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Molecular and morphological characterization of new promising black pepper (*Piper nigrum* L.) lines

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Abstract

Seven high yielding, new, promising lines of black pepper (*Piper nigrum*) were characterized using molecular markers (randomly amplified polymorphic DNA) and morphological features. Out of the 14 random decamer primers studied, 9 could generate unique bands in 6 lines. Maximum unique bands of the primers were observed in the line OPKm followed by HP-1411 and HP-105. HP-780 could not be discriminated by any of the primers. Sixteen unique bands were produced by the nine primers making an average of 1.7 bands per primer. The lines OPKm, HP-1411 and HP-105 exhibited distinct morphological features also.

Key words: black pepper, characterization, *Piper nigrum*, randomly amplified polymorphic DNA.

Introduction

Characterization of elite lines and varieties of high-value crops like spices is important for protection of biowealth in the present WTO era. Conventionally, characterization of biodiversity has been done using morphological features, and visually scorable morphological markers that correspond to quantitative traits are used for morphological characterization. Black pepper accessions and varieties have been traditionally classified based on plant characters such as leaf length and breadth, shoot tip colour, leaf shape and size, features of leaf tip and base, berry size, spike length, spike composition (bisexual, female and male), fruit set, number of fruits spike^{-1}, 1000 fruit volume, 1000 fruit weight, yield vine^{-1} and dry recovery, besides quality characters such as piperine, oleoresin and essential oil (Ratnambal *et al.*1985; Pillai *et al.* 1987; Ravindran *et al.* 1992; Ravindran & Sasikumar 1993; Sasikumar *et al.* 1999; Mathew *et al.* 2001). However, with the advent of biotechnology, molecular markers such as randomly amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), etc., are also being used to describe black pepper accessions and varieties (Kumar *et al.* 2001, 2003; Babu *et al.* 2003; George *et al.* 2003). Molecular markers in conjunction with morphological markers will be the ideal method to characterize any line or variety. The present work is an attempt to characterize seven newly developed promising black pepper lines based on molecular and morphological features.

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Materials and methods

The study was conducted at the Genetic Resources and Molecular Breeding Laboratory, Indian Institute of Spices Research, Calicut. The experimental material comprised of seven promising black pepper lines namely, HP-105, HP-728, HP-780, HP-813, Coll. 1041, HP-1411 and OP Karimunda (OPKm) (Table 1).

Fresh, tender, fully opened leaves of the seven promising lines of black pepper were collected from the Experimental Farm of Indian Institute of Spices Research at Peruvannamuzhi (Kerala). The genomic DNA was isolated by cTAB method (Doyle & Doyle 1987) and amplified with 14 random decamer primers (Table 2). RAPD reaction was carried out in 25 µl volume containing 25 ng genomic DNA, 1 U Taq DNA polymerase (Biogene, USA), 200 µM dNTPs, 2 mM $MgCl_2$ and 10 pico moles of random decamer primer according to Williams *et al.* (1990). Amplification condition consisted of pre-denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 37° C for 1 min, extension at 72°C for 1 min and final extension at 72° C for 10 min; number of cycles was 35. The amplified products were visualized in a 2% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and documented by a gel documentation system (Alpha Imager 2200, USA). The bands were scored based on the molecular weight marker (Eco RI/Hind III double digest).

The various morphological characters of the black pepper lines were recorded using the black pepper descriptor (IPGRI 1995). The morphological characters recorded were lateral branch habit, lateral branch length, number of nodes lateral $branch^{-1}$, leaf petiole length, leaf length, leaf width, leaf lamina shape, leaf base shape, spike length, peduncle length, number of male, female and bisexual flowers, number of berries $spike^{-1}$, fruit set percentage, threshing percentage, fresh weight and dry weight of berries and berry size. These observations were recorded from 5 year old vines.

Table 2. Sequences of the random decamer primers used for molecular characterization of black pepper lines

Primer	Sequence 5′-3′
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-17	GACCGCTTGT
OPC-07	GTCCCGACGA
OPC-09	CTCACCGTCC
OPC-13	AAGCCTCGTC
OPE-05	TCAGGGAGGT
OPE-06	AAGACCCCTC
OPE-18	GGACTGCAGA
OPE-20	AACGGTGACC

Table 1. Black pepper lines utilized for molecular and morphological characterization

Line	Remarks
HP-105*	Hybrid, suited to high altitude areas
HP-728	Hybrid, early maturing, high yielding
HP-780	Hybrid, high dry recovery, high yielding
HP-813*	Hybrid, high quality (oleoresin)
Coll. 1041*	Selection from cultivar, high yielding, tolerant to foot rot disease, suited to high altitude areas
HP-1411	Hybrid, high yielding
OP Karimunda	Open pollinated progeny of Karimunda, high yielding, tolerant to drought

*Proposed for release

Results and discussion

Molecular characterization

The number of bands produced by each primer in different black pepper lines and their size range are given in Table 4. The mean number of amplified products per primer ranged from 2.9 (OPA-13) to 6.4 (OPA-17) with a molecular weight of 292 to 2415 bp (OPA-17) (Table 3 & Fig. 1a–d).

Among the 14 primers utilized, only 9 produced unique bands in 6 of the black pepper lines studied. No primers produced unique bands in the line HP-780. A total of 16 unique

Table 3. Mean number of amplified products and their size generated by different primers in black pepper lines

Primer	Sequence-5′-3′	Mean no. of amplified products	Size range of amplified products (bp)
OPA-02	TGCCGAGCTG	5.1	426-1584
OPA-03	AGTCAGCCAC	3.0	398-808
OPA-05	AGGGGTCTTG	4.1	564-1041
OPA-06	GGTCCCTGAC	3.3	528-1725
OPA-07	GAAACGGGTG	5.1	482-1326
OPA-08	GTGACGTAGG	4.3	473-1360
OPA-17	GACCGCTTGT	6.4	292-2415
OPC-07	GTCCCGACGA	4.0	477-1307
OPC-09	CTCACCGTCC	3.9	583-1442
OPC-13	AAGCCTCGTC	2.9	497-1128
OPE-05	TCAGGGAGGT	5.0	408-1091
OPE-06	AAGACCCCTC	3.6	426-1293
OPE-18	GGACTGCAGA	5.8	462-1173
OPE-20	AACGGTGACC	3.4	447-778

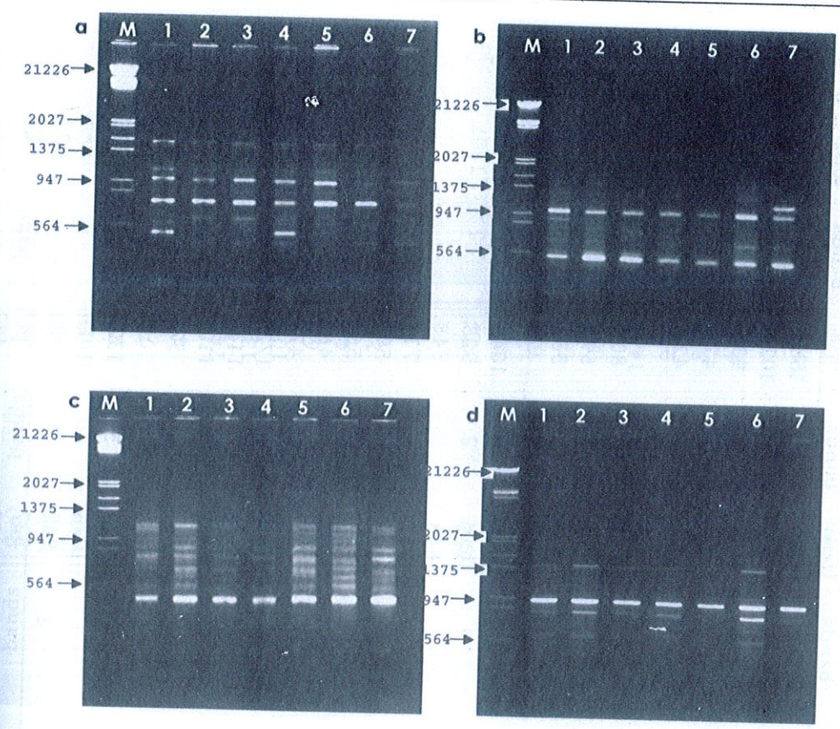

Fig. 1. RAPD profile of seven promising black pepper lines using primers (a) OPA 17 (b) OPA 06 (c) OPE 05 (d) OPC 09. M-Marker Eco RI/Hind III double digest, 1-HP 105, 2-HP 728, 3-HP 780, 4-HP 813, 5-Coll. 1041, 6-HP 1411, 7-OP Karimunda

Table 4. Number of bands produced by different primers in black pepper lines

Primer	HP-105		HP-728		HP-780		HP-813		Coll. 1041		HP-1411		OPKm	
	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)
OPA-02	5	1584	5	1584	5	1584	6	1584	5	1584	5	1584	5	1584
		1250		1026		1250		1026		1250		1250		1250
		936		936		936		936		936		936		936
		477		477		477		477		477		477		477
		426		426		426		473		426		426		426
								426						
OPA-03	2	708	3	808	3	808	3	808	2	694	4	808	4	808
		533		694		694		694		398		694		694
				533		398		398				533		533
												398		398
OPA-05	7	1041	8	1041	0	0	4	848	0	0	7	1041	3	848
		923		923				717				923		717
		848		848				599				848		471
		717		717				471				717		
		599		599								599		
		564		564								465		
		471		465								471		
				471										
OPA-06	5	1725	4	1725	4	1725	2	1102	2	1102	3	1102	3	1228
		1445		1445		1445		529		529		657		1102
		1102		1102		1102						729		529
		949		573		573								
		528												
OPA-07	5	1326	5	1326	5	1326	5	1326	6	1326	5	1326	5	1326
		1073		1073		1073		1073		1073		1073		1073
		922		922		922		922		922		922		922
		527		527		527		527		672		527		527
		482		482		482		482		527		482		482
										482				

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Table 4. Continued from previous page

Primer	HP-105		HP-728		HP-780		HP-813		Coll. 1041		HP-1411		OPKm	
	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)
OPA-08	5	1360	5	1360	5	1360	4	1099	1	473	5	1360	5	1360
		1099		1099		1099		717				1099		1099
		717		772		717		648				754		717
		648		717		648		473				717		600
		473		473		473						473		473
OPA-17	6	2415	6	2415	6	2415	7	2415	8	2415	7	2415	5	1094
		1375		1375		1375		1375		1375		1375		1333
		1094		1094		1094		1094		1094		1094		680
		680		680		680		680		680		680		487
		430		487		487		487		487		487		292
		348		292		292		367		438		438		
								292		367		292		
										292				
OPC-07	5	1307	5	1307	5	1307	3	762	1	477	5	1307	4	1307
		762		762		762		534				762		762
		671		671		671		477				671		671
		534		590		590						534		477
		477		477		477						477		
OPC-09	4	1442	4	1442	4	1442	4	1442	1	956	5	1442	4	1442
		956		956		956		956				966		966
		823		823		823		823				956		956
		618		618		618		618				823		823
												583		583
OPC-13	3	1128	3	1128	3	1128	1	772	1	772	4	1128	4	772
		950		950		950						950		627
		772		772		772						627		555
												772		497

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Table 4. Continued from previous page

Primer	HP-105		HP-728		HP-813		Coll. 1041		HP-1411		HP-780		OPKm	
	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)	No. of bands	Size (bp)
OPE-05	7	1091	3	1009	5	1009	5	1009	7	1009	3	1009	4	1009
		1009		939		939		939		939		939		769
		939		769		769		769		769		769		633
		769				452		452		633		633		501
		633				408		408		501		501		408
		501								452		452		
		452								408		408		
OPE-06	4	1293	4	1293	1	875	4	1293	4	1293	4	1293	1	
		875		875				875		875		875		
		620		620				620		620		620		
		426		426				426		426		426		
OPE-18	5	1173	6	1173	4	1173	6	1173	8	1173	6	1173	4	
		977		977		977		977		1079		977		
		787		787		787		787		1010		787		
		666		666		666		666		977		666		
		536		536		536		536		787		536		
		462		462		462		462		666		462		
OPE-20	4	778	4	778	2	812	4	778	4	778	4	778	2	812
		667		667		572		667		667		667		572
		564		564				564		564		564		
		477		477				477		477		477		

bands were produced by 9 of the primers studied making an average of 1.7 bands per primer. Out of the 14 primers studied, the primers that discriminated the different black pepper lines are given in the Table 5. Maximum number (three) of unique bands were produced by OPA-06 and OPA-08. The primer OPA-06 produced unique bands of size OPA-$06_{(1228)}$ in OPKm while the primer OPA-08 produced three bands i.e., OPA-$08_{(600)}$ in OPKm, OPA-$08_{(754)}$ in HP-1411 and OPA-$08_{(772)}$ in HP-728. The primer OPA-17 produced one unique band each of size OPA-$17_{(430)}$ in HP-105, and OPA-$17_{(1333)}$ in OPKm. OPE-18 also produced two bands of sizes OPE-$18_{(1079)}$ and OPE-$18_{(1010)}$ in HP-1411. Rest of the primers produced only one unique band per line such as OPA-$02_{(473)}$ in HP-813, OPA-$03_{(708)}$ in HP-105, OPA-$07_{(672)}$ in Coll. 1041 and OPE-$05_{(1091)}$ in HP-105. Thus, all the black pepper lines except HP-780, were clearly discriminated by the different primers with a maximum number of unique bands in OPKm (5 unique bands) followed by HP-1411 and HP-105 (4 unique bands), whereas among all the primers, OPA-06 and OPA-08 were useful in discriminating maximum number of lines. Kumar *et al.* (2001) discriminated the land races and cultivars of black pepper using random decamer primers (RAPD). In their study, 24 primers generated 372 RAPD markers of which 367 were polymorphic. Genetic proximity among the cultivars could be related to their phenotypic features or geographical distribution. They could observe greater divergence among the land races as compared to the advanced varieties.

Morphological characterization

The morphological features of black pepper lines studied are presented in Table 6. Among the lines studied, OPKm was unique with maximum leaf length (16.9 cm), long peduncle length (1.3 cm), absence of male flowers, maximum percentage of female flowers (6.8%), long spike length (16.6 cm), highest threshing percentage (93.4%), highest mean number of berries spike^{-1} (64.9), highest fruit set percentage (83.4%), and percentage of bold berries (67.2%). The distinct morphological markers and the number of unique bands observed in different lines are given in Table 7.

In the present study, maximum distinct morphological features were observed in OPKm, followed by HP-1411 and HP-105. Interestingly, these lines also exhibited more num-

Table 5. Discriminatory primers and unique bands specific to different black pepper lines

Line	Discriminatory primers	No. of unique bands	Size (bp)
HP-105	OPA-03, OPA-06, OPA-17, OPE-05	4	OPA-$03_{(708)}$ OPA-$06_{(949)}$ OPA-$17_{(430)}$ OPE-$05_{(1091)}$
HP-728	OPA-08	1	OPA-$08_{(772)}$
HP-780	0	0	0
HP-813	OPA-02	1	OPA-$02_{(473)}$
Coll.1041	OPA-07	1	OPA-$07_{(672)}$
HP-1411	OPA-06, OPA-08, OPE-18	4	OPA-$06_{(657)}$ OPA-$08_{(754)}$ OPE-$18_{(1079)}$ OPE-$18_{(1010)}$
OPKm	OPA-06, OPA-08, OPA-17, OPC-13	5	OPA-$06_{(1228)}$ OPA-$08_{(600)}$ OPA-$17_{(1333)}$ OPC-$13_{(555)}$ OPC-$13_{(497)}$

Table 6. Morphological features of black pepper lines

Morphological/ metric trait	HP-105	HP-728	HP-780	HP-813	Coll.1041	HP-1411	OPKm
Lateral branch habit	Erect	Erect	Erect	Erect	Erect	Erect	Hanging
Length of lateral branch (cm) (n=5)	55.6	65.0	58.0	60.4	33.4	63.0	63.3
No. of nodes lateral branch^{-1} (n=5)	44	21	30	29	29	24	28
Leaf petiole length (cm) (n=5)	1.4	2.5	2.4	1.2	1.2	1.7	2.5
Leaf length (cm)	12.9	13.3	14.0	15.4	14.2	15.6	16.9
Leaf width (cm) (n=5)	8.6	5.7	7.6	8.0	7.0	10.5	10.0
Leaf lamina shape	Ovate-elliptic	Ovate-lanceolate	Ovate-cordate	Elliptic-lanceolate	Ovate-elliptic	Ovate-cordate	Ovate-lanceolate
Leaf base shape	Round	Slightly acute	Round	Acute	Round	Acute	Round
Spike length (cm) (n=10)	9.2	9.1	11.0	8.8	8.2	12.0	16.6
Length of peduncle (cm) (n=5)	1.1	1.2	0.97	1.0	1.1	1.2	1.3
Percentage of male flowers spike^{-1} (n=10)	3.4	0.8	4.5	0.6	1.0	13.9	0
Percentage of female flowers spike^{-1} (n=10)	3.3	1.3	5.5	2.5	2.1	4.7	6.8
Percentage of bisexual flowers spike^{-1} (n=10)	94.3	98.0	90.0	96.8	96.9	81.3	93.2
No. of berries spike^{-1} (n=10)	50.9	53.5	46.4	40.3	31.1	30.3	64.9
Fruit set percentage	80.0	84.0	70.3	70.0	80.0	44.4	83.4
Threshing percentage	-	-	92.3	90.7	91.0	85.9	93.4
Fresh wt. of 100 berries (g)	11.2	-	16.6	10.3	15.6	14.6	12.9
Dry wt. of 100 berries (g)	4.00	-	7.0	5.0	5.0	5.8	6.8
Berry size - Above 3 mm (%)	-	-	36.8	65.1	43.0	28.4	31.9
Berry size - Above 4.75 mm (%)	-	-	60.6	28.6	42.0	63.7	67.2

Table 7. Distinct morphological markers and number of unique bands observed in different black pepper lines

Line	Distinct morphological features	No. of unique bands
OPKm	Leaf length Peduncle length Percentage of male flowers Percentage of female flowers Spike length Threshing percentage Mean number of berries spike^{-1}	5
HP-1411	Fruit set percentage Leaf length Leaf width Mean number of male flowers Threshing percentage	4
HP-105	Leaf petiole length Leaf length Percentage of male flowers spike^{-1} Percentage of bisexual flowers spike^{-1}	4
HP-728	Spike length Fruit set percentage Percentage of bisexual flowers spike^{-1}	1
HP-813	Percentage of female flowers spike^{-1} Percentage of bisexual flowers spike^{-1} Fruit set percentage	1
Coll.1041	Percentage of bisexual flowers spike^{-1} Threshing percentage Dry weight of berries	1

ber of unique RAPD bands may not be exactly corresponding to the distinct morphological features of the lines, it is supportive to the distinct identity of the lines from other lines, implying the usefulness of RAPD markers in characterizing lines and varieties of black pepper.

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