C. The reaction products obtained after PCR were analyzed on 1.2 or 1.8% agarose gels. The gel was stained in ethidium bromide and visualized and imaged on Nighthawk™ gel documentation system (pdi Inc., USA). Only distinct and well-separated bands repeated in replicated experiments were included in the analysis.

*SPAR with minisatellite primers (DAMD)*: The reaction was carried out essentially according to Zhou *et al.* (1997) for the primers 33.6, HBV and HVR(-) while for primer M13 the reactions were essentially according to Lorenz *et al.* (1995). The optimum annealing temperature was determined by carrying out DAMD at different annealing temperatures in the range 40°C to 55°C. The PCR parameters were as follows: First cycle of incubation at 94°C for 60 s followed by 30 cycles of incubation at 94°C for 20 s, at annealing temperature (41°C in case of M13 primer and 50°C in case of the other three primers) for 30 s and at 72°C for 45 s. The final cycle allowed an additional incubation at 72°C for 240 s. The reactions contained 100 ng template DNA, 40 pmoles of primer, 1.5 mM $Mg^{2+}$ ions, 200 µM each dNTP, 0.3 U *Taq* DNA polymerase in 1x assay buffer in a final volume of 15 µl in case of the primers 33.6, HBV and HVR(-). In case of the M13 primer, the template, primer and enzyme and the reaction volumes were 60 ng, 10 pmoles, 0.6 Units and 10 µl respectively. All amplification reactions were carried out in Air Thermal Cycler (Model ATC1605, Idaho Technology, Inc.) and the products were separated by electrophoresis (at constant current of

5A) through 1.2% agarose gels in 0.5X TBE buffer, visualized and imaged using Nighthawk™ gel documentation system (pdi Inc., USA) after staining with ethidium bromide. Only distinct and well-separated bands repeated in replicated experiments were included in the analysis.

*SPAR with SLXY primer (SLXY-PCR)*: The primers used for profiling, were synthesized from the prior known sequences specific to the X- and Y-chromosomes of the dioecious perennial plant *Silene latifolia* (Filatov *et al*., 2000). The reaction conditions were optimized for these primers also in the same way as for the RAPD primers. The final reactions included 100 ng of template, 20 pmoles of primer, 1.5 mM $Mg^{2+}$ ions, 200 μM each dNTP, 0.9 U *Taq* DNA polymerase in 1x assay buffer in a final volume of 15 μl. All PCR were carried out in Air Thermal Cycler (Model ATC1605, Idaho Technology, Inc.). The Air Thermal Cycler was programmed to include pre-denaturation at 94°C for 60 s. This was followed by 30 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s. The final cycle allowed an additional 240 s period of extension at 72°C. The PCR products were separated by electrophoresis (at constant current of 5A) through 1.4% agarose gels in 0.5X TBE buffer, visualized and imaged using Nighthawk™ gel documentation system (pdi Inc., USA) after staining with ethidium bromide. Only distinct and well-separated bands repeated in replicated experiments were included in the analysis.

*Data Analysis*: Data (fragment sizes of all amplification products, estimated from the gel by comparison with standard molecular weight marker, λ DNA double digested with *Hin*d III and *Eco*R I) were scored as discrete variables, using "1" to indicate presence and "0" to indicate absence of a band. A pair wise matrix of distances between genotypes was determined for the band data from each method using Jaccard coefficient (Jaccard, 1908) in the **FreeTree** program (ver. 0.9.1.5; Pavlicek *et al.* 1999). These pairwise distance data were used to compare the average distances estimated within and between the male and female betelvine landraces for each method. Additionally, a cumulative distance matrix, for the band data of all four methods considered together, was also computed separately to generate a single NJ tree after allowing a 1000 replicate bootstrap test using the same program. The tree was viewed, annotated and printed using **TreeView** (ver. 1.6.5; Page 2001). The robustness of the SPAR methods was tested in each case with a suitable non-*Piper* outgroup DNA included in the analysis, and in all cases outgroup was resolved as distinct from the betelvine DNAs (data not shown).

*Comparison of the different SPAR methods*: To determine the utility of each of the marker systems used, diversity index (**DI**), effective multiplex ratio (**E**) and marker index (**MI**) were calculated according to Powell *et al.* (1996).

**DI** for genetic markers was calculated from the sum of the squares of the allele frequency:

$$\mathbf{DI_n = 1-\sum p_i^2}$$

where '$\mathbf{p_i}$' is the allele/band frequency of the '$i^{th}$' allele and '**n**' is the total number of loci. **DI** for polymorphic markers ($\mathbf{DI_p}$) was calculated from:

$$\mathbf{DI_p = \sum DI_n / np}$$

where '**np**' is the number of polymorphic loci analyzed. Effective Multiplex Ratio (**E**) is the product of the fraction of polymorphic loci and the number of polymorphic loci for an individual assay and was calculated from:

$$E = np(np / n)$$

**MI** is defined as the product of the average diversity index for polymorphic bands in any assay and the effective multiplex ratio (**E**) for that assay. It was calculated as:

$$MI = DI_p \times E$$

The Mantel matrix correspondence test (Mantel, 1967) was used to compare distance matrices for each SPAR method with the help of the program **Mantel 2** (Liedloff, 1999).

## 3. Results

**Optimization of the different PCRs:** Screening of the genotypes and various optimization experiments was carried out so as to identify the reaction parameters and conditions including concentrations of template, $Mg^{2+}$ ions, primers and dNTPs used as well as the annealing temperatures in the PCRs that enabled the most repeatable results. Likewise, consistency of the profiles was judged by using duplicate or triplicate template samples for the same primers such that all the prominent bands were consistently produced with each of the template replicates. In all 22 male and 24 female betelvine variety DNAs were used in the present study along with *Piper hamiltonii* and mulberry DNAs as outgroups. The last named mulberry DNA outgroup was used just to determine the robustness of the PCR reactions and data from this was not scored for the analysis. On the basis of the preliminary optimizations (data not shown) template DNAs of male varieties, 211, 301 and 310 (Table 1) were excluded from data analysis since profiles with these were not consistent with all primers and all replicates tested.

**RAPD analysis of the gender set of betelvine landraces:** A total of 15 primers (Table 2) were used for RAPD analysis and all the primers resulted in discrete amplification products. Three landraces 211, 301 and 310 failed to give discrete profiles with some of the primers so data for these landraces was not scored from the profiles for final calculations and analysis**.** The RAPD data for all 15 primers were considered cumulatively and included a total of 219 bands (17.13 bands per primer) for the analysis of the relationship amongst the betelvine landraces. Of these some 98% bands were polymorphic and only four bands were present in all betelvine and *Piper hamiltonii* DNAs. Jaccard coefficients were computed from the band data and the highest (0.86) in female group was between two Bangla Ramtek accessions 239 (from Chinthalapudi) and 610 (from Sirugamani) while the least (0.22) was between 604 (Kuljedu) and 620 (Kallipati) and 427 (Nav bangla). The corresponding values in case of the male landraces were 0.96 between 213 (Kapoori Tuni) and 214 (Kapoori Peddachapelli) and 0.23 between 307 (Kapoori Indapur) and 218 (Kapoori Doddipatla). Between male and female, the highest (0.40) was between 326 (Kapoori Solapur) and 612 (Bangla) whereas least (0.18) was between both 305 (Kapoori Viddi) and 326 (Kapoori Solapur) and 427 (Nav Bangla). The distance data computed from the Jaccard coefficients were analyzed further by NJ method, using the FreeTree program, as described earlier in the materials and methods, to describe the relationship between betelvine landraces. The NJ tree after 1000 replicate bootstrapping revealed two major clusters respectively for the female and male landraces and the out group 501 (*Piper Hamiltonii*) was separated from these two clusters (NJ tree not shown).

**ISSR-PCR profiles of gender set of betelvine landraces:** Here five primers resulted in discrete patterns of 73 closely spaced bands (16.20 bands per primer). Of these some 93%

bands were polymorphic across the betelvine landraces and only 5 bands were present in all landraces as well as *P. hamiltonii*. The Jaccard coefficients were computed and the highest (0.94) in female group was between 223 (Bangla U.P.) and 235 (Bangla M.P.) while the least (0.31) was between 409 (Bangla Jal) and 612 (Bangla). In male group the corresponding values were (0.97) between 219 (Kapoori Chinnachapelli) and 226 (Kapoori Vuyyur) and (0.47) between 307 (Kapoori Indapur) and 618 (Karpuri) and between 616 (Tellaku) and 304 (Kapoori Bolvad). Among female and male groups the highest was 0.50 between 618 (Karpuri), 410 (Bangla Ramtek), 223 (Bangla U.P.), 609 (Sreenivasa Nallur) and 619 (Sangli Kapoori), 608 (SGM-1) and 304 (Kapoori Bolvad) while the least (0.28) was between 616 (Tellaku) and 427 (Kapoori Bihar). The NJ tree after 1000 replicate bootstrapping revealed the betelvine landraces grouped together in two major clusters of the male and female landraces and here also as in the case of RAPD data 501 (*Piper Hamiltonii*) was separated out distinctly (NJ tree not shown).

**DAMD profiles of the gender set of betelvine landraces:** The DAMD profiles consisted of distinctly polymorphic banding patterns and the four DAMD primers resulted in a total of 68 polymorphic bands (19.00 bands per primer). Jaccard coefficients in the female group here revealed highest (0.83) was between 410 (Bangla Ramtek) and 610 (Bangla Ramtek). In male group the highest (0.95) was between 305 (Kapoori Viddi) and 326 (Kapoori Solapur). Between female and male, the highest value is 0.44 between 606 (Kapoori Bihar) and 604 (Kuljedu) and 617 (Tellaku Uttukuru). The distance data analyzed by NJ method using the FreeTree program, as described earlier revealed the NJ tree after 1000 replicate bootstrapping was divided into two major clusters here also.

**SLXY primer PCR profiles of the gender set of betelvine landraces:** PCR with SLXY primers used singly in the amplification reactions resulted in distinctly polymorphic banding patterns. A total of 60 bands (all polymorphic) were scored with the three SLXY primers with the average number of 17.25 bands per primer. The highest (0.91) between 207 (Bangla Nagaram) and 235 (Bangla M.P.) and least (0.22) between 234 (Godi Bangla), 426 (Bangla Desi) and 427 (Nav Bangla) coefficients were computed in the female group. In male group the corresponding values are 1.0 between 218 (Kapoori Doddipatla) and 213 (Kapoori Tuni); 0.26 between 619 (Sangli Kapoori) and 614 (Tellaku Ponnur). Between female and male, the highest value is 0.45 between 617 (Tellaku Uttukuru) and 606 (Kapoori Bihar) and least 0.95 was between 427 (Nav Bangla) and 619 (Sangli Kapoori). The NJ tree after 1000 replicate bootstrapping reveals *Piper Hamiltonii* (501) were clearly separated from the rest of the betelvine landraces that were in turn divided into two major clusters of male and female landraces respectively.

**Comparison of the different PCR methods used:** Four different PCR-based methods were used to assess diversity and to distinguish between the genders. In order to determine the utility of each of these PCR-based methods used, a comparative statistical assessment was done according to Powell *et al*., (1996). Diversity Index (**DI**) or Heterozygosity Index, Marker Index (**MI**) and Effective Multiplex Ratio (**E**) were calculated in case of each of the methods used as described in the materials and methods. By RAPD method **DI** value was 0.29, **E** value was 17.13 and **MI** value was 4.91. For the same set of landraces with ISSR-PCR, **DI** was 0.33, **E** was 16.2 and **MI** was 5.3. When DAMD primers were used for the analysis, **DI** value was 0.23, **E** was 19.0 and **MI** was 4.4. The corresponding values for the SLXY primer PCR were 0.25, 17.25 and 4.37 respectively (Table 3).

| Comparison | Average similarity index (SI) | Method | Average # of bands per primer | Diversity Index (DI) | Effective Multiplex Ratio (E) | Marker Index (MI) |
|---|---|---|---|---|---|---|
| Within Male | 0.544 | RAPD | 14.60 | 0.29 | 17.13 | 4.914 |
| Within Female | 0.492 | | | | | |
| Between Male and Female | 0.282 | | | | | |
| Within Male | 0.671 | **ISSR** | 16.20 | 0.33 | 16.20 | 5.289 |
| Within Female | 0.606 | | | | | |
| Between Male and Female | 0.400 | | | | | |
| Within Male | 0.389 | **DAMD** | 19.00 | 0.23 | 19.00 | 4.423 |
| Within Female | 0.245 | | | | | |
| Between Male and Female | 0.183 | | | | | |
| Within Male | 0.545 | **SLXY** | 17.25 | 0.25 | 17.25 | 4.366 |
| Within Female | 0.489 | | | | | |
| Between Male and Female | 0.266 | | | | | |

Table 3. The comparison of different PCR methods for the assessment of genetic diversity in the gender set of betelvine landraces.

| Methods compared Matrix 1 / Matrix 2 | Standard Normal Variate (g) | Mantel Coefficient for 100 random iterations (Z) | Correlation coefficient (r) |
|---|---|---|---|
| RAPD / ISSR | 21.179** | 673.781 | 0.8432+++ |
| RAPD / DAMD | 9.968** | 970.869 | 0.5009+ |
| RAPD / SLXY | 18.021** | 828.749 | 0.7853+++ |
| ISSR / DAMD | 8.895** | 790.384 | 0.4619+ |
| ISSR / SLXY | 17.562** | 680.878 | 0.7854+++ |
| DAMD / SLXY | 6.399** | 978.962 | 0.3850+ |

** Highly significant values (Critical value $\mathbf{p}_{0.005} = 2.575$)

+++: High correlation

+: Moderate or low correlation

Table 4. Correlations among the distance matrices for the RAPD, ISSR, DAMD and SLXY primer PCR band data in case of the gender based set of the betelvine landraces. A significant, (p = 0.005), standard normal variate **(g)** was obtained among the matrices generated by all the four methods in all possible pairs of matrix comparison.

Another parameter for comparing the different profiling methods is to correlate the respective distance matrices (computed from the Jaccard coefficients) by a Mantel test. Such a correlation analysis was carried out (Table 4) and resulted in highly significant correlation (standard variate $\mathbf{g > p}_{0.005}$ value) in all cases. However, for the four methods the highest correlation was between RAPD and ISSR distance matrices (**r** = 0.8432, Table 4). This clearly indicates that relative distances of the landraces to each other were almost similarly estimated by both RAPD and ISSR methods. On the other hand, in case of the DAMD method, it was less strongly correlated (**r** = 0.5009, 0.4619 and 0.3850) to the other three methods (RAPD, ISSR and SLXY respectively). The correlation between RAPD and ISSR with SLXY primer data was almost equally strong in both cases (**r** = 0.7853, 0.7854 respectively).

## 4. Discussion

Dioecy is a widespread condition in flowering plants. Despite their recent evolutionary origin, 6% of the 240,000-angiosperm species are dioecious and 7% of 13,000 genera of angiosperms include dioecious species, suggesting that it has arisen many times during flowering plant evolution (Renner and Ricklefs, 1995). Dioecy is correlated with perennial climbing growth, wind or water pollination and has a preponderence in tropical flora. Natural selection, development of complex physiological and morphological traits, male fitness, ecological context, sex ratio, phylogenetic perspective and nuclear-cytoplasmic gynodioecy are some of the factors affecting distribution of dioecy. The plant taxa can offer insights into level of dimorphism that exists prior to the evolution of complete dioecy and the tradeoffs or constraints faced by hermaphrodites. The betelvine is one such interesting dioecious plant with an almost obligate vegetative propagation, lianaceous habit and perennial growth that provides a good system for studying molecular aspects of dioecy in general and functional dioecy in particular.

The study with PCR profiles was the first step towards resolving gender differences, if any, in betelvines. In an earlier study with 53 landraces that included only a few landraces with known genders, the RAPD method had provided a distinction between male and female betelvines (Verma *et al.*, 2004). In the present study with a larger sampling specifically amongst the betelvines with known genders, the bootstrap NJ tree for the RAPD data clearly differentiated the male and female landraces into two separate broad clusters, thereby supporting our original grouping of the landraces in terms of male and female vines based on known or actual flowering data. Banerjee *et al.* (1999) have reported a similar study of RAPD profile variation in another dioecious species, *Piper longum* L. and have further shown that at least two RAPD fragments were consistently associated with male plants. The clear separation of the betelvines on the basis of gender provides important leads for the identification and development of gender specific primers and probes. This work, however, has an important caveat that the economically most important product of the betelvines are the leaves and leaves of both male and female vines have a market value. Therefore the gender specific detection and or diagnosis will have only an academic value in case of betelvines unlike that in plants such as Papaya (Deputy *et al.*, 2002; Ma *et al.*, 2004). ISSR-PCR has been successfully utilized to distinguish gender or gender-specific markers in dioecious plants like hops and datepalm (Jakse et al. 2008; Younis et al. 2008). In the present

study one ISSR primer each with dinucleotide and trinucleotide motifs and three primers with tetranucleotide motifs were tried in PCR and revealed distinct profiles that were however broadly similar across all the landraces. This result indicated that the ISSR regions were apparently conserved at least in length if not in sequence. The polymorphic bands helped to resolve the NJ tree into two major clusters with two groups of male and female betelvine clearly separating out. DAMD method has not been specifically used for any gender distinction studies in plants. For betelvine landraces, the NJ tree separated the male and female genders of the landraces and at the same time the DAMD method also revealed a relatively greater diversity amongst the male betelvines. The primers based on sequences specific to known X and Y chromosome of a dioecious plant *Silene latifolia* were tested with the dioecious betelvines in the gender set of landraces. Here the expectation was that the X and Y specific primers would reveal clear differences in the PCR profiles of the female and male betelvines respectively. These primers when used singly as well as in combination, however, did not reveal any such discrete profile differences. Surprisingly, these primers actually resulted in RAPD like multibanded profile and hence the band data for this was also scored in the same way as for the other three methods. This kind of study has never been done for any other plant to our knowledge. The conclusion about the most similar or most dissimilar landraces was based on cumulative data for all the SLXY primers. The result is actually interesting. Primarily the results indicate that multiple primer binding sites were present in all the DNAs. If these primers were generating multiple products it would appear that they have no co-relation with gender determining sequences in betelvines. This however is not entirely true since the NJ tree from the cumulative data can be clearly resolved into two major clusters, for the male and female betelvine respectively. Thus the PCR products resulting from these primers seemingly differs between the two genders and since several products are formed. It would appear that these primers are amplifying sequences from more than one gender determinant. Further since primers from both X and Y specific sequences gave multiple amplification products from the both male and female betelvines, our results indicate that gender determination in betelvine may either not be dependent on specific sex chromosomes or if such chromosome do exist male and female betelvines have similar sets of sequences on these chromosomes. Considering that all dioecious plants do not always have heteromorphic and distinct sex chromosomes, on the basis of our results with SLXY primers we infer that betelvine is one of those dioecious plants that lack distinct sex chromosomes.

In a novel approach, the fourth group of primers used was actually a heterologous set of primers were derived from X and Y chromosome specific sequences of the dioecious plant *Siliene latifolia*. The use of such primers for the dioecious betelvines was expected to reveal specific information about chromosomal basis for dioecy, if any. Interestingly, the SLXY primers resulted in multibanded profiles of several distinct bands even under stringent PCR conditions clearly indicating that several dispersed sequences homologous to the primers used were present. Though the primers collectively generated data that segregated the male and female vines, no single primer gave a sharply defined dimorphic profile for the two genders. This observation leads us to the conclusion that dioecy in betelvine may not follow the same chromosomal basis as in the case of *Silene latifolia*. Of course it is possible that such a lack of dimorphic profile could also be attributed to lack of strong homology of the primers to the appropriate regions of the betelvine. In this situation however, it would be

very interesting to determine the identity and sequence of genomic regions of betelvines that have resulted in the discrete multibanded profiles even under high PCR stringency.

**Comparison of the different methods used to assess the genetic diversity in betelvines:** The gender based set of betelvine landraces was systematically analyzed with four different types of primers. For each set of primers used, the profile data were used for the calculation of the Diversity Index (DI), Effective Multiplex Ratio (E) and Marker Index (MI) and Mean probability (p). These calculations allowed a comparison of the four PCR-methods. The values calculated in each case, are given in Table 3. Parson *et al.* (1997) suggested that differences in the chromosomal location of the three types of markers could influence the diversity assessment. Kojima *et al.* (1998), indicated that in wheat RAPDs were more representative of chromosomal regions enriched in repeated sequences, while ISSRs were related, as RFLPs, to coding sequences. A similar case was seen in case of lentil (Sonnate and Pignone, 2001) where the authors could not find any congruence between the RAPD and ISSR method. Carvalho and Schaal (2001) also obtained different levels of polymorphism in cassava, where the SSR-primed markers showed less polymorphism than the RAPD markers. In their case also grouping of varieties from different geographical habitats varied between the RAPD and ISSR techniques. In absence of any pedigree information about the varieties, we could not address the issue of concordance between the molecular profiling based estimates of genetic similarity and pedigree but, we may expect greater genetic information about genetic similarity from the molecular profiling based estimates in accordance with Russell *et al.* (1997). From their result also it was apparent that the different techniques reveal information about distinct regions of the genome. Moreover, the rate of evolution of the primer target site sequence is most likely different for the two types of markers. So depending on this aspect, the divergence shown amongst the genotypes by the different techniques would also differ. Powell *et al.* (1996) suggested, that any estimation of genetic relationships between individual genotypes was affected by, the number of markers, the distribution of markers in the genome (genome coverage) and the nature of evolutionary mechanisms underlying the variation measured.

The four methods do not reveal polymorphism within gender set to the same extent. Under our experimental conditions ISSR method was found to have the higher Marker index as well as PIC. This is clearly reflected in the bootstrapped Neighbour Joining tree for the ISSR data where the entire female vines clustered together in one broad group while the male vines were separated in at least three distinct sub-groups. In general during the course of the present study we have observed that the male betelvines are invariably more heterogenous than the female vines. Such a result is of significance for the application of breeding methods for the improvement of betelvines. Unfortunately betelvine as a crop is cultivated by vegetative means ever since it was first domesticated. There have been only sporadic attempts at the improvement of betelvines through controlled process. The present study has resulted in the assessment of range of diversity in the betelvines for a breeder who can now exploit the diversity judiciously.

In our study we found that ISSR method, which showed the highest Diversity and Marker Index, can be the method of choice for diversity analysis type of studies, in so far as polymorphism or Marker Index is the criterion. In the present study actually all four methods were almost equally useful for the analysis of the betelvine landraces (MI values in the range 4.366 to 5.289, Table 3). In fact the Mantel Test (distance matrix correlation)

analysis (Table 4) also resulted in highly significant normal standard variate $g > p_{0.005} = 2.575$ in all cases. However, here RAPD and ISSR have resolved the landraces almost equally similarly since the correlation (r = 0.8432, Table 4) was strong between the distance matrices of the two methods. This is an interesting observation and it is suggestive of the possibility that the landraces have more differences amongst themselves in their minisatellite and related tandem repeat sequences. This is actually supported by the observation that all DAMD bands in betelvine were polymorphic.

Sex detemination systems based on heteromorphic X and Y sex chromosomes are particularly interesting to study from both a developmental and evolutionary perspective. There are many paralleles between the sex chromosomes, in different species even between animals and plants. The evolution of heteromorphic sex chromosome systems in widely differing species suggests that similar forces may have been involved in each case (Charlesworh, 1992; Ellis, 1998; Charlesworth and Guttman, 1999). The *Silene* genus is an example of how the evolution of an XY system contributes to morphological change and speciation. The Y chromosome differs from all other chromosomes not only in that it is the only chromosome that does not recombine along majority of its length, but also is being present only in the male sex in a permanent haploid condition (Y genetic isolation), in having a common ancestry and persistent meiotic relationship with the X, and the tendency of its genes to degenerate during evolution (Y genetic erosion). The Y becomes a specialized male chromosome, which essentially behaves like a single recombination unit. The functional coherence of the Y can be achieved relatively early during Y evolution, which might be an essential condition for the maintenance of an XY system. Filatov *et al.* (2001) found several differences in polymorphism of the regions of X and Y chromosomes. In another study done by Lebel-Hardenack *et al.* (2002) for genetically mapping of the sex-determination loci on the male-specific Y chromosome, it was found that *S.latifolia* has three dispersed male-determining loci on the Y chrosome. The sorrel *Rumex acetosa* (Polygonaceae) is a perennial dioecious weed, which possesses sex chromosomes (XX in females, XY1 Y2 in males). These studies indicated that it is only in the recent years that some details are emerging about molecular mechanisms and profiles vis-à-vis gender discrimination in plants.

The four methods tested with the gender set of betelvines have resulted in separation of the male and female betelvines. Though the individual sub-clusters in the four methods are not congruent however the primary separation of the male and female betelvines is clear and unambiguous. Thus the gender distinction of the betelvine landraces in terms of flowering is strongly supported by molecular profiling with four different PCR methods. This is an important and an interesting result. The four methods result in discrete profiles that reflect different genomic regions and in spite of that, the methods allow the landraces to be segregated on the basis of gender. Thus, it appears that there are several levels of genomic differences between the male and female betelvines. In other words, it appears that gender distinction in betelvine may not be confirmed to a few chromosomes or chromosomal regions. This inference is actually supported by lack of male specific and female specific PCR profiles when Y and X chromosome specific primers were respectively used. This further strengthens our inference that dioecy in betelvines is not apparently determined by distinct heteromorphic sex chromosomes. In order to have a comprehensive distribution of landraces, the data generated by all four methods combined and considered cumulatively.

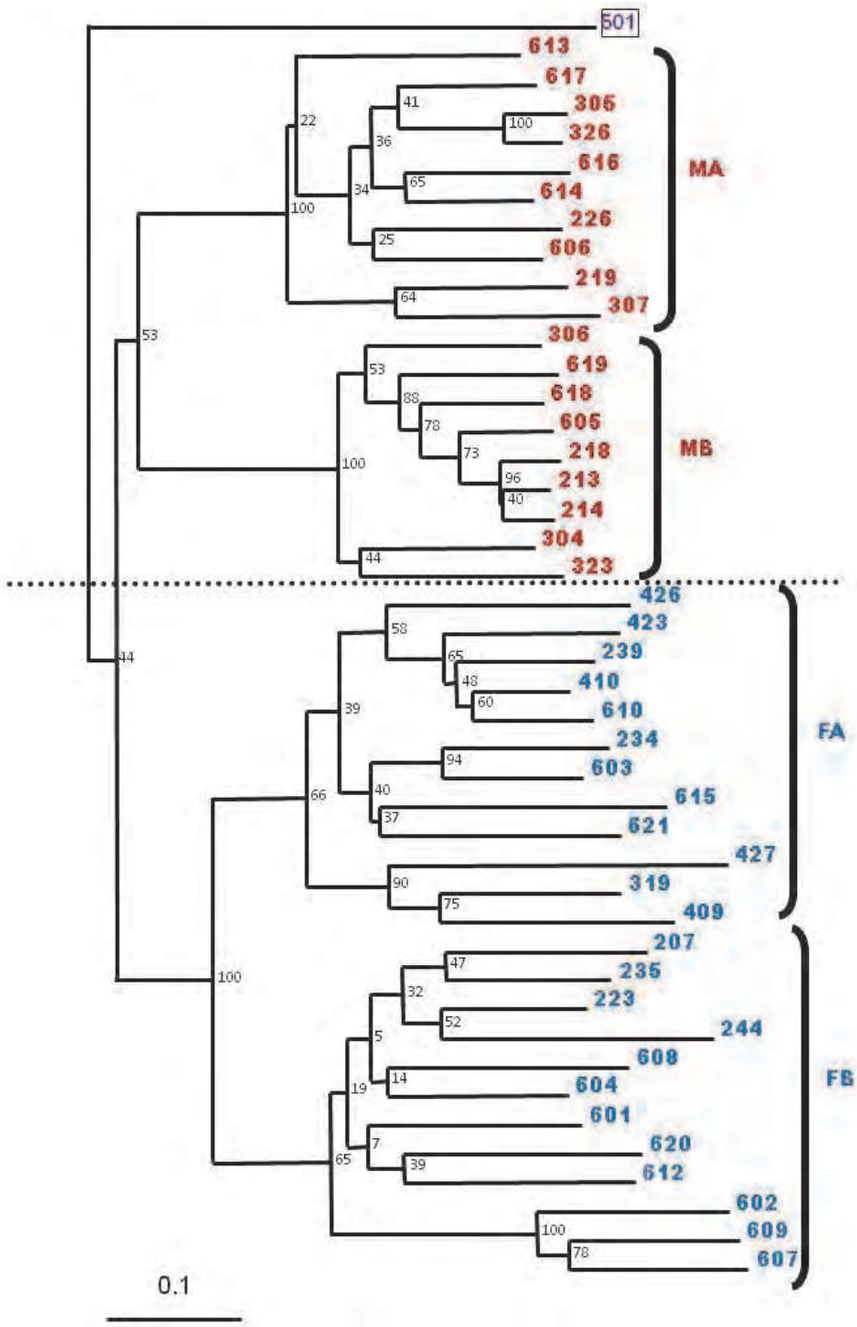


Fig. 4. The NJ tree for the cumulative band data for all the four PCR methods after 1000 replicate bootstrap analysis clearly depicts the separation of the out group taxon, *Piper hamiltonii* (501) from the betelvines which in turn are clearly distributed into separate clusters of male and female landraces. Interestingly each cluster of male or female landraces is further resolved into two sub clusters each, demarked in the figure with smaller labeled parenthesis. The scale at the bottom of the figure is for the Jaccard coefficients. Each landrace is identified by its number and color code as in Table 1. The dashed line through the figure separates the NJ tree into the respective gender halves.

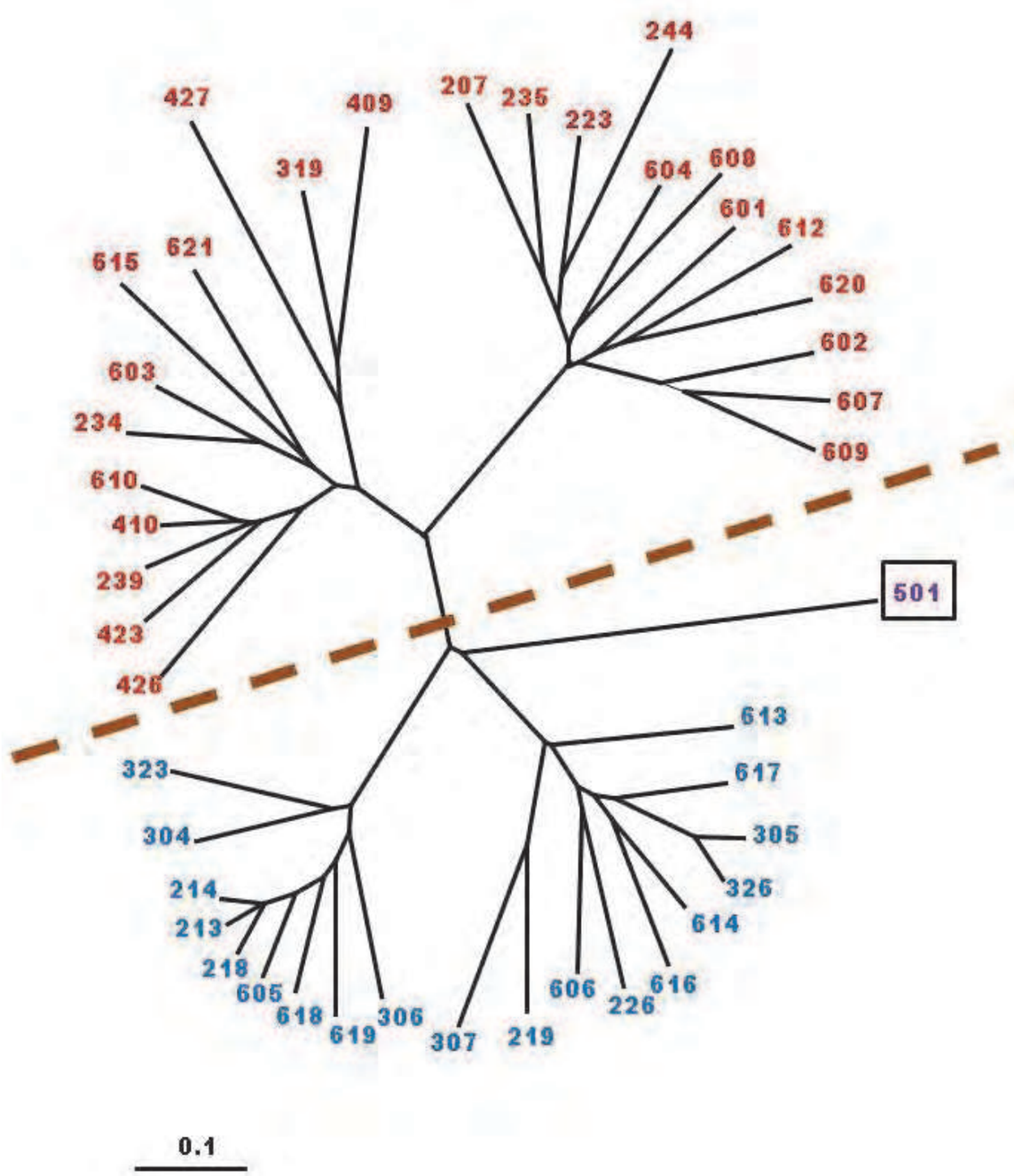


Fig. 5. The radial NJ tree for the cumulative band data for all the four PCR methods after 1000 replicate bootstrap analysis clearly depicts the separation of the out group taxon, *Piper hamiltonii* (501) from the betelvines which in turn are clearly distributed into separate clusters of male and female landraces. Each cluster of male or female landraces is very distinctly resolved into two sub clusters each. The scale at the bottom of the figure is for the Jaccard coefficients. Each landrace is identified by its number and color code as in Table 1. The dashed line through the figure separates the NJ tree into the respective gender halves.

The NJ tree from such a combined data for all methods is given in Figure 4. This tree also clearly separates the male and female betelvines. Interestingly the tree can be resolved into two subclusters within both male and female clusters as in the figure. MA, MB, FA and FB are the subclusters for males and females betelvines respectively (illustrated best by a radial tree form of the NJ tree as shown in Figure 5). We infer possibility of having at least two distinct ancient lineages for the male and female betelvines. In the absence of historical data and chronology of cultivation of these landraces it is not possible to confirm that there were actually a few discrete lineages of the cultivated betelvines and that the present day betelvine landraces are descendants of these lineages. The primary interest in all these studies is fuelled by the fact that dioecy as a proportion of accounts for only a small fraction of the numbers of flowering plants, yet in distribution across the plant families it is wide. Further, at the applied level, for plants of economic importance, detection and diagnosis of plant sex as an early event is desirable when the economically viable and important plant part is gender associated such as for example the fruits. In this context, betelvine is an exception in that for both male and female plants, the economically important plant part is the leaf.

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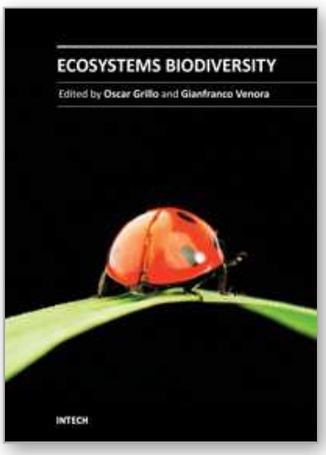


**Ecosystems Biodiversity**

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Ecosystems can be considered as dynamic and interactive clusters made up of plants, animals and micro-organism communities. Inevitably, mankind is an integral part of each ecosystem and as such enjoys all its provided benefits. Driven by the increasing necessity to preserve the ecosystem productivity, several ecological studies have been conducted in the last few years, highlighting the current state in which our planet is, and focusing on future perspectives. This book contains comprehensive overviews and original studies focused on hazard analysis and evaluation of ecological variables affecting species diversity, richness and distribution, in order to identify the best management strategies to face and solve the conservation problems.



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