Genetic relation of *Coffea* and Indian *Psilanthus* species as revealed through RAPD and ISSR markers

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Abstract

Coffee plants belong to the genus *Coffea* and *Psilanthus* of the tribe *Coffeeae* in the family Rubiaceae and are mostly distributed in tropical and subtropical regions. Over a period of time, *Psilanthus* was considered as a section *Paracoffea* under *Coffea* but recent taxonomic reports favor *Psilanthus* as separate genera. However, biochemical and molecular studies indicate close genome similarity of *Psilanthus* with *Coffea*. To address this question we selected RAPD and ISSR markers to assess the genome similarity between the two genera. The two marker systems showed significant difference in the genome of Indian *Psilanthus* species from *Coffea*. Further clustering of *Psilanthus* species correlated with their geographical distribution showing diversity of Peninsular Indian species from the Northeast Indian species. In case of *Coffea*, analysis of species relationship using RAPD and ISSR markers indicated correlation between the similarity of the genomes and the geographical distribution. The three East African species showed close relationship with each other but having high level of diversity from West and Central African species. Rest of the accessions of *Pachycoffea* members (tree coffee species) showed significant divergence from *C. liberica* and *C. dewevrei*.

Keywords: *Coffea*, RAPD, ISSR markers, genetic diversity, *Psilanthus*.

INTRODUCTION

*Coffea* and *Psilanthus* are distinguished in the tribe *Coffeeae* of the family Rubiaceae based on floral characters (Leroy, 1980; Bridson 1987). The genus *Coffea* consists of approximately 105 taxa (Davis *et al* 2006) and all species are woody, ranging from simple shrubs to robust trees originated in inter tropical forest of Africa and Madagascar. *C. arabica* L. (2n=4x=44) and *C. canephora* Pierre. (2n=2x=22) are the two cultivated species of economic importance. *C. arabica* is a natural allotetraploid and is self fertile, while other species are diploid and generally self-incompatible. The species belongs to the genus *Psilanthus* originate from either Asia or Africa and again divided into 2 subgenera. *P. subg. Psilanthus* with only two species is restricted to Western and Central Africa, where as, *P. subg. Afrocoffea* with 17 species has wide distribution in Africa, Asia, Philippines and Australia (Leroy 1980; Bridson 1987; Bridson and Verdcourt 1988).

Coffee trees differ greatly in morphology, size and ecological adaptation, leading to the description of a large number of species. Particular attention has been paid to the subgenus *Coffea* (genus *Coffea* L.) which includes two cultivated species of economic importance. Intergeneric classifications have been proposed based on morphological characters (Lebrun 1941; Chevalier 1947). However, grouping criteria have become very complex rather confused and to date they are considered of low value (Bridson and Verdcourt 1988). Complementary investigations are therefore required to classify the phylogenetic relationships among these taxa (Charrier and Berthaud 1985). Biochemical
components have been investigated (Anthony et al. 1993). However, such characteristics reveal fractional information and can be phylogenetically misleading due to parallel evolution and rapid adaptive radiation. Furthermore, genetic relationships among Coffea species have been assessed through molecular markers such as RAPD (Lashermes et al. 1993), ITS sequence of nuclear ribosomal DNA (Lashermes et al. 1997), chloroplast DNA polymorphism (Cross et al. 1998; Lashermes et al. 1996a), RAPD and organellar specific PCR (Orozco Castillo et al. 1996) chloroplast and mitochondrial DNA variation (Berthou et al. 1983). On the other hand, Psilanthus has not received much attention. Traditionally the generic relationship of Psilanthus and Coffea has puzzled the coffee taxonomists. Over a period of time Psilanthus was considered as a section Paracoffea under Coffea. Even though there are more than 20 Psilanthus species, only 3 species are studied extensively at DNA level and P. travancorensis showed close resemblance with Coffea (Lashermes et al. 1996a). The combined molecular analysis by nuclear and plastid sequence analysis (Maurin et al., 2007) also not resolved the taxonomic relationship of Coffea and Psilanthus. Davis et al. (2007) showed clear distinction within Coffeeae (Psilanthus and Coffea), but is only weakly supported by their combined molecular–morphological analysis and authors claim that further data, including wider sampling in these genera, are urgently required to ascertain whether two genera can be upheld. More recently seven species are identified under Psilanthus originating from India (Sivarajan et al. 1992). To address the genetic relation between Coffea and Indian Psilanthus species we have analyzed 4 species of Psilanthus originally from India and 15 species of Coffea originally from Africa by RAPD and ISSR markers. The main objective was to use the data to resolve the divergence and phylogenetic relationship of Indian Psilanthus species with Coffea species.

MATERIALS AND METHODS

Study material comprised of 19 samples, which included 15 species of Coffea and 4 species of Psilanthus (Table 1 [Supplementary data]). Leaf samples of Coffea species, were taken from the plants maintained at Central Coffee Research Institute (CCRI) germplasm collection at Chickmagalur, India. Fully expanded, first pair of fresh leaves were harvested and used for DNA isolation. For each sample, DNA was isolated from two individual plants separately and pooled for PCR amplification. MATAB two-step method as reported by Ky et al. (2000) was followed for DNA isolation with slight modification (Kumar and Sreenath 2006).

RAPD and ISSR amplification

RAPD amplification was performed according to Lashermes et al. (1996b), except that 0.6 units of Taq DNA polymerase, 10 pmol of primer and 40 ng of genomic DNA were used in a final reaction volume of 20 μl with annealing temperature of 38°C. Totally 47 random decamer primers were selected, which gave considerable number of bands with C. arabica and P. wightianus (data not shown) and used for RAPD amplification (Table 2 [Supplementary data]). For ISSR amplification, 21 ISSR primers were used and the reaction conditions were the same as those used for RAPD, except for annealing temperature. After initial optimization, different annealing temperatures were used for different sets of ISSR primers (Table 3 [Supplementary data]). Both RAPD and ISSR-amplified products were separated on 1.5% agarose gel and visualized by ethidium bromide fluorescence.

Data analysis

RAPD and ISSR products were scored separately for presence (1) or absence (0) of the bands. The data was boot-strapped to get 200 replicate data sets which were used to calculate Nei’s genetic distance (Nei 1978). 200 UPGMA trees were constructed based on genetic distance and strict consensus UPGMA tree was generated out of 200 trees using Felsenstein’s (2002) PHYLIP 3.6a3 statistical package. Dice similarity (Dice 1945) matrix was calculated using RAPD and ISSR binary data and multidimensional scaling was performed separately using NTSYSpc, version 2.01 statistical package. ‘Mantel-T’ test (Mantel 1967) was carried out for significance of RAPD, and ISSR similarity matrices.

RESULTS

RAPD

Highly polymorphic fingerprints were obtained in Psilanthus and Coffea species with the 47 primers tested. From the species studied, total 846 bands were amplified with 47 primers, out of
which 840 were polymorphic. The number of amplified products varied between 12 and 24, depending on the primer used, with an average of 17 polymorphic bands per primer with PIC value ranging from 88.2 to 100. The size of the amplified products ranged from 250 bp to 3.0 kb. Six monomorphic bands were present across all species of Psilanthus and Coffea (AT-18\textsuperscript{800}, D-05\textsuperscript{300}, X-09\textsuperscript{700}, UBC-217\textsuperscript{10}, 250 and UBC-266\textsuperscript{400}). Out of 840 polymorphic bands scored, 159 (18.9%) were specific to the genus Psilanthus and 681 (81%) were specific to Coffea.

In Psilanthus species studied, eight common bands were scored from the four species. Seventeen fragments were common between P. travancorensis and P. wightianus, where as, only 4 bands were shared by P. bababudanii and P. bengalensis. Thirty-eight unique bands were recorded in P. travancorensis, where as, P. wightianus produced 29, P. bababudanii showed 11 and P. bengalensis showed two unique bands (Fig. 1).

In Coffea, high level of polymorphism was observed among the 15 species studied. Out of 681 Coffea specific fragments scored, 91 unique bands were recorded from 14 species. Number of unique bands ranged from the highest of 12 in C. stenophylla to the least of 1 in C. arabica. No unique bands was observed in C. dewevrei.

ISSR

In ISSR fingerprints also, high level of polymorphism in banding pattern was observed in four species of Psilanthus and 15 species of Coffea studied. Totally 464 fragments were scored from Psilanthus and Coffea species from 21 primers, out of which 461 fragments were polymorphic among the species. The number of polymorphic fragments varied between 17 and 31 depending on the primer used, with an average of 22 polymorphic bands per primer with PIC values ranging from 94.4 to 100 (data not shown). The size of the amplified products ranged from 200 bp to 3.0 kb. Three monomorphic ISSR fragments {\((GA)_{6}CC^{900}\), \((GAG)_{3}GC^{320}\) and \((GTG)_{3}GC^{320}\)} were recorded across all the species of the genus Coffea and Psilanthus. Out of 461 polymorphic bands scored, 117 (25.3%) were specific to the genus Psilanthus and 344 (74.6%) were specific to Coffea species.

In the four Psilanthus species studied, six fragments were shared by all the four species, where as, 20 bands were shared between two Northeast Indian species (P. bengalensis and P. bababudanii) and 30 bands were shared between two South Indian species (P. travancorensis and P. wightianus). Unique bands found in P. travancorensis was 22 and the number decreased to 12 in P. wightianus, 05 in P. bababudanii and only one in P. bengalensis. Interestingly, eight (P. travancorensis, P. wightianus and P. bengalensis), seven (P. travancorensis, P. bengalensis and P. bababudanii), three (P. wightianus and P. bengalensis) and two (P. travancorensis and P. bababudanii) bands were shared between South and Northeast Indian species (Fig. 2).

Figure 1: Picture showing the distribution of RAPD markers amplified in Psilanthus species. Data shows genome resemblance of P. travancorensis with P. wightianus and P. bababudanii with P. bengalensis.

Figure 2: Picture showing the distribution of ISSR markers amplified in Psilanthus species. Data shows genome resemblance of P. travancorensis with P. wightianus and P. bababudanii with P. bengalensis.

Out of 344 Coffea specific fragments, 37 unique bands were recorded from 13 species except, C. canephora and C. dewevrei which showed no unique bands. A maximum of eight unique bands were scored in C. stenophylla, where as, only one unique band was scored in C. zanguebariae, C. liberica, C. arnoldiana and C. congensis.
Genetic relationship of Psilanthus with Coffea species

The dendrogram generated after UPGMA using genetic distance obtained from RAPD and ISSR markers showed similarities in the tree topology. RAPD and ISSR-generated dendrograms showed maximum similarity with each other (Fig. 3).

RAPD

The genus Psilanthus was separated from Coffea taxa forming a separate cluster with highly significant level of boot-strap values. P. travancorensis and P. wightianus showed similarity values of 0.5625 with each other and formed a small sub-cluster, where as, P. bababudanii and P. bengalensis with similarity values of 0.8288 formed another sub-cluster within the Psilanthus cluster.

In Coffea taxa, two main clusters resulted corresponding to their natural geographical distribution. The first cluster involved West and Central African species and the other cluster comprised of three East African species. Within the first main cluster, three sub-clusters were formed, in which C. canephora, C. liberica, C. dewevrei, C. congensis, C. arabica and C. arnoldiana formed one sub-cluster. Two species, C. kapakata and C. eugeniodes formed second sub-cluster within first cluster and four species of West African species of Pachycoffea members (C. abeokutae, C. aruwemiensis, C. excelsa and C. stenophylla) formed third sub-cluster. C. canephora exhibited close relationship with C. liberica, where as, C. abeokutae showed close relationship with C. aruwemiensis supported by maximum boot-strap values. Second cluster within Coffea taxa included three East African species viz., C. salvatrina, C. racemosa and C. zanguebariae belonging to Mozambicoffea section. C. racemosa showed close relationship with C. salvatrina than C. zanguebariae with moderate level of boot-strap values.

ISSR

The genus Psilanthus was separated from Coffea taxa forming a separate cluster with highly significant level of boot-strap values. P. travancorensis and P. wightianus showed similarity values of 0.6805 with each other and formed a small sub-cluster, where as, P. bababudanii and P. bengalensis with similarity values of 0.8182 formed another sub-cluster within the Psilanthus sector. Clustering of Coffea species was similar to the dendrogram obtained from RAPD data. However, branching of second main cluster was much clear from RAPD data, where as, branching of these species was independent from three different nodes of the phenetic tree indicating presence of diversity within these East African species. This branching pattern did not affect the tree topology. Both RAPD and ISSR trees were consistent with the clustering of the species with moderate to significant level of boot-strap values. In RAPD and ISSR-derived dendrograms, clustering of the
species was in accordance to their natural geographic distribution.

Figure 4: Non-metric multidimensional scaling analysis of Psilanthus and Coffea species from RAPD (a) and ISSR (b) markers. Numbers 1 to 19 refer to the sample identification number as per the Table 1. In both the marker systems Psilanthus species showed clear differentiation from Coffea species. RAPD markers detected more diversity among the Coffea species than ISSR markers. C. zanguebariae and C. racemosa showed more divergence from other Coffea species based on ISSR markers.

Non-metric multidimensional scaling based on Eigen values obtained from RAPD and ISSR separated out the members of the genus Psilanthus from Coffea (Fig. 4). C. congensis was observed to be close to C. canephora and C. liberica from ISSR and RAPD data. Considerable diversity was observed in all the Coffea species studied, which showed clear spatial distance from each other. A highly significant score of ‘r’ = 0.91644 (p=1.0000) was recorded for ‘Mantel-T’ test for cophenetic correlation between two similarity matrices obtained from RAPD and ISSR data.

DISCUSSION

Phylogenetic relation of Psilanthus with Coffea

In the present investigation, RAPD and ISSR markers are used to study phylogenetic relation of Psilanthus with Coffea. The two marker systems established clear differences between the two genera at the DNA level. Clustering of Coffea and Psilanthus species generally correlated with their geographical distribution. Considerable diversity was observed between the two South Indian and the two Northeast Indian Psilanthus species studied. Further, close affinity among two Indian Psilanthus species having same geographical distribution indicated genome similarity is related with geographical distribution of the species.

Successful inter-generic hybridization of P. ebracteolatus and C. arabica (Couturon et al. 1998) indicated close affinity of Psilanthus with Coffea. However, genetic divergence of the two genera was supported from the unsuccessful attempt towards intergeneric crosses between Psilanthus and Coffea at CCRI (unpublished data).

Diversity among Coffea species

RAPD and ISSR markers have been successively used for fingerprinting germplasm collections and phylogenetic studies in many plant species (Fang et al. 1997; Joshi et al. 2000; Rajesh et al. 2003; Arnau et al. 2003). In the present study, analysis of species relationship in Coffea using RAPD and ISSR markers indicated close similarity of the genomes of the species distributed in the same geological area. Species from West and Central Africa differed from those from East Africa. C. canephora accessions of India belong to diversity group ‘E’ representing Congolese genepool of Central Africa (Prakash et al. 2005). This Congolese group has close affinity with C. dewevrei which is a Central African species. However, in the present study C. canephora accession showed close relationship with C. liberica rather than C. dewevrei. C. dewevrei was introduced to India later during 1950’s from Guatemala (Ram et al. 1994) and this accession might be distinct from the C. liberica from West Africa.

Harrer (1957) considered C. canephora as a biotype of C. congensis and further genetic proximity between C. canephora and C. congensis was demonstrated in previous studies using RFLPs of cpDNA and mtDNA (Berthou et al. 1983) and with SSR and AFLP markers (Prakash et al. 2005). However, in our study C. congensis showed less similarity with C. canephora when compared to C. liberica and C.
dewevrei in RAPD markers. ISSR markers detected much diversity between C. congensis and C. canephora in which two more species, C. arnoldiana and C. arabica showed more similarity with C. canephora than C. congensis. The two marker systems showed close relationship of C. eugenioides with C. kapakata. This is similar to earlier results by Ruas et al. (2003) who reported that above two species are close to each other based on ISSR markers. Further, close affinity was reported between C. eugenioides and C. kapakata based on chemotaxonomic studies in 17 Coffea species available India (unpublished data). Srinivasan and Vishveshwar (1980a) reported three groups in Coffea and Psilanthus species available in India based on caffeine content in seeds, in which, East African species clustered with Psilanthus species. In our results also, genus Psilanthus is closely placed with three Coffea species from East Africa.

C. liberica and C. dewevrei are distinguished into two species according to their geographical distribution in two separate areas of Africa, i.e. Central and Western Africa (Porteres 1936). Lebrun (1941), on the basis of botanical data, pooled 12 species of the Pachycoffea taxa in a single species with two varieties: C. liberica var. liberica and C. liberica var. dewevrei which was later supported by extensive morphological description by Bridson and Verdcourt (1988). However, these two are maintained as separate species at CCRI (Ram et al. 1994). Present investigation with molecular markers also showed differentiation of C. dewevrei from C. liberica as the latter was much closer to C. canephora. Further, three species of Pachycoffea members (C. abeokutae, C. aruwemiensis and C. excelsa) exhibited high level of diversity from C. liberica and C. dewevrei. Berthou et al. (1983) reported clear differences between C. excelsa and C. liberica based on mtDNA variation. Further Ahmed and Srinivasan (1984) reported divergence of C. dewevrei from other 10 species available in India based on 15 morphological traits. This indicated that all Pachycoffea members are not exactly varieties of C. liberica and C. dewevrei. A detailed study covering all Pachycoffea members is required to assess genetic diversity and determine the taxonomy of this complex coffee group.

From RAPD and ISSR markers, three East African species showed close relationship with each other and high level of diversity from West and Central African species. This agrees with Lashermes et al. (1997) who reported close affinity of C. salvatrix, C. racemosa and C. pseudozanguebariae based on ITS2 region. Morphologically, these three species can be differentiated from West and Central African species with short fructification period (2 months as against 8 to 10), low caffeine content and lesser number of flowers on the nodes. Early ripening was reported in case of Indian Psilanthus species (Narasimhaswamy and Vishveshwar 1963). Based on caffeine content, Srinivasan and Vishveshwar (1980b) clustered C. salvatrix with Psilanthus. However, in our study C. salvatrix is clustered with C. racemosa and C. zanguebariae which are having low level of caffeine. Further DNA content in these species is reported to be lower than West and Central African species (Noiriot et al. 2003).

**Consistency of the two fingerprinting methods**

Dominant RAPD and ISSR markers are used to study interspecific relationship in various crops (Campos et al. 1994; Brummer et al. 1995; Lanner et al. 1995; Rieseberg, 1996; Fahima et al. 1999). Compared to RAPD markers, ISSR profiling is reported to be more reliable and reproducible (Tsumura et al. 1996; Fang & Roose 1999; Fang et al. 1997; Jones et al. 1997; Powell et al. 1996; Russel et al. 1997; Bohn et al. 1999). Considerable number of polymorphic loci was observed between Psilanthus and Coffea species from RAPD and ISSR.

In the present study, 13 di, 4 tri and tetra nucleotide repeats each, were used as ISSR primers (Table 2). Except for tetra nucleotide repeats, all other primers were anchored at 3’ region with one or two bases to prevent incidental annealing of primers within SSRs which may lead to smear formation on agarose gels. We did not consider primers anchored at 5’ region since these may impose selection of long simple sequence repeats within amplified region (Blair et al. 1999). Only 4 di nucleotide repeats (CT, CA, GA and GT) were used with different base combination at 3’ anchored region. Number of amplified fragments varied within the primers having same repeat motifs, but differed only in anchored bases. This indicated that ISSR-amplified products mainly depended on the anchored base, rather than the di-nucleotide repeat motifs. Compared to RAPD, more number of fragments were amplified per primer in case
of ISSR. This indicated that, abundant simple sequence repeats (microsatellites or SSRs) might be present on Coffea and Psilanthus genomes. GA repeat was reported to produce excellent banding pattern in mulberry (Vijayan and Chatterjee, 2003), rice (Blair et al. 1999; Joshi et al. 2000), orange (Fang et al. 1997), Douglas fir and sugi (Tsumura et al. 1996) and chickpea (Ratnaparkhe et al. 1998). In case primers with tri-nucleotide repeat motifs, additional GC was anchored at 3’ region. Of all the species tested, four tri-nucleotide repeat primers detected polymorphic bands, out of which GAG repeat detected maximum number of polymorphic bands. Even though primers having tetra-nucleotide as repeat motifs were not anchored at 3’ region, these primers also detected considerable number of polymorphic bands. This may be due to lower copy number of the tetra-nucleotide repeat motifs present within amplifiable distance and thus can be easily picked by the unanchored primers. Tri and tetra nucleotide repeats are reported to be less frequent in plant genomes (Reddy et al. 2002). Non-anchored tetra nucleotide primers were successively used to study polymorphism in tomato (Yu et al. 2003).

47 random primers having different levels of GC content (60 to 90 %) were tested to assess genetic diversity and phylogenetic relationship of Coffea and Psilanthus species. RAPD markers established clear difference between the two genera showing less genome similarity between the two. RAPDs detected high amount of genetic diversity available within Indian collections of Coffea species. Since these decamer primers are random, if sufficient number is used, RAPDs cover major portion of the genome depicting clear picture of the genome. RAPDs are most popular method of fingerprinting in terms of cost and skill. However, there is a serious drawback with the technique when reproducibility is concerned (Jones et al. 1997).

From these observations it can be concluded that ISSR technique is superior over RAPD for fingerprinting of Psilanthus and Coffea. Two marker systems showed clear differentiation of East African species from West and Central African species of Coffea. Present molecular study established clear molecular divergence of Indian Psilanthus species with Coffea.

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References


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