Plant Omics Journal POJ

POJ 9(1):73-80 (2016) ISSN: 1836-3644

Morpho-molecular characterization of putative interspecific crosses in black pepper (*Piper nigrum* L. and *Piper colubrinum*)

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Abstract

The objective of the present study was to establish the hybridity or otherwise of the six putative hybrids produced by separate interspecific hybridization programmes between a common male parent, *viz*., *Piper colubrinum* (2n=2x=26) and six genotypes of black pepper (*Piper nigrum* L.) (2n=4x=52). Though the morphological and cytological features of the two parental species show drastic differences, the hybrids resembled their female parents with respect to morphological features only, except for the fruit setting character. In all the six putative hybrids, the fruit set was significantly low. Considering the heterozygous nature of the *P. nigrum* genotypes and the genetic difference between the parents, a true hybrid should show greater genetic diversity from both parents and it will be sterile due to triploidy. Hence, in addition to morphological features, parents and putative hybrids were evaluated using RAPD and SSR markers. The molecular markers were efficient in revealing the species level differences between the two parental *Piper* species as well as varietal difference of the various genotypes of *P. nigrum* used as female parents. However, with respect to molecular markers also, the six hybrids resembled their respective female parents only. Cluster analysis, based on Dice dissimilarity coefficient using unweighted pair group method based on arithmetic mean (UPGMA), revealed 96-100% similarity of hybrids with respective female parents and grouped each of the putative hybrid along with the respective genotype used as the female parent. Hence it is suspected that the progenies considered as putative hybrids in the present study might have originated by parthenocarpic development of ovaries in the respective female parents at the time of interspecific crossing. SSR primer PN_A5 produced a species specific band in genotype Karimunda.

Keywords: Hybrids, Parthenocarpy, *Piper colubrinum*, *Piper nigrum*, RAPD, SSR.
Abbreviations: AFLP_amplified fragment length polymorphism; IPGRI_International Plant Genetic Resources Institute; RAPD_random amplified polymorphic DNA; RFLP_restriction fragment length polymorphism; SSR_simple sequence repeats.

Introduction

Black pepper (*Piper nigrum* L.; 2n=2x=52) belongs to the family Piperaceae, and is one of the oldest and most widely used spices in the world (Srinivasan, 2007; Nair, 2011; Gordo et al., 2012). Having originated in the humid, tropical evergreen forest of the Western Ghats in India, it has characteristic pungency and flavour. It is an important ingredient in cooking and has medicinal properties. Due to its medicinal properties, it is used in traditional medicine for its antioxidant, anti-inflammatory and anticancer properties (Liu et al., 2010; Nishimura et al., 2011; Gordo et al., 2012). India is one of the major producer, consumer and exporter of black pepper in the world. Over 1000 species are reported in genus *Piper* among which about 110 are of Indian origin (George et al., 2005). As India is the primary centre of origin of black pepper, the indigenous genetic resources are reservoirs of useful genes for plant improvement programme. Majority of the released varieties and cultivars of black pepper in Kerala are susceptible to *Phytophthora* foot rot. Although only a narrow range of variability in *Phytophthora* tolerance is reported among the cultivated types, the exotic wild species, *P. colubrinum* (2n=2x=26), is reported to be resistant (Ravindran, 2000). With the objective of transferring *Phytophthora* resistance from *P. colubrinum* to *P. nigrum*, several attempts were made to cross these two species. However the results obtained are inconsistent and the success obtained is very low (Sasikumar et al., 1999). Since *P. nigrum* is a tetraploid and *P. colubrinum*, a diploid, the progeny from this cross is expected to be a triploid and hence sterile. Also the heterozygous nature of *P. nigrum* results in segregation of characters in its progenies, whether selfed or crossed. By crossing different *P. nigrum* genotypes with *P. colubrinum*, a partially fertile interspecific hybrid (*P. nigrum* × *P. colubrinum*) with partial resistance obtained from one of the crosses has been reported by Vanaja et al. (2008). Many of the reports suggested that identification of true hybrids based on morphology alone is not sufficient in perennial crops like black pepper. The reasons are, morphological markers are influenced by environmental factors and frequently lacks the resolving power to identify hybrids at the juvenile stage (George et al., 2005). Therefore, ambiguity in morphological characters necessitated to go for molecular markers to establish the hybridity. RAPD markers have been utilized by various workers for identification of hybrids in many crops [Chrysanthemum (Huang et al., 2000), Barley

(Hoffman et al., 2003), Chilli (Mongkolporn et al., 2004), Cotton (Mehetre et al., 2004)] and there is earlier report in black pepper also (George et al., 2005). Microsatellite markers are also markers of choice (Esselink et al., 2003) because they are abundant, uniformly distributed, highly polymorphic, codominant, rapidly produced by PCR and easily accessed through published primer sequences (Gupta and Varshney, 2000). Microsatellite markers have been used in identifying hybrids between red and cultivated rice (Gealy et al., 2002) and in Rose (Werlemark, 2000; Kaul et al., 2009). The present investigation is for testing the hybridity in the putative hybrids from six interspecific crosses of black pepper made earlier at Pepper Research Station, Panniyur, Kerala, India. The plants, though with lower fertility, were difficult to be distinguished from their female parents based on morphological features. This necessitated detailed characterization using molecular markers also in addition to morphological characters in order to establish the true hybrid nature or otherwise. Also identification of true hybrids at juvenile stage is advantageous in perennial crop breeding. Identifying genotype specific molecular markers will be useful in varietal identification as well. Hence RAPD and SSR markers were used in the present study in addition to the morphological descriptor for black pepper.

Results and Discussion

Comparison of morphological characters of putative hybrids with respective parents

Several vegetative, reproductive and qualitative characters were identified as varietal characters in black pepper. Among the vegetative characters, plant growth habit and leaf area were found suitable to distinguish the hybrids and parental cultivars. Leaf area characters have varietal significance as reported earlier (Ibrahim et al., 1985; Sujatha and Namboothiri, 1995). The biometric observations recorded in the present study included 15 vegetative characters (Table 4) and 8 reproductive characters (Table 5). Among the 15 vegetative characters studied, there was no significant difference between hybrids and respective female parent with respect to 9-10 traits (Table 4). However, the fruit characters *viz*., fresh and dry weight of spikes, number of berries per spike, weight and volume of thousand fresh and dried berries, number and percentage of well-developed and under developed berries per spike and fruit setting percentage were significantly different in the interspecific hybrids (Table 5).
Out of the 28 qualitative characters examined, plant growth habits, shape, tip and base of young and mature leafs, leaf texture, arrangement and venation, spike colour, orientation and shape were similar to the respective female parents and differed from that of the male parent. Though shoot tip colour was similar in female parents and respective hybrids in five crosses, for the hybrid P5PC, it differed from female parent (Panniyur 5 has light purple shoot tips while the hybrid P5PC has pale green shoot tip).

Characterization of putative hybrids and parents using molecular markers

Ten decamer primers were selected for DNA amplification of the parents and putative hybrids in RAPD analysis and 11 SSR markers were selected for SSR analysis. Each hybrid and the respective parents were compared based on the total bands produced by the different primers. According to their presence or absence bands were classified into seven types of markers (Table 6) as per Huang et al. (2000) and Mehetre et al. (2004). The bands common in both parents and their hybrid were included in marker type I. These are monomorphic bands and indicate the similarity between the three genotypes. The bands common in any one of the parents and the respective hybrid were included in marker type II and III and were good markers to confirm hybridity or otherwise in *P. nigrum* × *P. colubrinum* crosses. The non-parental bands expressing uniquely in hybrids were included in type IV whereas the bands found in both parents but absent in hybrid were included in type V. Marker type VI and type VII were band specific to female and male parents respectively.

Fingerprinting the genotypes by RAPD

There were a maximum of 80 RAPD markers among which type I (monomorphic) included 22-27 bands in all the six different crosses (Table 6). Among the amplicons found in male parent (*P. colubrinum*) 22-28 nos belonged to type VII (Table 6) (Fig 1), which were distinct from all the *P. nigrum* species and hence can be classified as species specific bands of *P. colubrinum*. Female parent specific bands were obtained for Panniyur 1 (OPA_08-1100bp), Panniyur 3 (OPA_10-950bp, OPAH_09-1400bp) and Panniyur 5 (OPA_28-1200bp, OPC_08-1100bp) (Fig 1). One non-parental band (type IV) was observed in hybrid P2PC; observations with respect to the remaining amplicons showed that, in all the crosses, the hybrids possessed bands similar to respective female parent only (type II) and none were similar to male parent (type III). RAPD is reported to be an efficient marker in distinguishing black pepper genotypes (Pradeepkumar et al., 2003) and for identification of hybrids and testing genetic purity of hybrid in black pepper (George et al., 2005). The pattern of bands produced was identical in hybrid and respective female parent. This indicates a possibility of somatic origin of the embryo rather than by union of two different gametes, in which case the hybrid should have possessed a few bands specific to male parent. However, since RAPD is a dominant marker, the information on hybrid nature is limited. The RAPD profiles in the present study displayed a high degree of inter-varietal and inter-specific polymorphism of *Piper* which confirms suitability of RAPD markers for discrimination of the *Piper* species. In brief, the study yielded highly reproducible RAPD fingerprints, which proved as reliable and useful tool for hybridity testing and the analysis of genetic variation in *Piper* plants. The non-parental bands (included in type IV) expressing uniquely in some progenies with specific RAPD markers has also been reported in black pepper (George et al., 2005) and in other perennial crops like rose (Werlemark, 2000; Kaul et al., 2009).

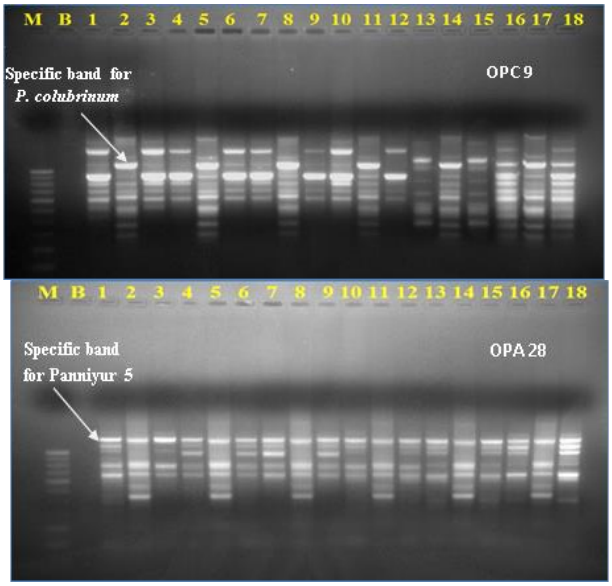
Fingerprinting the genotypes by SSR

SSR analysis with eleven SSR markers amplified a total of 32 alleles with sizes ranging from 50 to 1200bp (Table 6). Four loci were monomorphic in both the parents and hybrids (type I). Out of eleven markers, three (PN_A5, PN_H4 and Pn_GATA10) completely failed to distinguish even between the two different species. The remaining eight SSR markers amplified 15 loci of type VII, specific to male parent *P. colubrinum*. The marker PN_F1 amplified 3 loci; while PN_B5, PN_E3, Pn_CA9, Pn_AG30 and Pn_GT2, each amplified two loci and PN_G11 and PN_D10, one locus each. These could clearly distinguish between *P. nigrum* and *P. colubrinum* and can be used for species identification. These eight SSR markers also amplified 13 loci of type II (common in hybrid and respective female parent). However,

Table 1. Interspecific hybrids in black pepper (*P. nigrum* × *P. colubrinum*) and their respective parents.

Hybrid	Female	Male
P5PC	P5	PC
P3PC	P3	PC
P2PC	P2	PC
P1PC	P1	PC
KMPC	KM	PC
UKPC	UK	PC

P. nigrum genotypes (P1 to UK): P1_Panniyur 1; P2_Panniyur 2; P3_Panniyur 3; P5_Panniyur 5; KM_Karmunda; UK_Uthirankotta; PC_ *P. colubrinum* **(**common male parent in all crosses).



Lanes **M:** DNA standard, **B:** Control, **1:** Panniyur 5, **2:** *P. Colubrinum*, **3:** P5PC, **4:** Panniyur 3, **5:** *P. Colubrinum*, **6:** P3PC, **7:** Panniyur 2, **8:** *P. Colubrinum*, **9:** P2PC **10:** Panniyur 1, **11:** *P. Colubrinum*, **12:** P1PC, **13:** Karimunda, **14:** *P. Colubrinum*, **15:** KMPC, **16:** Uthirankotta, **17:** *P. Colubrinum*, **18:** UKPC

Fig 1. Amplification of parents and putative hybrids with RAPD primers exhibiting similarity between putative hybrid to female parent and difference from male parent.

Table 2. List of selected random decamer primers used for characterization of hybrids and parents.

Primer	Nucleotide Sequence
OPA_8	5'-GTGACGTAGG-3'
OPA_10	5'-GTGATCGCAG-3'
OPA_17	5'-GACCGCTTGT-3'
OPA_28	5'-GTGACGTAGG-3'
OPA_30	5'-AGGTGACCGT-3'
OPC_08	5'-TGGACCGGTG-3'
OPC_09	5'-CTCACCGTCC-3'
OPC_14	5'-TGCGTGCTTG-3'
OPP_08	5'-ACATCGCCCA-3'
OPAH_09	5'-AGAACCGAGG-3'

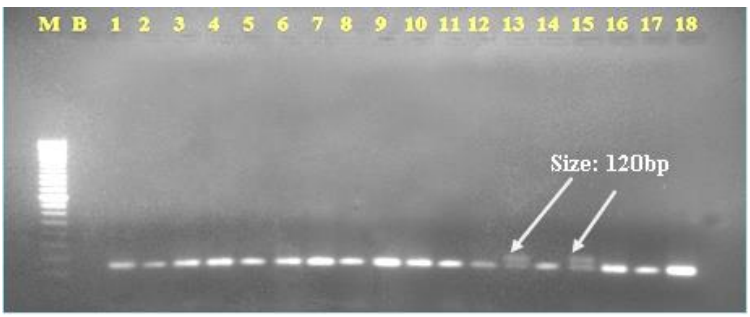


Fig 2. SSR marker (PN_A5) specific to Karimunda and its putative hybrid.

Lanes **M:** DNA standard, **B:** Control, **1:** Panniyur 5, **2:** *P. Colubrinum*, **3:** P5PC, **4:** Panniyur 3, **5:** *P. Colubrinum*, **6:** P3PC, **7:** Panniyur 2, **8:** *P. Colubrinum*, **9:** P2PC **10:** Panniyur 1, **11:** *P. Colubrinum*, **12:** P1PC, **13:** Karimunda, **14:** *P. Colubrinum*, **15:** KMPC, **16:** Uthirankotta, **17:** *P. Colubrinum*, **18:** UKPC.

Table 3. List of selected SSR primers used for characterization of hybrids and parents.

Name of Primers	Annealing temperature	Sequence
PN_A5	58 ºC	F 5'-CTTCCAGACCAATAATCAACTT-3'
		R 5'-ATCCCAAAATACACAACAATTC-3'
PN_B5	58 ºC	F 5'-GTTTTGAATGGGTCGGTGAT-3'
		R 5'-ATTGTTCTGATTTCTTCGTTATTG-3'
PN_E3	58 ºC	F 5'-TTTGTGTCCTCTCCCTCTCC-3'
		R 5'-AAGACTAAATAGGCAAGGCAAA-3'
PN_F1	58 ºC	F 5'-ACTTCAGTGCTATTTTTATCTTCC-3'
		R 5'-CCAACGCCCACTCTCAT-3'
PN_G11	58 ºC	F 5'-TTACTAGTGTCCACCCCCACT-3'
		R 5'-TCGATGGAAAGTCACCCTCT-3'
PN_H4	53 ºC	F 5'-CTTTTCCCACAATTCAGTCTCG-3'
		R 5'-ACCCATGCGTGTATCTTCTCAG-3'
PN_D10	58 ºC	F 5'-GTGTTACCTTTGGGGCATTCA-3'
		R 5'-TGTGTCAGGGCATCAAACC-3'
Pn_AG30	53 ºC	F 5'-ACTAAGGCTAATGTGATAACCTGAGG-3'
		R 5'-ATCCCTGGATGGAAATTTGAAGGCTTGC-3'
Pn_CA9	53 ºC	F 5'-TCATCAATCACACCTAAAAGAAGGCTATCC-3'
		R 5'-ATGTGGCTATGGGGAACGGTCAGGGGT-3'
Pn_GATA10	53.7ºC	F 5'-CTCCCAGTTATACAACATCACAACCTAGCAC-3'
		R 5'-AGAGGCTTGTCTTAGTTGTGCTCGGGA-3'
Pn_GT2	50 ºC	F 5'-CTAGAGAGTAACAGTTATCACTTCACAGC-3'
		R 5'-CTA GCAAATTTGTTCTCTAATTCACATGT-3'

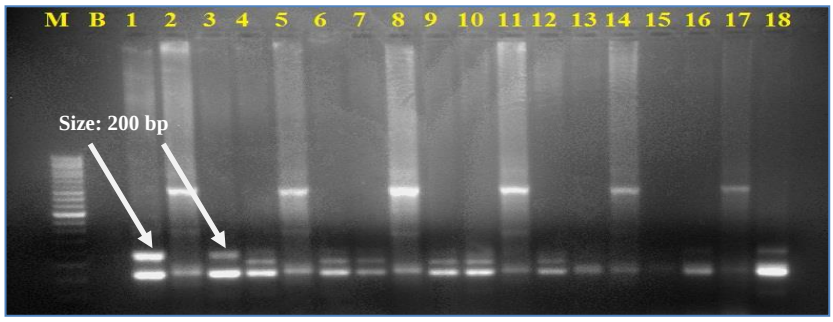


Fig 3. SSR marker (PN_D10) specific to Panniyur 5 and its putative hybrid.
Lanes **M:** DNA standard, **B:** Control, **1:** Panniyur 5, **2:** *P.Colubrinum*, **3:** P5PC, **4:** Panniyur 3, **5:** *P.Colubrinum*, **6:** P3PC, **7:** Panniyur 2, **8:** *P.Colubrinum*, **9:** P2PC **10:** Panniyur 1, **11:** *P.Colubrinum*, **12:** P1PC, **13:** Karimunda, **14:** *P.Colubrinum*, **15:** KMPC, **16:** Uthirankotta, **17:** *P.Colubrinum*, **18:** UKPC.

only PN_A5 and PN_D10 showed inter-varietal and interspecific polymorphism. Also the remaining six markers could distinguish between *P. colubrinum* from *P. nigrum* and their corresponding hybrids, but produced uniform fingerprints for all the six female parents and their hybrids.

Marker PN_A5 was unique for Karimunda and its putative hybrid (120bp) (Fig 2) while marker PN_D10 was unique for Panniyur 5 and its putative hybrid (200bp) (Fig 3). Apart from the additional band, PN_D10 failed to amplify a 150 bp amplicon in female parent Panniyur 5 and its putative hybrid, which were present in all the other five female parents and their corresponding putative hybrids. So these markers clearly proved that for Panniyur 5 and Karimunda, the putative hybrid is homozygous in the particular locus and clearly different from the male parent *P. colubrinum* as well as the remaining genotypes. This uniqueness of Karimunda and *P. colubrinum* was in exact correlation with the already reported distinctiveness based on morphological data (Jose and Sharma, 1984; Ravindran et al., 1997; Sen et al., 2010) and AFLP fingerprint analysis (Joy et al., 2007).

Also the fact that none of the male specific bands were present in any of the putative hybrids as in the case of RAPD markers again points to the possibility of somatic origin of the putative hybrids. But since the SSR markers available in black pepper are limited, the selected makers could not bring out the inter-varietal polymorphism as in the case of RAPD.

Cluster analysis

In the dendrogram (Fig 4), based on Dice dissimilarity coefficient using UPGMA, all the six interspecific hybrids showed highest similarity with their respective female parents and value of similarity ranges from 96 (in Panniyur 1, Panniyur 2, Panniyur 3 and Panniyur 5) to 100 per cent (in Karimunda and Uthirankotta). All interspecific hybrids showed greater diversity (52-72%) from the male parent *P. colubrinum* (Fig 4).

Materials and Methods

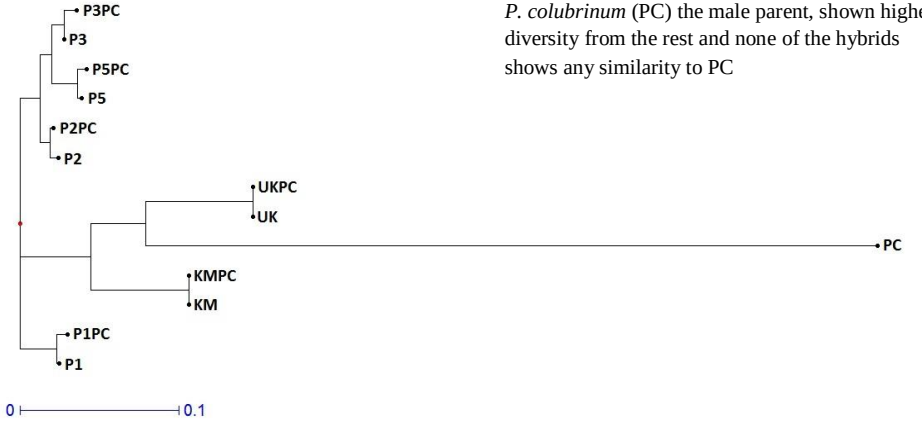
Plant materials

Six *P. nigrum* genotypes, *P. colubrinum* and their hybrid populations maintained at the Pepper Research Station, Panniyur, Kerala, India were used in this study. Six cross-combinations involving six different *P. nigrum* genotypes (four released varieties and two cultivars) and one common male parent *P. colubrinum* were included in the study (Table 1). The morphological observation of various quantitative and qualitative characters was recorded from field grown vines of parents and putative hybrids, as per descriptor of black pepper (IPGRI- Biodiversity International, 1995).

Table 4. Mean of putative interspecific hybrids and their respective parents for various quantitative traits of leaf and stem.

Parents and respective hybrids		Young leaf characters				Mature leaf characters					Stem characters					
											Orthotropes			Plagiotropes		
No. of cross	Genotypes	Length (cm)	Width (cm)	L/W ratio	Leaf area (cm^2)	Length (cm)	Width (cm)	L/W ratio	Leaf area (cm^2)	Petiole length (cm)	Internode length (cm)	Thickness at node (cm)	Thickness at internode (cm)	Internode length (cm)	Thickness at node (cm)	Thickness at internode (cm)
I. M	P. colubrinum	8.5 [a]	4.4 [a]	1.9 [a]	31.8 [a]	16.4 [a]	6.6 [a]	2.5 [a]	91.1 [a]	1.3 [a]	15.0 [a]	6.5 [a]	4.5 [a]	6.8 [a]	2.0 [a]	1.1 [a]
I. F	Panniyur 5	9.7 [b]	4.5 [a]	2.2 [b]	37.1 [b]	16.1 [a]	8.5 [b]	1.9 [b]	116.3 [b]	2.5 [b]	10.0 [b]	6.0 [b]	4 [b]	6.1 [a]	2.1 [a]	1.1 [a]
I. H	P5PC	9.5 [b]	4.3 [a]	2.2 [b]	34.5 [b]	15.2 [b]	8.0 [c]	1.9 [b]	103.4 [c]	2.4 [b]	11.0 [c]	6.0 [b]	3.5 [c]	6.8 [a]	2.6 [a]	1.5 [a]
II. M	P. colubrinum	8.5 [a]	4.4 [a]	1.9 [a]	31.8 [a]	16.4 [a]	6.6 [ab]	2.5 [a]	91.1 [a]	1.3 [a]	15.0 [a]	6.5 [a]	4.5 [a]	6.8 [a]	2.0 [a]	1.1 [a]
II. F	Panniyur 3	8.6 [a]	4.0 [b]	2.2 [b]	29.2 [a]	14.3 [b]	6.7 [a]	2.1 [b]	81.4 [b]	1.4 [a]	7.2 [b]	2.3 [b]	2.0 [b]	5.3 [b]	2.6 [a]	1.6 [a]
II. H	P3PC	8.4 [a]	4.2 [b]	2.0 [b]	29.9 [a]	13.9 [c]	6.2 [b]	2.2 [ab]	73.3 [c]	1.3 [a]	7.5 [b]	5.0 [c]	3.0 [c]	5.1 [b]	2.3 [a]	1.4 [a]
III. M	P. colubrinum	8.5 [a]	4.4 [a]	1.93 [b]	31.8 [a]	16.4 [a]	6.6 [b]	2.5 [a]	91.1 [a]	1.3 [b]	15.0 [a]	6.5 [a]	4.5 [a]	6.8 [a]	2.0 [b]	1.1 [a]
III. F	Panniyur 2	8.2 [b]	3.9 [b]	2.10 [a]	27.1 [b]	14.2 [b]	7.2 [a]	2.0 [b]	89.9 [a]	1.9 [a]	7.6 [b]	3.0 [c]	2.5 [c]	6.7 [a]	2.4 [ab]	1.3 [a]
III. H	P2PC	8.0 [b]	3.9 [b]	2.05 [a]	26.5 [b]	12.8 [c]	6.5 [b]	2.0 [b]	70.7 [b]	1.7 [a]	7.8 [b]	5.0 [b]	4.0 [b]	6.4 [a]	2.7 [a]	1.3 [a]
IV. M	P. colubrinum	8.5 [a]	4.4 [b]	1.93 [b]	31.8 [a b]	16.4 [a]	6.6 [b]	2.5 [a]	91.1 [c]	1.3 [b]	15.0 [a]	6.5 [a]	4.5 [a]	6.8 [a]	2.0 [b]	1.1 [a]
IV. F	Panniyur 1	7.8 [b]	5.0 [a]	1.56 [a]	33.1 [a]	14.8 [b]	11.5 [a]	1.3 [b]	144.7 [a]	5.3 [a]	7.5 [b]	2.5 [b]	2.0 [b]	5.4 [b]	2.9 [a]	1.7 [a]
IV. H	P1PC	7.5 [c]	4.9 [a]	1.53 [a]	31.2 [b]	13.5 [c]	11.2 [a]	1.2 [b]	128.5 [b]	5.5 [a]	7.3 [b]	2.5 [b]	2.0 [b]	5.0 [b]	2.5 [ab]	1.5 [a]
V. M	P. colubrinum	8.5 [a]	4.4 [a]	1.9 [a]	31.8 [a]	16.4 [a]	6.6 [a]	2.5 [a]	91.1 [a]	1.3 [b]	15 [a]	6.5 [a]	4.5 [b]	6.8 [a]	2.0 [a]	1.1 [a]
V. F	Karimunda	8.7 [a]	4.5 [a]	1.9 [a]	33.2 [a]	13.5 [b]	6.5 [a]	2.1 [b]	74.58 [b]	1.7 [a]	6.5 [b]	6 [b]	4.0 [c]	6.4 [a]	2.2 [a]	1.1 [a]
V. H	KMPC	8.5 [a]	4.7 [a]	1.8 [a]	33.9 [a]	13.1 [b]	6.3 [a]	2.1 [b]	70.15 [b]	1.6 [a]	6.5 [b]	6 [b]	5.0 [a]	6.7 [a]	2.6 [a]	1.4 [a]
VI. M	P. colubrinum	8.5 [ab]	4.4 [a]	1.9 [b]	31.8 [a]	16.4 [b]	6.6 [c]	2.5 [a]	91.1 [c]	1.3 [b]	15 [a]	6.5 [a]	4.5 [a]	6.8 [a]	2.0 [a]	1.1 [a]
VI. F	Uthirankotta	8.7 [a]	3.4 [b]	2.6 [a]	25.1 [b]	17.4 [a]	10.9 [a]	1.6 [b]	161.2 [a]	1.6 [a]	7.7 [b]	2.9 [b]	2.4 [b]	6.2 [ab]	2.2 [a]	1.0 [a]
VI. H	UKPC	8.2 [b]	3.2 [b]	2.6 [a]	22.3 [b]	14.4 [c]	9.1 [b]	1.6 [b]	111.4 [b]	1.4 [ab]	7.5 [b]	2.7 [b]	2.4 [b]	5.5 [b]	2.0 [a]	1.2 [a]

M_male; F_female; H_hybrid; [abc] Values in the same row with a different superscript differ significantly at $p<0.05$.



P. colubrinum (PC) the male parent, shown highest diversity from the rest and none of the hybrids shows any similarity to PC

Fig 4. Dendrogram (UPGMA) pattern of combined RAPD and SSR analysis using DARwin.

Table 5. Berry characters of putative interspecific hybrids and their respective female parents*.

Female Parent and respective hybrid		No. of berries per spike	1000 berries weight		1000 berries volume		Developed berries (%)	Undeveloped berries (%)	Fruit setting (%)
No. of cross	Genotypes		Fresh wt. (g)	Dry wt. (g)	Fresh volume (cc)	Dry volume (cc)			
I. F	Panniyur 5	102 [a]	140 [a]	47 [a]	120 [a]	60 [a]	79 [a]	21 [b]	84.6 [a]
I. H	P5PC	97 [b]	110 [b]	39 [b]	105 [b]	45 [b]	23 [b]	77 [a]	23.0 [b]
II. F	Panniyur 3	92 [a]	120 [a]	44 [a]	115 [a]	50 [a]	83 [a]	17 [b]	89.3 [a]
II. H	P3PC	84 [b]	110 [b]	33 [b]	110 [b]	50 [a]	11 [b]	89 [a]	11.5 [b]
III. F	Panniyur 2	71 [a]	130 [a]	46 [a]	120 [a]	60 [a]	75 [a]	25 [b]	74.2 [a]
III. H	P2PC	65 [b]	100 [b]	45 [b]	105 [b]	60 [a]	32 [b]	68 [a]	32.7 [b]
IV. F	Panniyur 1	125 [a]	170 [a]	72 [a]	149 [a]	70 [a]	93 [a]	7 [b]	96.0 [a]
IV. H	P1PC	101 [b]	140 [b]	39 [b]	135 [b]	60 [b]	21 [b]	79 [a]	26.6 [b]
V. F	Karimunda	62 [a]	120 [a]	47 [a]	105 [a]	60 [a]	81 [a]	19 [b]	80.7 [a]
V. H	KMPC	50 [b]	110 [b]	30 [b]	105 [a]	50 [b]	33 [b]	67 [a]	33.6 [b]
VI. F	Uthirankotta	76 [a]	160 [a]	51 [a]	160	79 [a]	72 [a]	28 [b]	82.4 [a]
VI. H	UKPC	55 [b]	120 [b]	34 [b]	136	70 [b]	24 [b]	83 [a]	21.5 [b]

*Male parent data not provided as berry setting is not there in P. colubrinum. M_male; F_female; H_hybrid; [abc]Values in the same row with a different superscript differ significantly at $p<0.05$.

Table 6. Classification of amplicons observed in interspecific hybrids of P. nigrum × P. colubrinum using selected RAPD and SSR primers.

Type	Property of marker			P5PC		P3PC		P2PC		P1PC		KMPC		UKPC	
	F	H	M	RAPD	SSR	RAPD	SSR	RAPD	SSR	RAPD	SSR	RAPD	SSR	RAPD	SSR
I	+	+	+	24	4	22	4	25	4	24	4	26	4	27	4
II	+	+	-	27	11	27	12	26	12	24	12	22	13	24	12
III	-	+	+	0	0	0	0	0	0	0	0	0	0	0	0
IV	-	+	-	0	0	0	0	1	0	0	0	0	0	0	0
V	+	-	+	0	0	0	0	0	0	0	0	0	0	0	0
VI	+	-	-	2	0	2	0	0	0	1	0	0	0	0	0
VII	-	-	+	27	15	28	15	28	15	27	15	24	15	22	15
Total				80	30	79	31	80	31	76	31	72	32	73	31

F_female; H_hybrid; M_male; '+' indicates presence of band and '-' indicates absence of band.

Isolation of DNA and marker analysis

Genomic DNA was isolated from fresh juvenile leaves from parental lines and the putative hybrids by CTAB extraction method (Rogers and Bendich, 1994) with slight modification using increased concentration of CTAB (4%), β-mercaptoethanol (3%) and PVP (2%) in a high salt concentration extraction buffer. Quantification of DNA was accomplished by analyzing the DNA on 0.8% (w/v) agarose gel, the approximate DNA yields were calculated using a spectrophotometer and the DNA samples were stored at -20°C. For RAPD analysis, ten selected decamer primers (Operon Technologies Inc.) were used (Table 2). The PCR reactions were performed using a 20 µl reaction mixture containing 30 ng template DNA, 1X *Taq* assay buffer B (Bangalore Genei, India), 25 mM $MgCl_2$ (Bangalore Genei, India), 10 mM of each dNTP mix (Bangalore Genei, India), 10 pmol decamer random primer, 1 U *Taq* DNA polymerase (Bangalore Genei, India) and sterile distilled water. For DNA amplification, the Eppendorf Master Cycler (Eppendorf, USA) was programmed as follows: initial denaturation at 94°C for 4 min followed by 40 cycles of incubation at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. The final extension was done at 72°C for 8 min. The amplification products were separated by electrophoresis in 1.4% (w/v) agarose gel with 1X TAE buffer, stained with 0.5 mg/ml ethidium bromide and visualised under the UV transilluminator in gel documentation system (Biorad, USA).

Sixteen microsatellite primer pairs developed for *P. nigrum* (Menezes et al., 2009; Joy et al., 2011) were screened for parental polymorphism and 11 primer pairs (Table 3) were selected for verifying the hybridity of interspecific hybrids. The sequences of the selected polymorphic primer pairs and their annealing temperature are presented in Table 3. The PCR reactions was performed in a volume of 20 µl reaction mixture containing 30 ng of template DNA, 10 µM of each dNTPs, 1X *Taq* assay buffer with $MgCl_2$ (Bangalore Genei, India), 1 U of *Taq* polymerase (Bangalore Genei, India), 10 µM each of the SSR primer pair (Sigma Aldrich, USA) and sterile distilled water, using a Veriti Thermal Cycler (Applied Biosystem, USA). Amplification reactions were initiated by 1 min pre-denaturation at 94°C and followed by 35 cycles each at 94°C for 1 min, corresponding annealing temperature for 1 min, 72°C for 1 min. A final extension step at 72°C for 5 min was performed after 35 cycles. The amplification products were separated by electrophoresis in 2% (w/v) agarose gel with 1X TAE buffer, stained with 0.5 mg/ml ethidium bromide and visualised under the UV transilluminator in gel documentation system (Biorad, USA).

Data analysis

The significant difference for all measured morphological (vegetative and reproductive) parameters was analysed statistically using analysis of variance (ANOVA), followed by Duncan's Multiple Range Test at the probability level of $p<0.05$. All RAPD assays were repeated thrice and only the

reproducible bands were scored as present (1) or absent (0), likewise the amplified SSR products were scored for each primer genotype combination. The data entry was done into a binary data matrix and Dice coefficient of dissimilarity was generated using unweighted pair group method based on arithmetic mean (UPGMA) through the computer package DARwin (Version 5.0.158).

Conclusion

In present investigation, the morphological similarity of the inter-specific hybrids to the respective female parent were further tested based on the amplification pattern with selected RAPD and SSR primers. The results reveal that all the six putative interspecific hybrids resemble their female parent suggesting a somatic origin of the putative hybrids from the female parent. In black pepper, George et al. (2005) reported banding pattern of hybrids exactly similar to the female parent in populations derived from crosses. They reported that in controlled pollination studies during the course of hybrid development in black pepper, chances like the formation of progenies without the paternal relationship like apomixis could occur and the developed seeds will give false results. In present study also, a banding pattern exactly similar to the female parent was observed in the populations derived from interspecific crosses, which also may point to the apomictic property of selected lines of black pepper. Therefore, the present study may also be extended to confirm the mode of reproduction by apomixis versus self-pollination, or cross-fertilization. Also, result indicates that cultivar Panniyur 5 and Karimunda are genetically distinct in species *P. nigrum*. Joy et al. (2011) also reported that Karimunda and wild black pepper (*P. colubrinum*) are highly diverse in 35 studied cultivars, and this diverse nature might have been contributed by the mutational events that accumulated during the course of its evolution or by any external factors.

Acknowledgements

Research work was supported by Department of Biotechnology, New Delhi, India under the Post graduate degree programme. The authors also gratefully acknowledge the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University for providing the excellent facilities for conduct the research work.

Conflict of Interest

The authors declare that they have no conflict of interest.

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