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Interspecific hybridization in vanilla and molecular characterization of hybrids and selfed progenies using RAPD and AFLP markers

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Abstract

Vanilla, *Vanilla planifolia* Andrews, is native to Mexico and Central America, but is now cultivated in other parts of the tropics. Continuous clonal propagation has resulted in very little variability for crop improvement programmes in vanilla. In this study, an attempt has been made to increase the spectrum of variation by interspecific hybridization with *Vanilla aphylla*, an Indian species which is tolerant to *Fusarium*. Interspecific hybrids were successfully produced and morphological characters and molecular profiles revealed the true hybridity of the progenies. Ten seedling progenies of *V. planifolia*, and four interspecific hybrids obtained from crosses between *V. planifolia* (female) and *V. aphylla* (male) using a number of different loci as markers were evaluated and 319 amplified fragment length polymorphisms (AFLPs) and 83 random amplified polymorphic DNAs (RAPDs) loci were marked. The profiles indicate similarity between the parents, selfed progenies and interspecific hybrids and that all the progenies tested were variable when compared to each other, which can be exploited for crop improvement in vanilla. This is the first report in vanilla, indicating that RAPD and AFLP profiles coupled with morphological characters can be utilized to assess the variability and hybrid nature of genotypes and of successful interspecific hybridization and production of hybrids between *V. planifolia* and *V. aphylla*.

Keywords: Interspecific hybridization; Molecular characterization; *Vanilla aphylla*; *V. planifolia*

1. Introduction

Cultivated Vanilla, *Vanilla planifolia* Andrews, (Syn. *Vanilla fragrans* Salisb.) Ames belongs to the family Orchidaceae. Native to Mexico and Central America, but now cultivated in other parts of the tropics too, it is now considered the aroma of the planet (Anon., 2000). It is an introduced crop in most of the countries of cultivation, but since much of the planting material originated from limited clonal propagation, practically no variability is available for crop improvement. This leads to monoculture making vanilla susceptible to diseases and pests (Purseglove et al., 1981). The leafless species of vanilla viz., *Vanilla aphylla*, *V. wightiana* and *V. walkerie* also occur in India (Hooker, 1973). The cultivation of vanilla is threatened by root rot caused *by Fusarium batatis* var. *vanillae* Tucker and anthracnose caused by *Calospora vanillae* and breeding programmes are greatly hampered by the lack of variability in the germplasm. More recently *Phytophthora* infection has also been reported (Joseph and Bhai, 2001). *V. aphylla*, a leafless Indian species was found tolerant to *Fusarium* (Minoo, 2002). Attempts have been made to increase the variability by successfully generating segregating progenies and interspecific hybridization using Central American *V. planifolia* (♀) and *V. aphylla* (♂) to produce interspecific hybrids. Comparative studies were made between *V. planifolia* and its selfed progenies with that of the interspecific hybrids. This study evaluates these interspecific hybrids to estimate and confirm the extent of hybridity using both morphological as well as molecular characterization. Vanilla being perennial, phenotypic evaluation takes a long time to draw useful conclusions.

Abbreviations: AFLP, amplified fragment length polymorphism; BAP, benzyl aminopurine; DNA, de-oxy ribonucleic acid; dNTPs, di-nucleotide triphosphates; IBA, Indole-3-butyric acid; MS, Murashige and Skoog; NAA, napthalene acetic acid; RAPD, randomly amplified polymorphic DNA

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2. Materials and methods

2.1. Materials

The genotypes used in the present study included two species of Vanilla viz., *V. planifolia* (V.p) and *V. aphylla* (V.a), seedling progenies of *V. planifolia* viz., V1, V2, V4, V6, V7, V8, V10, V11, V12, V24 and interspecific hybrids produced between the two species viz., VH1, VH4, VH5, VH6.

2.2. Production of seedling progenies and interspecific hybrids

V. planifolia flowers were hand pollinated by raising the rostellum and pressing the pollinial mass onto the receptive stigma facilitating passage of the pollen mass into the style. Seeds from such pods pollinated were cultured in vitro to generate seedling progenies.

Desired *V. planifolia* flowers were emasculated early in the morning. Pollen mass (pollinia) collected from freshly opened flowers of *V. aphylla* (endangered species) was used to hand pollinate *V. planifolia* flowers by inserting in to the column of the female flower (*V. planifolia* vine).

Pods were harvested 7 months after pollination and surface sterilised under aseptic conditions by 95% ethanol dip and subsequent flaming. This was repeated twice. The capsules were then split open and the seeds transferred to sterilised nutrient medium.

2.3. Culture media and conditions

Murashige and Skoog (1962) medium (MS) fortified with 2% sucrose and gelled with 0.65% agar was used as basal medium. Growth regulators; cytokinins (BAP and Kinetin) and auxins (IBA and NAA) were supplemented to the basal medium singly or in combination (0.5 and 1.0 mg l^{-1}) at two levels. MS medium supplemented with 0.5 mg l^{-1} kinetin was used for seed germination. The germinating seeds were transferred to different combinations of MS medium supplemented with cytokinins (BAP and Kinetin) and auxins (IBA and NAA) at the concentration of 0.5–1.0 mg l^{-1} fortified with 3% sucrose and 0.7% agar as a gelling agent to select out the best suitable combination for multiplication.

The pH of the culture medium was adjusted to 5.8 in all cases prior to autoclaving at 15 lbs pressure and 120 °C temperature for 20 min. All cultures were incubated at 22 ± 2 °C with a photoperiod of 14 h and a light intensity of 3000 lx, provided by 'Philips' cool white fluorescent tubes.

2.4. Hardening and planting out

Plantlets, developed in vitro, with good roots were taken out and washed carefully for removing the traces of medium sticking to the roots. They were then dipped in 0.3% Dithane-M45 for 5–10 min and transplanted in polybags containing a mixture of garden soil, sand and vermiculite in equal proportions. The transplanted plantlets were kept in humid chamber for 3–4 weeks for hardening and establishment. Forty plantlets each of the different genotypes were multiplied in vitro for different studies. The plantlets were kept in the nursery for the first year, and transferred to earthen pots (12 in. diameter), the subsequent years, for evaluation.

2.5. Characterization of interspecific hybrids

Morphological data on plant type, leaf and stem characters were recorded.

CTAB method (Ausubel et al., 1995) was used to isolate high molecular weight DNA from all the genotypes. RAPD and AFLP protocols were standardized (Williams et al., 1990; Vos et al., 1995) and profiles were developed.

2.5.1. Developing RAPD profiles

2.5.1.1. PCR reaction components. The reaction mixture 20 μl was prepared with, Sterile distilled water: 9.5 μl; 10× PCR buffer: 2 μl; dNTPs (1.25 mM): 3 μl; Primer (5 pmoles (l^{-1}): 2 μl; $MgCl_2$ (10 mM): 2 μl; Taq polymerase (3 U μl^{-1}): 0.5 (l; Template DNA (ng μl^{-1}): 1 μl.

Cycle 1	94 °C: 2 m	40 °C: 1 m	72 °C: 1 m	Repeat 1
Cycle 2	94 °C: 1 m	40 °C: 30 s	72 °C: 1 m	Repeat 30
Cycle 3	94 °C: 1 m	40 °C: m	72 °C: 15 m	Repeat 1

2.5.1.2. Primer screening. RAPD profiles were developed as per the method suggested by Williams et al. (1990) with minor modifications. The dNTPs, Taq polymerases and other chemicals were procured from Amersham Pharmacia Biotech, Sweden. Sixteen arbitrary primers with 60–70% GC content and no self-complementary ends from Operon Technologies Inc. Alameda, California were used for PCR reaction. They are OPA 04, OPA 10, OPA 20, OPB 02, OPB 10, OPB 20, OPB 14, OPC 09, OPC 19, OPD 03, OPD 19, OPE 05, OPE 09, OPE 14, OPF 03 and OPF 12.

2.5.2. Developing AFLP profiles

AFLP profiles were developed as per the method suggested by Vos et al. (1995). The primers and stock solutions used are as follows.

2.5.2.1. Primers and corresponding adaptors. Both primers and adapters were obtained from Life Technologies, USA. *Eco*RI and *Mse*I adapters were used at 5 and 50 pmoles μl^{-1} concentration, respectively. *Eco*RI and *Mse*I primers were used at 50 ng μl^{-1} concentration. Four primer combinations EAC-MTG, ETG-MAC, EGG-MTG and EGG-MGC were used for developing AFLP profiles.

2.5.2.2. Amplification. Primers were labeled for selective AFLP amplification by phosphorylating the 5′ end of the primers with gamma-33P-ATP and polynucleotide kinase. Amplification was done in a thermocycler with the following cycle regime, a first cycle of 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C, followed by 12 cycles with a stepwise decrease of the annealing temperature in each subsequent cycle by 0.7 °C, and

23 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. The reaction was started at a high annealing temperature to obtain optimal primer selectivity. In the following steps the annealing temperature is lowered gradually to a temperature for optimal primer annealing.

Amplification products were analyzed on 5% denaturing polyacrylamide-sequencing gels at 110 W and TBE (1×) was used as running buffer. Autoradiographic exposure of the 33^{P}-gels to standard X-ray film for 2–3 days with intensifying screens gave good autoradiogrammes.

2.6. Analysis of data

The presence and absence of bands were scored, similarity index estimated and dendrograms were drawn using NTSyS-pc Version 2.0 (Rohlf, 1997) softwares. Similarity/differences between the genotypes were estimated as Paired Affinity Indices (PAI) and expressed as percentage.

$$\text{PAI} = \frac{\text{No. of similar bands} \times 100}{\text{Total no. of bands}}$$

Bootstrap analysis was conducted using WinBoot (Yap and Nelson, 1996) which provides a method for determining (and comparing) confidence intervals in these cases. Dice's coefficient was used for determining bootstrap values.

3. Results

3.1. Interspecific hybridization between V. planifolia and V. aphylla

Interspecific hybridization was attempted between *V. planifolia* and its related species to increase the spectrum of variations by bringing desirable characters from wild species into the cultivated vanilla. Pollinia from *V. aphylla* flowers (Fig. 1e) were used to pollinate *V. planifolia* flowers (Fig. 1d). Eleven crosses were made, but after 4 days, perianth of six of the flowers fell off indicative of unsuccessful pollination. The remaining five fruits were left to mature (Fig. 1f) and harvested at different maturity periods to study germination of the 'hybrid' embryos.

3.2. Culture of seeds and development of plantlets

Though approximately 500 'hybrid seeds' obtained from *V. planifolia* (♀) and *V. aphylla* (♂), were cultured in vitro, only 20 germinated. Seventy percent were albinos and could not develop further. Only six seeds germinated of which two were albinos and did not survive. The remaining four expressed characters segregating between the two parents and were named VH1, VH4, VH5 and VH6.

3.3. Morphological similarities of hybrids with parents

The distinctive features of *V. planifolia* and *V. aphylla* and the variation exhibited by their interspecific progenies are given (Tables 1 and 2).

These progenies were indexed cytologically and using molecular markers. *V. aphylla* is a wild leafless form while *V. planifolia* is a cultivated plain leaved type (Fig. 1a–c). The progenies showed segregation of leaf characters. VH4 and VH5 express partial albinism (light green plants) and are 'aphylla' types, leafless in nature (Fig. 1g), i.e., morphologically similar to male parent (*V. aphylla*) while the other two, VH1 and VH6, were 'planifolia' type normal green plants with leaves, resembling female parent (*V. planifolia*)

Table 1
Morphological characters of interspecific hybrids between *Vanilla planifolia* and *V. aphylla*, at the time of hardening

No.	Interspecific parents/hybrids	Leaf	Plant height (cm)	Internodal length (cm)	Leaf size	
					Length (cm)	Breadth (cm)
1	*V. planifolia*	Subsessile	7.00	1.36	2.36	1.28
2	*V. aphylla*	Leafless	6.62	1.53	0.30[a]	0.30[a]
3	VH1	Vp	5.85	1.59	2.25	1.12
4	VH4	Va	8.57	1.61	0.6[a]	0.3[a]
5	VH5	Va	9.25	1.50	0.5[a]	0.2[a]
6	VH6	Vp	4.26	1.40	3.05	1.65

[a] Scale leaves.

Table 2
Important fruit characters of *V. planifolia* and *V. aphylla* and their hybrids

No.	Characters	*V. planifolia*	*V. aphylla*	Interspecific hybrids
1	Fruit set (%)	95	90	80
2	Fruit size (cm)	12–15	8–10	9
3	Maturity time (months)	6–9	6–9	[a]
4	Germination rate (%)	87	80	0.2
5	Multiplication rate (seed)/60 days	1:9	1:9	1:6
6	Development of chlorophyll	Normal	Normal	50%-Achlorophyllous
7	Chromosome number[b]	32	64	18–58

[a] Could not be studied.
[b] Most commonly observed '2*n*' number.

Fig. 1. Interspecific hybridization in *Vanilla*. (a) *Vanilla planifolia* in flower, (b) *V. planifolia* and *V. aphylla* plants, (c) *V. aphylla* in flower, (d) *V. planifolia* flower – close-up view, (e) *V. aphylla* floral parts, (f) fruit set after interspecific hybridization, (g) an interspecific hybrid showing male plant type and (h) interspecific hybrids (VH1, VH4, VH5, VH6) in comparison with female and male parents (Vp, Va).

in leaf characters (Fig. 1h). The presence of characters of pollen donor parent indicates that the progenies are hybrids and not accidental selfed progenies of *V. planifolia*. Comparison of plant characters among interspecific hybrids between *V. planifolia* and *V. aphylla* along with their parents at the time of hardening are given in the Table 2.

3.4. Molecular characterization of progenies and hybrids

3.4.1. RAPD profiles

RAPD profiles were developed for *V. planifolia*, *V. aphylla*, interspecific hybrids and seven selfed progenies of *V. planifolia*. The presence and absence of bands were scored, Paired Similarity Indices were estimated and the dendrogram was drawn (Fig. 2).

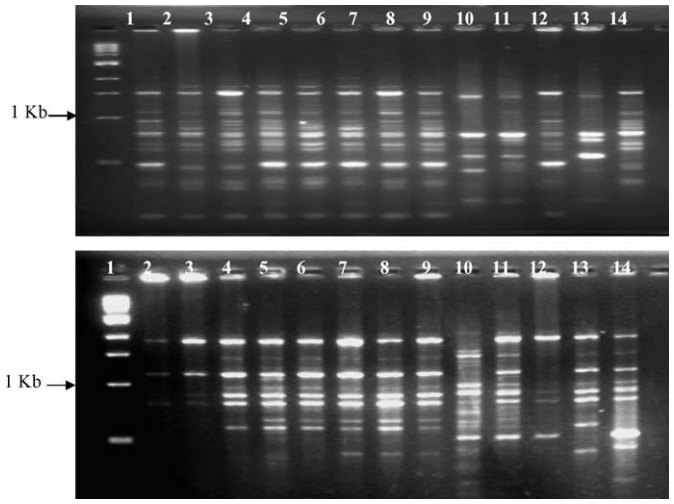

Fig. 2. RAPD profiles of selfed and interspecific hybrids of vanilla using operon primer. Upper panel, OPA 10; lower panel, OPD 03, *V. planifolia* and *V. aphylla*. 1, 1 kb ladder; 2, V1; 3, V2; 4, V4; 5, V6; 6, V7; 7, V8; 8, V10; 9, *V. planifolia*; 10, *V. aphylla*; 11, VH1; 12, VH4; 13, VH5; 14, VH6.

The four interspecific hybrids displayed banding pattern intermediate to *V. planifolia* and *V. aphylla*. The similarity expressed by interspecific hybrids VH1, VH4, VH5 and VH6 ranged from 58.5 to 68.3% to female parent *V. planifolia* and 50.0–72.0% to *V. aphylla*. Of the four, VH1 and VH5 clustered along with *V. aphylla* indicating their similarity, while VH4 and VH6 clustered along with *V. planifolia*. Morphologically VH1 and VH6 are *V. planifolia* types and VH4 and VH5 are *V. aphylla* types, with regard to leaf nature. The RAPD profiles showed banding pattern differences among the selfed progenies of *V. planifolia* indicating genetic variability among them (Fig. 2). Thus the RAPD profiles coupled with morphological characters indicate the true hybrid nature of these four genotypes and there is considerable variation among the selfed progenies of *V. planifolia*.

The selfed progenies of vanilla are more similar to their parent *V. planifolia* and to each other. The percentage similarity ranged from 35.4 to 95.1%. Among progenies V4 and V6 are nearest to each other with 95.1% similarity. Among the progenies, V1 is farthest (82.9%), while V8 is the nearest to *V. planifolia*, with 92.7% similarity and V10 is in general least similar to other selfed progenies. Studies confirm that *V. aphylla* is the farthest from *V. planifolia* with 42.7% similarity. When the selfed progenies of *V. planifolia* are compared with their parents, the % of similarity ranged from 75.6 to 95.1% in *V. planifolia* (Table 5) whereas all the progenies were less than 47% similar to *V. aphylla*. The present study thus indicated a good amount of variation among the selfed progenies of *V. planifolia* as expressed by RAPD polymorphisms.

The interspecific hybrids expressed differences in grouping, in the molecular profiles when compared to the morphology. Though VH4 and VH5 were morphologically similar to *V. aphylla*, RAPD profiles showed that they were more similar to VH1 (68.3 and 69.5%) and only 52.4% similar to each other. VH5 was the least similar to *V. aphylla* (50%) in its RAPD profile. The percentage similarity expressed by the hybrids to both the parents indicated their hybrid nature.

3.5. Amplified fragment length polymorphism (AFLP) profiles

The AFLP profiles were developed for the first time in *V. planifolia*, *V. aphylla*, interspecific hybrids and a few selfed progenies of *V. planifolia*, using four different primer combinations (Fig. 3). The profiles of *V. planifolia* and *V. aphylla* indicated that they are widely distant and banding patterns consistent with the species indicating species-specific bands were observed. The selfed progenies of *V. planifolia* showed the banding pattern similar to that of *V. planifolia* parent and did not show the species-specific bands of *V. aphylla*. These progenies showed variation in banding pattern within them indicating that there is considerable variation among the selfed progenies. AFLP profiles also indicated V10 to be least similar to other selfed progenies. Interspecific hybrids showed the banding patterns inbetween the parents in that segregation of species-specific bands were noticed among the progenies further confirming hybrid nature of the progenies. Among these hybrids, VH1 (*V. planifolia* type) was least similar (Table 6) to *V. planifolia* and most similar to *V. aphylla* in its AFLP profile, while VH5 was least similar to *V. aphylla*.

Combined analysis of the data obtained from RAPD and AFLP profiles revealed that among the selfed progenies, V1 and V10 showed highest number of parental bands, whereas V7, V6 showed highest frequency of non-parental bands, the frequency of non-parental bands ranged from 2.36 to 3.93% (Table 3). About 0.72–1.72% markers were found to reveal additivity among the hybrid progenies of *Vanilla* (Table 4). Moreover, there were no significant difference as to whether markers in offspring were more similar to female (similarity ranging from 32.02 to 86.94%) or to male parents (ranging from 17.64 to 79.78%) by similarity analysis.

The dendrograms of divergence also supported the above findings (Figs. 4 and 5). The polymorphism observed between the seedlings and hybrids was scored and the Paired Affinity

Table 3
Segregation of bands expressed in molecular profiles by selfed progenies of *V. planifolia*

No. of bands	V1	V2	V4	V6	V7	V8	V10
Parental	272 (97.14)	281 (96.89)	289 (97.6)	285 (96.61)	293 (96.06)	277 (96.85)	274 (97.5)
Non-parental	8 (2.85)	9 (3.1)	7 (2.36)	10 (3.38)	12 (3.93)	9 (3.14)	7 (2.49)
Total	280	290	296	295	305	286	281

Figures in parenthesis indicate the frequency of occurrence.

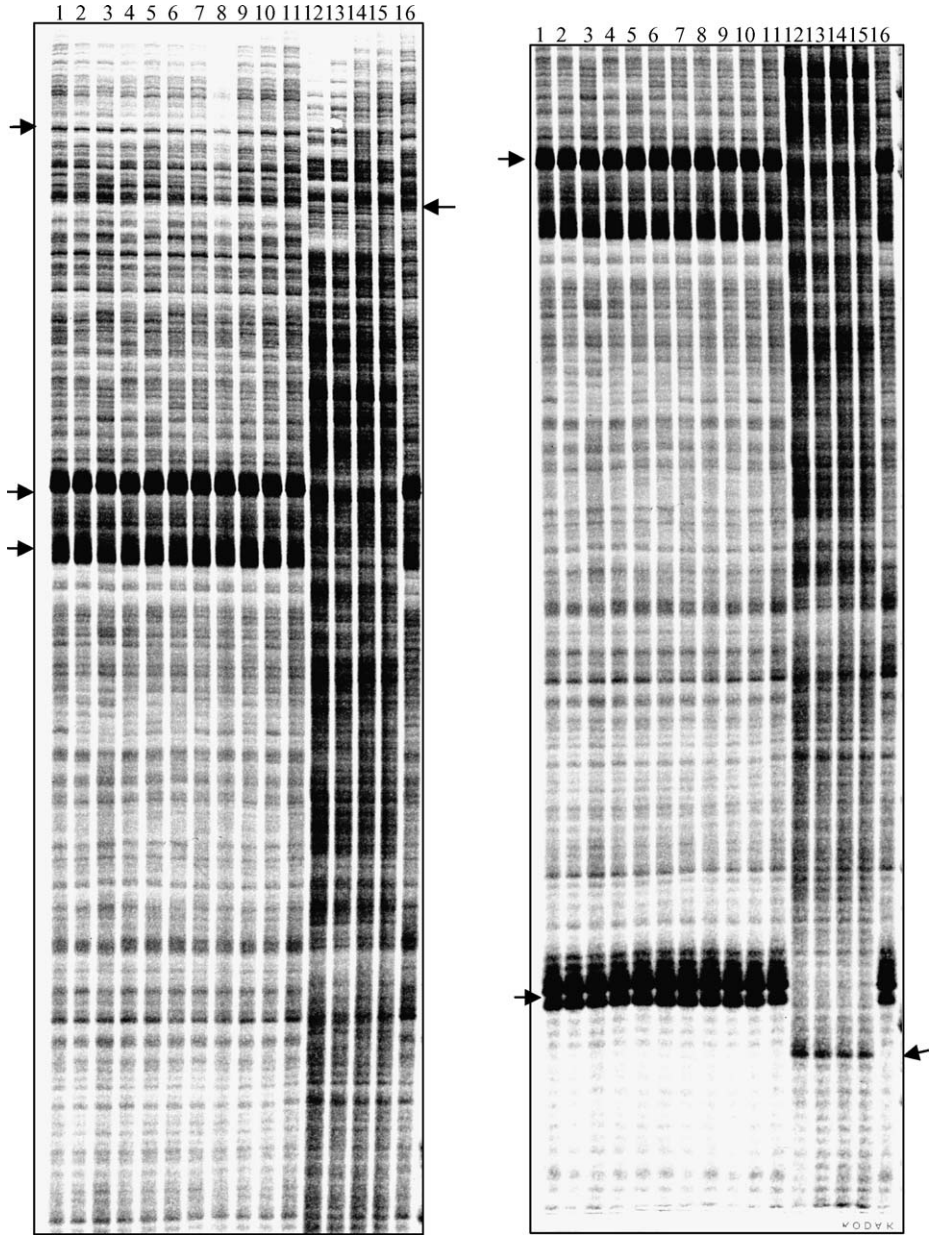

Fig. 3. AFLP profiles of vanilla seedling progenies and interspecific hybrids developed by primer combination EAC-MTG (lanes 1–10: seedling progenies of *V. planifolia*, 14–16: interspecific hybrids of *V. planifolia* and *V. aphylla*). 1, V1; 2, V2; 3, V4; 4, V6; 5, V7; 6, V8; 7, V10; 8, V11; 9, V12; 10, V24; 11, *V. planifolia*; 12, *V. aphylla*1; 13, *V. aphylla*2; 14, VH1; 15, VH4; 16, VH5. Arrows indicate species specific bands.

Indices was calculated based on similarity (Tables 5 and 6). The bootstrap analysis further proved the confidence of the grouping of VH1 and VH4 with *V. aphylla*, rather than being an artifact of the clustering process (Fig. 6).

4. Discussion

Cultivated *V. planifolia* is characterized by subsessile leaves and pale greenish yellow flowers with a chromosome number of

Table 4
Comparison of bands expressed in molecular profiles by interspecific hybrids to its parents

No. of bands	VH1	VH4	VH5	VH6
Parental (♀)	208 (75.09)	219 (77.38)	253 (86.94)	49 (32.02)
Parental (♂)	221 (79.78)	188 (66.43)	162 (55.67)	27 (17.64)
Non-parental	2 (0.72)	3 (1.06)	5 (1.72)	2 (1.31)
Total	277	283	291	153

Figures in parenthesis indicate the frequency of occurrence.

Table 5
Paired affinity Indices of selfed progenies and interspecific hybrids in Vanilla as expressed by RAPD markers

	V1	V2	V4	V6	V7	V8	V10	V.p	V.a	VH1	VH4	VH5	VH6
V1	–												
V2	89.0	–											
V4	82.9	91.5	–										
V6	82.9	86.6	95.1	–									
V7	79.3	90.2	91.5	89.0	–								
V8	82.9	86.8	87.8	87.8	91.5	–							
V10	75.6	79.3	82.9	78.0	81.7	85.4	–						
V.p	82.9	89.0	90.2	85.4	89.0	92.7	90.2	–					
V.a	40.2	43.9	45.1	47.6	43.9	42.7	35.4	42.7	–				
VH1	51.2	57.3	56.1	56.1	54.9	53.7	51.2	58.5	72.0	–			
VH4	63.4	69.5	63.9	63.4	67.1	61.0	58.5	65.8	62.2	68.3	–		
VH5	57.3	61.0	64.6	59.7	61.0	62.2	69.5	64.6	60.9	69.5	52.4	–	
VH6	70.7	69.5	68.3	68.3	67.1	68.3	68.3	68.3	50.0	58.5	65.9	59.8	–

V1–V10: selfed progenies of *V. planifolia*; V.p: *V. planifolia*, V.a: *V. aphylla*, VH1–VH5: interspecific hybrids of *V. planifolia* × *V. aphyll*.

Table 6
Paired affinity indices of selfed progenies and interspecific hybrids as expressed by AFLP markers

	V1	V2	V4	V6	V7	V8	V10	V11	V12	V24	V.p	V.a1	V.a2	VH1	VH4	VH5
V1	–															
V2	89.2	–														
V4	89.2	90.7	–													
V6	89.8	91.1	91.4	–												
V7	88.8	93.3	89.8	92.0	–											
V8	90.7	92.0	91.1	92.6	93.6	–										
V10	88.5	88.5	88.8	90.4	88.2	87.6	–									
V11	94.6	88.2	86.6	88.2	88.5	87.3	88.2	–								
V12	88.8	90.7	90.4	90.7	93.0	91.7	92.0	87.3	–							
V24	97.7	90.1	88.5	91.4	90.4	89.8	88.8	95.5	90.4	–						
V.p	91.7	91.7	93.3	93.6	91.4	91.4	90.4	88.2	92.0	92.0	–					
V.a1	45.7	43.1	44.7	43.8	44.1	44.7	46.3	44.7	43.4	43.4	44.4	–				
V.a2	46.0	44.1	44.4	44.1	44.4	44.4	47.3	45.7	44.4	44.4	44.7	99.0	–			
VH1	59.3	56.1	57.1	56.8	55.8	56.5	60.6	59.0	56.5	57.7	58.0	83.8	84.7	–		
VH4	60.0	59.3	59.0	58.0	58.4	56.7	63.8	59.0	59.0	58.4	60.0	80.6	81.5	93.6	–	
VH5	85.0	85.7	86.0	85.0	86.6	85.3	84.4	86.0	86.0	86.0	86.9	48.6	48.8	59.6	60.9	–

V1–V24: selfed progenies of *V. planifolia*; V.p: *V. planifolia*; V.a1, V.a2: *V. aphylla*, VH1–VH5: interspecific hybrids of *V. planifolia* ♀ × *V. aphylla* ♂.

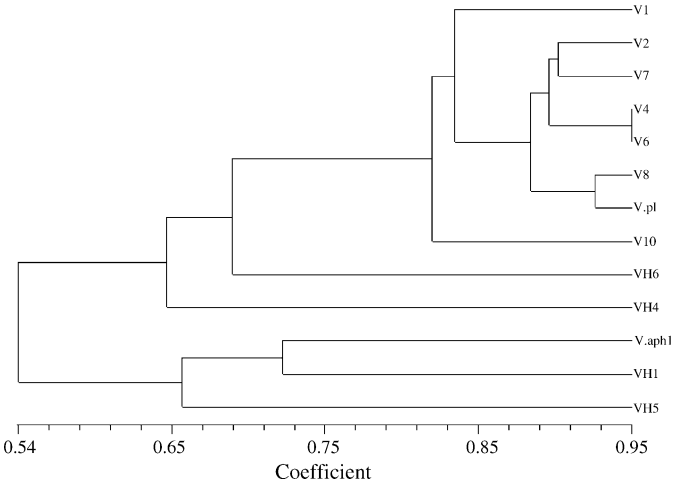

Fig. 4. Dendrogram showing linkage groups between selfed progenies and interspecific hybrids as expressed by RAPD markers.

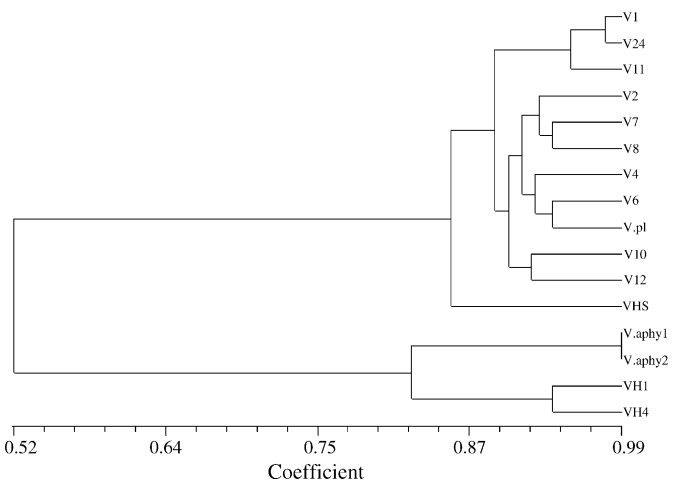

Fig. 5. Dendrogram showing linkage groups between selfed progenies and interspecific hybrids as expressed using AFLP markers.

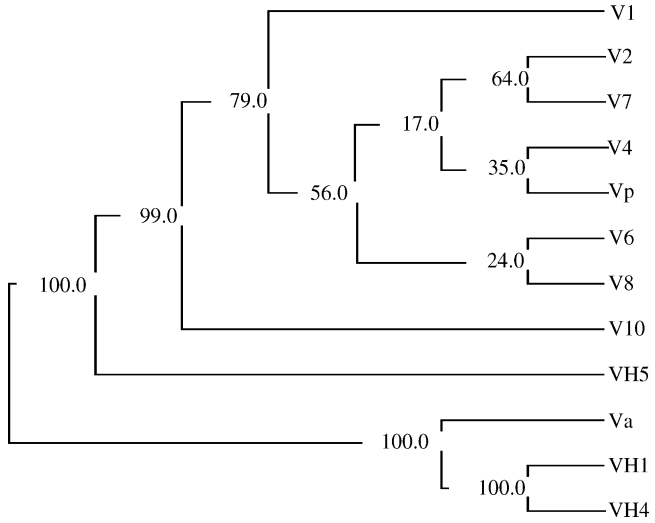

Fig. 6. Cluster generated using combined data of both AFLP and RAPD data, with bootstrap values indicating interrelationships between *V. planifolia* (Vp) selfed progenies (V1, V2, V4, V6, V7, V8, V10), interspecific hybrids (VH1, VH4, VH5 and VH6) and *V. aphylla*.

$2n = 32$, whereas wild relative, *V. aphylla* is characterized by absence of leaves/presence of scale leaves, greenish yellow flowers with a tuft of violet tipped hairs on throat of lip and $2n = 64$.

In the present study, generating selfed progenies and interspecific hybridization between *V. planifolia* and its related species *V. aphylla* was successfully achieved which can be used to increase the spectrum of variations by bringing desirable characters from wild species into the cultivated vanilla. Though only a few cases of genetic incompatibility between closely related species in orchids have been reported, occurrence of natural interspecific hybridization is rare and has been reported between *V. barbellata*, *V. claviculata* and *V. dilloniana* based on the enzyme assay of the vanilla populations in Caribbean Islands (Nielsen and Siegismund, 1999).

V. aphylla, the wild leafless species, gave tolerant reactions to infections with *Fusarium oxysporum* which is the causative organism of the major threat, root rot, to cultivations (Minoo, 2002). The interspecific hybrids produced between *V. planifolia* (♀) and *V. aphylla* (♂) showed segregation in morphological characters with regard to leaf type. Two of the hybrids, VH4 and VH5 express partial albinism and were leafless 'aphylla' types, i.e., morphologically similar to male parent while the other two, VH1 and VH6, were 'planifolia' type plants with normal chlorophyll and leaves, resembling female parent in leaf characters. The presence of characters of pollen donor (male) parent indicates that the progenies are hybrids and not accidental selfed progenies of *V. planifolia*.

Vanilla is reported to have chromosomal associations and reports of meiotic and post-meiotic chromosomal abnormalities have been made (Ravindran, 1979). Hybrids showed somatic associations and segmental allopolyploidy, indicating the hybrid origin of the progenies and that the parents are related closely. VH6 had chromosome numbers nearer to *V. planifolia*, but no cell with $2n = 32$, i.e., a variation frequency of almost 100% in the cells observed. VH1 had chromosome numbers nearest to *V. planifolia*, however it also showed a variation frequency of 61.53%. High rate of somatic associations was observed making counting extremely difficult in most of the cells.

This study investigated the use of random amplified DNA fragments as genetic markers in Vanilla. Arbitrary oligonucleotides were used as primers to amplify genomic DNA of the different species, seedling progenies and interspecific hybrids by polymerase chain reaction. Extensive variations were observed in the RAPD profiles and genetic relationships between the different genotypes are deduced from the degrees of similarity expressed in amplified product profiles. Random amplified DNA markers appeared to be of immense value for characterization and analysis of *Vanilla*. Development of interspecific hybrids and utilization of RAPD markers to characterize them is being done in many crops of importance like *Carica* (Drew et al., 1998; Magdalita et al., 1998). Molecular characterization of inter- and intra-specific somatic hybrids of potato, which eliminated the difficulty of unequivocally identifying nuclear hybrids, has been done (Baird et al., 1992). Markers that flank a gene determining a trait of agronomic interest can be used to track the trait in genetic crosses and also to estimate the genetic contribution of each parent to each member of a segregating population. Examples of such cases are RAPD assisted genetic segregation in diploid cultivated alfalfa, genetic diagnosis of F1 hybrid of *Rehmania* sp. for segregating characteristics from both parents (Hatano et al., 1997).

RAPD profiles were supplemented with AFLP profiling for the genotypes since AFLP technology has the added advantage of its capability to detect various polymorphisms in different genomic regions simultaneously, while being highly sensitive and reproducible. As a result, AFLP has become widely used for the identification of genetic variation in closely related species. *Eco*RI/*Mse*I digested genomic DNA was used to generate AFLP bands and 319 markers were identified, that segregated among the different individuals. Genetic aspects of vanilla are poorly understood. AFLP employs the polymerase chain reaction (PCR) to produce rapid and reproducible anonymous DNA markers for mapping purposes. Using this technique it was possible to generate large numbers of markers without any prior knowledge of the vanilla genome. AFLP maps have been developed for many species of agricultural importance (Mackill et al., 1996) and, more recently, for organisms of ecological and/or evolutionary significance (Kim and Rieseberg, 1999). AFLP markers in this study were chosen on the basis of the presence/absence of bands and therefore represent species-specific, parental and hybrid specific markers and can be used for genotyping in all interspecific hybrid crosses.

The molecular profiling utilizing RAPD and AFLP studies, indicated that *V. aphylla* is the most divergent from *V. planifolia* with a similarity index of only 44.4% in AFLP profiles. This wide variation between these two species is expected because *V. planifolia* has originated in Central America while *V. aphylla* originated in South Asia with very wide geographical isolation.

Both RAPD and AFLP data indicated similar patterns of similarity between the parents, selfed progenies and interspecific hybrids and all the progenies tested and were variable

when compared to each other, which can be exploited for crop improvement in vanilla. This is the first report in vanilla, of successful interspecific hybridization and production of hybrids between *V. planifolia* and *V. aphylla*, and that molecular profiles, RAPD and AFLP, coupled with morphological characters can be utilized to assess the true hybrid nature of genotypes. A combined analysis of the molecular data revealed that among the selfed progenies, V1, V10 and among the interspecific hybrids, VH5, VH1 showed highest number of parental bands. The frequency of non-parental bands ranged from 2.36 to 3.93% in the former. About 0.72–1.72% markers were found to reveal additivity among the hybrid progenies of *Vanilla*. Moreover, there were no significant difference as to whether markers in offspring were more similar to female or to male parents by similarity analysis, which might be due to the complex and diversified nature of the genus.

The results of the present investigation is particularly significant for future studies for the expression of disease resistance character which has a complex genetic basis, being the result of recessive and additive genes inherited from both parents. The data demonstrates that even when moderate population sizes are used, the level of segregation and recombination observed is sufficient to facilitate efficient introgression of important characters which may be of agronomic significance, from wild germplasm.

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