Table 1. Morphological description of purple colour accessions

Descriptors	RG 2008	RG 1930
Stem colour and bloom	Purple, zero bloom	Purple, zero bloom
Number of nodes on main stem	12	16
Plant height (cm)	38	56
Leaf shape	Semi-cup	Flat
Leaf colour	Purple	Purple
Primary raceme length (cm)	32	18
Shape and compactness of raceme	Conical, loose	Umbrella, loose
Capsule colour and nature	Purple, spiny	Purple, spiny
Seed shape	Oval	Oval
Seed colour	Dark brown	Brown
Mottling on seed coat	Conspicuous	Less conspicuous
100-seed weight (g)	8	13
Days to 50% flowering	40	47
Days to 50% maturity	105	117
Seed yield (g/plant)	48	30
Oil content (%)	50	45

colour was uniform in all the progenies. The inheritance studies being conducted at the Directorate showed that this character is easily heritable. The hybrid between purple colour type female and other colour type male was purple while when the purple-coloured morphotype was a male parent the F_1 was intermediate. This indicates that this morphotype could also be used as a genetic marker in hybrid development programme. As purple-coloured castor looks ornamental and attractive, it could also be promoted as an ornamental plant.

Mahalanobis's D^2-statistics and canonical analysis[4] were used to assess the magnitude of divergence between the purple colour accessions along with other 89 castor accessions collected from north-eastern India. The analysis of dispersion for the test of significance of differences in the mean values based on Wilk's criterion[5] revealed highly significant differences between the genotypes ($v = 961$) for the aggregation of eight characters studied. Interestingly the two purple-coloured genotypes were placed in two diverse clusters, RG 1930 in cluster II and RG 2008 in cluster VI. The highest intra-cluster distance (5.1) between cluster II and VI indicates high genetic divergence between these two accessions. These purple-coloured morphotypes could serve as diverse resistant sources for leafminer and wilt disease with a distinct genetic marker, in castor improvement programmes in order to breed diverse resistant breeding cultivars.

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Identification of hybrids in black pepper (*Piper nigrum* L.) using male parent-specific RAPD markers

Black pepper (*Piper nigrum* L.) ($2n = 52$) belongs to the family Piperaceae, and is one of the oldest and most widely used spices in the world. 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. India is a 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[1].

The development of improved cultivars through hybridization has made a major contribution to increased productivity and quality of plants in different crop plants. Hybridization of genetically different parents is followed for hybrid cultivar (F1) development and molecular marker techniques are often used for fastening plant improvement[2].

Majority of the black pepper fields are now cultivated with land races or with the most popular hybrid variety, 'Panniyur-1'. Most of the varieties released for cultivation are clonal selections from the existing land races. F_1 hybrids with new gene combinations will probably replace the traditional low-yielding senile plants in a cultivators' gardens in the near future, but will depend on many factors, including availability of tools to speed up the improvement programmes. One of the problems faced by pepper breeders is the difficulty in identifying true hybrids from the crossed progenies before planting. The traditional method of hybrid identification based on morphological characters is influenced by environmental factors and frequently lacks the resolving power to identify hybrids at the juvenile stage. Therefore, black pepper plants are to be grown to maturity (i.e. 3 to 4 years) to confirm hybridity. A reliable method for identification of hybrid pepper at the early stage of the plants is thus essential. Molecular markers used to detect DNA polymorphism are the most direct answer to the problem. Sasikumar *et al.*[3] reported the use of isozyme technology in the identification of two interspecific hybrids of *Piper*. However, the level of polymorphism obtained using isozymes is often insufficient to distinguish cultivars[4]. Markers like RFLP have been used in other crops for marker-assisted selection and to guide the introgression of target genes from related species during the past several years[5]. However, RFLP is labour-intensive and costly.

Development of PCR has allowed the introduction of RAPD approach[6] for molecular analysis of genomes. The major advantage of this approach lies in the fact that it allows exploration of large genomic portions. RAPD markers are now widely used for the identification of artificial and natural hybrids in different crops[7].

The main objective of the study was to develop DNA markers for specific crosses and to identify true hybrids at the juvenile stage. Eleven black pepper accessions and their hybrid populations maintained at the Indian Institute of Spices Research (IISR), Calicut were used in this study. Eight cross-combinations involving different accessions, including wild relatives as male parents were included in the study (Table 1).

Total DNA from the leaves of black pepper was prepared by a modified CTAB extraction method[8] using increased concentration of CTAB (4%) and β-mercaptoethanol (0.5%). The quality of DNA was checked by agarose gel electrophoresis (1% agarose), the approximate DNA yields were calculated using a spectrophotometer and the DNA samples were stored at –20°C. Thirty-five decamer oligonucleotide primers (Operon Technologies Inc-Alameda, California) were screened by PCR. The PCR reactions were performed using a 20 µl mixture containing 1.5 mM $MgCl_2$ (Finnzymes, Finland), 0.25 mM dNTP mix (Bangalore Genei, India), 5 pmol random decamer, 1 U Taq DNA polymerase (Finnzymes) and 30 ng genomic DNA for DNA amplification. The thermocycler was programmed as follows: initial denaturation at 93°C for 4 min followed by 40 cycles of incubation at 93°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 1.2% agarose gel with 1X TAE buffer, stained with 0.5 µg/ml ethidium bromide and photographed under exposure to UV light. The size of each band was estimated using the DNA molecular weight marker (λDNA–*Eco*R1/*Hin*dIII double digest; Bangalore Genei).

Out of 35 decamer primers used for RAPD analysis, 13 primers, viz. OPE-01, OPE-02, OPE-03, OPE-05, OPE-08, OPE-11, OPE-13, OPE-16, OPE-17, OPE-18, OPE-19, OPE-20 and OPC-16 yielded the best amplification products. Amplified products were scored on the basis of their presence or absence of bands. Consistent and reproducible RAPD results were only considered for screening. Each RAPD band was treated as an independent character/locus. RAPD banding pattern among parents and their hybrid populations was compared to assess hybridity at the DNA level. Oligonucleotide primers that could result in the amplification of male parent-specific polymorphic bands are listed in Table 1. These primers were useful in generating at least one such band and to select true hybrids based on shared bands in male parent and offspring (Figure 1). Though we have observed considerable difference in band intensity, such qualitative differences were not considered in the present data analysis. The percentage of hybridity was also not estimated. This technique of hybrid selection tested with established hybrid plants in black pepper also yielded promising results to confirm its utility.

Non-parental bands observed in some progenies might have resulted from DNA recombination or mutation[9,10]. Chromosomal crossing-over during meiosis may result in the loss of priming sites and thus markers are present in parents but not in offspring[11]. Black pepper, being heterozygous and propagated through cuttings, segregation of characters can be expected in the hybrid progenies. Therefore, it is not surprising to find all bands from each parent not present in the hybrids of black pepper.

Table 1. Unique male parent-specific PCR products amplified with RAPD primers

Cross	Male parent-specific marker	Marker in F_1 plants			
		1	2	3	4
Aimpiriyan × Panniyur 1	OPE 11_{1120}	–	–	+	a
Panniyur 1 × P24	OPE 16_{1500}	–	+	+	–
HP780 × *P. nigrum* (wild)	OPE 01_{850}	–	+	a	a
HP813 × IISR 4182	OPE 01_{1290}	–	–	–	+
Neelamundi × *P. nigrum* (wild)	OPC 16_{2360}	–	+	+	–
Irumaniyan × Karimunda	OPE 18_{530}	+	–	+	–
Panniyur 1 × Karimunda	OPE 16_{490}	–	+	–	–
Aimpiriyan × *P. attenuatum*	OPE 13_{1790}	–	–	+	a

a, absence of samples.

Although the results given here pertain to identification of hybrids, the RAPD marker technique has potential applications in the identification, registration and protection[4] of black pepper accessions. Molecular characterization of black pepper cultivars using RAPD markers was reported by Pradeepkumar *et al.*[12]. The information obtained through germplasm characterization using RAPD will be useful also for the screening of duplicates, assessing genetic diversity and monitoring the genetic stability of conserved germplasm. In the genus *Piper*, this technique has been successfully utilized in identifying somaclonal variants of *P. longum* L[13].

Majority of black pepper cultivars are bisexual type but some are predominantly female. Controlled pollination studies indicated that 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[3]. A banding pattern exactly similar to the female parent was observed in some populations derived from certain crosses, which also indicates 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.

The technique can be adopted for large-scale screening of hybrids, but black pepper being heterozygous, markers diagnostic of each male parent are to be determined for each cross. Confirmation of the hybrid nature of seedlings at the juvenile stage by screening with RAPD markers would be practical and of economic significance in a perennial crop like black pepper. It will enable elimination of all doubtful

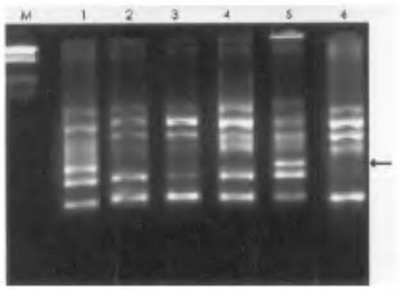

Figure 1. RAPD molecular marker profile generated with OPE 01 primer. Lane M, λDNA–*Eco*R1/*Hin*dIII double digest; lane 1, IISR4182 ( ); lanes, 2–5, Seedlings derived from cross (HP813 × IISR4182); lane 6, HP813 ( ), Arrow indicates male parent-specific band.

seedlings and can save labour, space and cost. The RAPD analysis can also be applied to a broad array of cultivars and wild accessions to get a more accurate picture of the genetic diversity within the genus *Piper*.

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Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate overt negative effects[1]. They are a largely unexplored component of biodiversity, especially in the tropics. Endophytic fungi have been isolated from leaves, stems and roots of woody plants in the temperate regions and the tropics[2–4]. They have a protective role against insect herbivory and many are potential producers of novel antimicrobial secondary metabolites[5]. Endophytes are constantly exposed to intergeneric–genetic exchange with the host plant. Isolation of a potent anticancer agent, taxol from *Pestalotiopsis microspora*, an endophyte of the yew tree and the phytohormone-producing fungus from rice plant, *Gibberella fujikuroi* suggests the potential of endophytes as a source of useful metabolites[6,7].

The current study was carried out to isolate and identify fungal endophytes from living symptomless inner bark tissues of neem (*Azadirachta indica* A. Juss), which is an indigenous medicinal plant in India and Africa. Neem is an evergreen tree of the tropics and sub-tropics belonging to the family Meliaceae. It is widely used in Indian traditional medicine for various therapeutic purposes as well as the source of agrochemicals for many centuries. The bark extract has been scientifically investigated from the past two decades for anti-bacterial, antipyretic[8,9], anti-inflammatory[10] effects and against skin diseases such as eczema, burns, ulcers, herpes, etc.[11]. Based on the recent claims that endophytic microbes may play a key role in therapeutic properties of plants, we postulate that the healing properties may be due to the secretion of metabolites from the endophytes residing in the bark.

Bark samples from a neem tree growing in Mysore were obtained by cutting the tree bark at 1.5 m above the ground level and 1–1.5 cm depth with ethanol-disinfected machete. Approximately 5 × 5 cm bark pieces were taken for the study. The samples were processed within 24 h of collection. Surface sterilization of bark sample was done by immersing the bark pieces in 70% (v/v) ethyl alcohol for 1 min and 3.5% (v/v) sodium hypochlorite for 2 min and rinsed three times in sterile distilled water for 1 min[12]. Excess water was blotted in an airflow chamber. The outer bark was removed and the inner portion containing the cortex was carefully dissected into bits (1.0 × 0.2 cm). 200 segments were plated on water agar medium (15 g l^{-1}) amended with streptomycin (100 mg l^{-1}) and incubated in a chamber for 21days at 12 h light/dark cycles at 22°C[13]. The plates were monitored regularly for the growth of endophytic fungi. The hyphal tips that grew on surface-sterilized bark pieces were isolated onto potato dextrose agar (PDA). Each fungus was assigned a number and stored at 4°C. Endophytic fungal strains were identified based on morphological characters using standard identification manuals. All the endophytic isolates were documented, maintained in cryovials on PDA layered with 15% glycerol (v/v) and stored in –80°C freezer (Cryo Scientific Pvt Ltd, Chennai) at the Department of Applied Botany and Biotechnology, University of Mysore.

The per cent frequency of occurrence[14] was calculated as the number of bark segments colonized by a specific fungus divided by total number of segments plated × 100 and dominant endophytes[15] were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes × 100.

A total of 77 endophytic fungal isolates belonging to 15 genera were isolated from the inner bark of *A. indica*. The colonization frequency was 38.5% (Table 1). The fungal composition included 71.4% of hyphomycetes, 18.2% of coelomycetes, 6.5% of ascomycetes and 3.9% of sterile mycelia.

In the tropics, only a few studies have been carried out on endophytes of tree species[16]. Rajagopal and Suryanarayanan[17] have investigated the endophytic fungi in the leaves of *A. indica*. These studies have shown the effect of leaf tissue type, site and seasonality on endophyte assemblages and colonization. They recorded only *Fusarium* spp. and some sterile fungi. We have recovered endophytic genera like