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ORIGINAL ARTICLE

Molecular characterization of reciprocal crosses of *Aerides vandarum* and *Vanda stangeana* (Orchidaceae) at the protocorm stage

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Abstract *Aerides vandarum* and *Vanda stangeana* are two rare and endangered vandaceous orchids with immense floricultural traits. The intergeneric hybrids were synthesized by performing reciprocal crosses between them. In vitro germination response of the immature hybrid embryos was found to be best on half-strength Murashige and Skoog medium supplemented with 20% (*v*/*v*) coconut water/liquid endosperm from tender coconut. Determination of hybridity was made as early as the immature seeds or embryos germinated in vitro, using randomly amplified polymorphic DNA (RAPD) markers. Out of 15 arbitrarily chosen decamer RAPD primers, two were found to be useful in amplification of polymorphic bands specific to the parental species and their presence in the reciprocal crosses. However, a decisive profile that can identify the reciprocal crosses could not be provided by RAPD. Amplification of the *trn*L-F non-coding regions of chloroplast DNA of the parent species and hybrids aided easy identification of the reciprocal crosses from the fact that maternal inheritance of chloroplast DNA held true for these intergeneric hybrids. Subsequent restriction digestion of the polymerase chain reaction (PCR) amplified *trn*L-F non-coding regions of chloroplast DNA also consolidated the finding. Such PCR-based molecular markers could be used for early determination of hybridity and easy identification of the reciprocal crosses.

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Keywords *Aerides vandarum* · Intergeneric hybrid · Orchidaceae · Randomly amplified polymorphic DNA · *trn*L-F non-coding regions · *Vanda stangeana*

Abbreviations

Av	*Aerides vandarum*
CW	Coconut water
DAI	Days after inoculation
MS	Murashige and Skoog medium
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
Vs	*Vanda stangeana*
VW	Vacin and Went medium

Introduction

Orchids are a group of flowering plants which are generally attuned to a long juvenile period before they mature and flower. The maturation time for vandaceous orchids ranges from three and a half (Kishor et al. 2006) to ten years (Teoh 1986). Such delayed flowering has been the main disadvantage to breeders as the final point of interest, i.e. the characteristics of the flowers of hybrids could not be determined within a short timespan. Hence, the development of molecular markers for rapid confirmation of hybridity using simple and reliable biotechnological tools is necessary. With advent of molecular biology, many breeders have employed polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) for determination of the hybridity of interspecific *Carica* hybrids (Drew et al. 1998), cotton hybrids (Dongre and Parkhi 2005), lily hybrids (Yamagishi 1995; Obata et al.

2000), potato hybrids (Baird et al. 1992), interspecific *Vanilla* hybrids (Divakaran et al. 2006), and intergeneric slipper orchid hybrids (Handa et al. 1998), etc. Lim et al. (1999) also suggested that RAPD could be used to indicate the genetic closeness of orchid species and hybrids quickly and efficiently and thus help to predict the outcome of a cross, based on genotypic information.

Unlike nuclear genes, the inheritance pattern of chloroplasts is predominantly maternal in angiosperms (Birky 1995; Corriveau and Coleman 1988). Inheritance pattern of chloroplast DNA has rarely been studied in the family Orchidaceae. However, in recent years chloroplast genome study has been applied for detection of interspecific gene flow in hybrid zones (Aceto et al. 1999, 2000). Maternal inheritance of chloroplast DNA was reported in the hybrids of *Phalaenopsis* and *Doritis* (Chang et al. 2000) and in *Anacamptis palustris* (Cafasso et al. 2005). Hence, the chloroplast DNA inheritance pattern can also be applied for identification of reciprocal crosses in an orchid breeding programme.

Aerides vandarum Reichb. f. (Syn. *Papilionanthe vandarum*) (Fig. 1a) and *Vanda stangeana* Reichb.f. (Fig. 1b) are two beautiful rare and endangered vandaceous orchids with immense floricultural traits. *A. vandarum* is an epiphytic orchid having terete (cylindrical) leaves. Two or three white and scented flowers are produced in each inflorescence. Flowers are 4–4.5 cm across with twisted sepals and petals. It flowers during February–April. *V. stangeana* has strap leaves which are recurved and long. Its long inflorescence has 15–20 flowers, which are 5–6 cm across and are yellowish green in colour with brown tessellation. Flowering time is March–May.

In this study, an attempt was made to confirm hybridity of the intergeneric reciprocal crosses of *A. vandarum* and *V. stangeana* by developing RAPD markers using DNAs extracted from in vitro-raised protocorms. Further, the *trn*L-F non-coding region of chloroplast DNA was employed for unequivocal identification of the reciprocal crosses by studying its inheritance pattern. Again, the PCR amplified product of the same region was subjected to

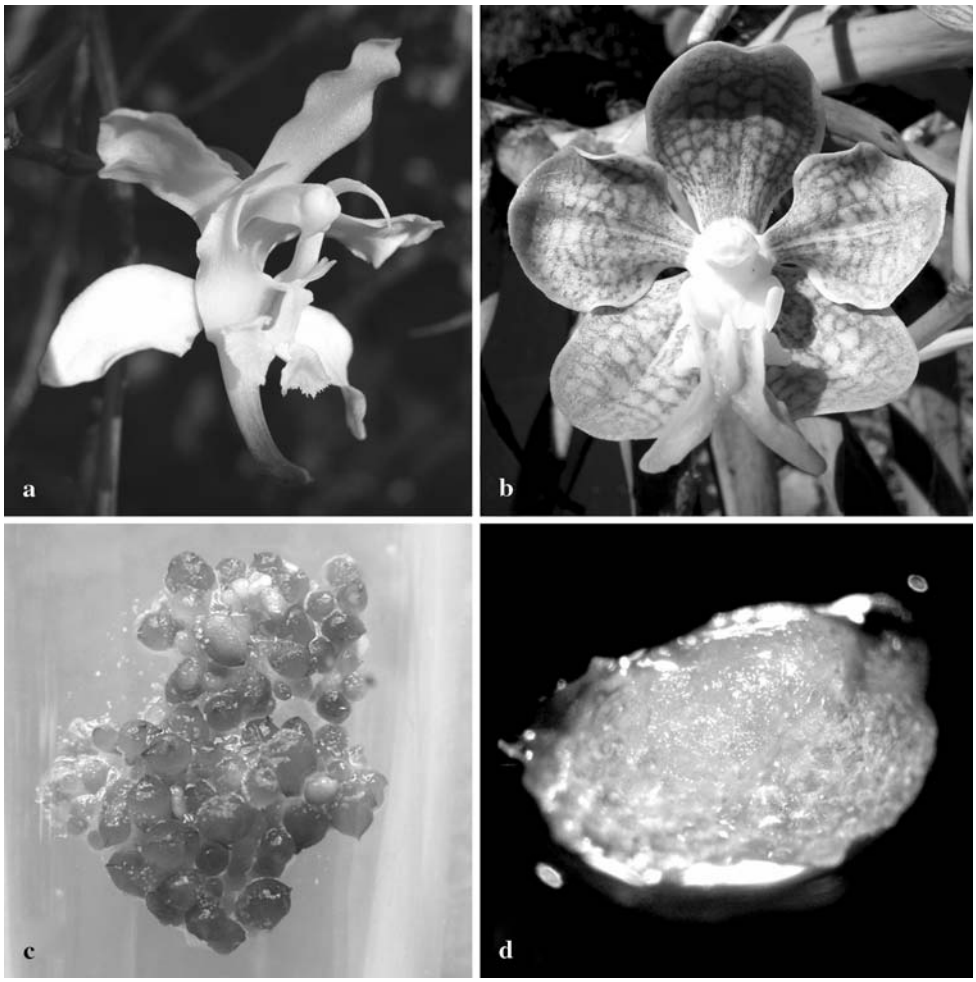

Fig. 1 Flowers of parent species and germination of hybrid embryos of *A. vandarum* × *V. stangeana*. **a** *A. vandarum*, **b** *V. stangeana*, **c** Immature embryo germination response of *V. stangeana* × *A. vandarum* on half-strength MS medium supplemented with 20% (*v*/*v*) coconut water (CW). **d** An isolated protocorm used for DNA extraction

restriction fragment length polymorphism (RFLP) to consolidate the finding.

Materials and methods

Plant materials

Both *A. vandarum* and *V. stangeana* were collected from their habitat in different locations in Manipur (23°84′70″–25°84′10″ north latitude and 93°86′10″–94°84′80″ east longitude) in north-east India. The plants were grown in the Experimental Orchidarium of the Institute of Bioresources and Sustainable Development, under ambient environmental conditions.

Hybridization and in vitro seed germination

Hybridization was performed during April 2005 when both *A. vandarum* (Av) and *V. stangeana* (Vs) were flowering synchronously. After 5–6 days of flower opening, pollinia of both the species were separately removed, using fine sterilized forceps, and deposited on the stigma of the respective female parents. Pollinia from the female parents were also removed to prevent self-pollination. The hand-pollinated flowers were bagged for seven days to prevent unwanted pollination and labelled individually with tags giving the date and the time of pollination. After hybridization, the capsules were allowed to develop by maintaining the plants in the orchidarium for 150 days. Five flowers were pollinated in each group. Capsules containing the hybridized seeds were collected from the plant 150 days after pollination. They were soaked in an aqueous solution of commercial detergent (Labolene, Qualigens, India) for 30 min. Solid dirt particles adhering to the surface of the capsules were removed using a fine brush followed by rinsing with sterile distilled water. Capsules were then surface-disinfected successively with 70% (*v*/*v*) ethanol for 30 s and 0.1% (*w*/*v*) aqueous mercuric chloride solution for 15 min. Afterwards, capsules were rinsed four times, each for 5 min, with sterile double-distilled water before air-drying in a laminar airflow cabinet for 5 min. Green capsules were dissected transversely with a sterile surgical blade. The immature seeds were scooped out of the sterilized capsules and approximately 100 seeds were sown on culture medium in a test tube (32 × 200 mm) for in vitro asymbiotic seed germination.

Culture medium and cultural conditions

For seed germination Vacin and Went (VW; 1949) medium containing 2% (*w*/*v*) sucrose and half-strength Murashige and Skoog (MS; 1962) medium containing 2% (*w*/*v*) sucrose were used with and without 20% (*v*/*v*) coconut water (CW). CW from green coconut was drained into a 500 ml glass beaker and boiled for 5 min. Afterward it was filtered through a Whatman 4 paper and added to the media. After adjusting the pH of the VW medium to 5.2 and the MS medium to 5.7 with 1 N NaOH/1N HCl the media were gelled with agar (Hi-Media, India). Approximately, 35 ml of the medium was dispensed into each culture tube (32 mm × 200 mm), closed with doubled layered aluminium foil, and sterilized by autoclaving at 15 psi and 121°C for 20 min. The scooped aggregated mass of seeds was placed on the medium in the culture tubes and incubated at 25 ± 2°C under 28–35 μmol m^{-2} s^{-1} illumination from fluorescent tubes for 16/8 h (light/dark) photoperiod daily.

DNA extraction

The germinating embryos or protocorms (Fig. 1d) were used as a source for DNA extraction from the hybrid whereas fresh leaves were used for the parent species. Total genomic DNA was extracted by following the procedure of Doyle and Doyle (1987), with minor modifications. The quality and quantity of the DNA samples were determined by observing the ratio of absorbance at A260/A280. Intactness of genomic DNA was further checked by subjecting it to 0.8% agarose gel electrophoresis.

PCR-RAPD for hybridity confirmation

Amplification was done in 25 μl reaction volume containing 1× Taq polymerase assay buffer (10 mM Tris–HCl pH 8.0, 50 mM KCl, and 3 mM $MgCl_2$), 0.2 mM dNTPs, 1 μM primer, 1.0 unit of Taq polymerase, and 25 ng genomic DNA. All reagents used were from Bangalore Genie (India). Fifteen arbitrarily chosen decamer random primers (synthesized at Bangalore Genie by providing the original sequences from Operon Technologies, Alameda, USA) were used. The sequences of OPA1 and OPA4 were 5′-CAGGCCCTTC-3′ and 5′-AATCGGGCTG-3′, respectively. Amplification was performed using a thermal cycler (Model: icycler, Biorad, USA) programmed for an initial denaturation at 94°C for 4 min for one cycle, then 94, 53, and 72°C for 1 min for 35 cycles with 5 min final extension at 72°C. The amplified DNA fragments were resolved in 2% agarose gel electrophoretically at 70 V, using 1× TAE buffer. A 1-kb ladder (Promega, USA) served as the standard molecular weight marker. The ethidium bromide-stained (0.5 μg/l) gels were visualized and photographed using a Gel Documentation System (Software: Quantity one, 4.5.1, Biorad, USA). The RAPD reaction was carried out at least three times to ensure the reproducibility of the amplified bands. Faint bands were not considered for analysis.

PCR amplification of *trn*L-F noncoding region of chloroplast DNA

The *trn*L-F noncoding region, *trn*L (UAA) intron and the intergeneric spacer between the *trn*L (UAA) 3′exon and *trn*F (GAA) gene of the chloroplast DNA of *V. stangeana*, *A. vandarum*, Vs × Av, and Av × Vs were amplified from genomic DNA extracted from leaves or protocorms using the primers *trn*L-Fc (5′-CGAAATCGGTAGACGCTACG-3′) and *trn*L-Ff (5′-ATTTGAACTGGTGACACGAG-3′) of Taberlet et al. (1991). PCR was performed in 25 µl reaction volume containing 1× Taq polymerase assay buffer (10 mM Tris–HCl pH 8.0, 50 mM KCl, and 3 mM $MgCl_2$), 0.2 mM dNTPs, 1 µM primer, 1.0 unit Taq polymerase, and 20–25 ng genomic DNA. Amplification conditions were as follows: an initial denaturation at 94°C for 4 min for 1 cycle, then 94°C for 30 s, 58°C for 45 s and 72°C for 1 min for 30 cycles, with 5 min final extension at 72°C. The amplified DNA fragments were resolved in 2% agarose gel electrophoretically at 70 V, using 1× TAE buffer. A 100-bp ladder (Bangalore Genie) served as the standard marker, and visualization was achieved by staining with ethidium bromide.

PCR–RFLP

The PCR amplified non-coding *trn*L-F regions of the chloroplast DNAs of the parental species and their hybrids were used for RFLP detection. Approximately 10 µl each of the PCR products were digested with one unit of the restriction endonuclease *Eco*R1 (five base cutter) according to the manufacturer's instructions (Promega). Restriction digested fragments were separated in 2% agarose gel at 70 V for 2.30 h and documented after staining with ethidium bromide.

Experimental design and data analysis

In vitro immature embryo germination experiments were set up in a completely randomized design. Fifteen culture tubes were used in seed-germination experiments. Each tube was considered as a replicate. Seed germination was assessed as the percentage of fertile seeds per tube which developed to green protocorms. Protocorms were counted using a stereozoom microscope (Model: SZII, Olympus). Data were subjected to standard analysis of variance (ANOVA).

Results

Hybridization and in vitro-seed germination

A. vandarum and *V. stangeana* were compatible for intergeneric hybridization as crossability between the two orchids showed 100% success (Table 1). A few days after crossing the ovary swelled and continued developing. The resulting hybrid embryos were allowed to develop for 150 days. The harvested pods revealed 100% full seed content as examined before inoculation, indicating success of the cross.

Table 1 Result of cross and reciprocal cross performed for the hybridization programme using *V. stangeana* and *A. vandarum*

No.	Parentage		No. of flowers pollinated	No. of pods formed	Successful cross (%)
	Female (♀)	Male (♂)			
1	*Vanda stangeana*	*Aerides vandarum*	5	5	100
2	*A. vandarum*	*V. stangeana*	5	5	100

Table 2 Effect of different media on asymbiotic seed germination of the hybrid *V. stangeana* × *A. vandarum*

Medium	Germination (%)	Time taken for development (days)			
		Green globular	Leaf primordial	First leaf	First root
1/2 MS	100	56.5[b]	66.2[b]	0.0[a]	0.0[a]
1/2 MS + 20% CW	100	42.8[c]	58.4[c]	63.3[b]	63.6[b]
VW	0	0.0[a]	0.0[a]	0.0[a]	0.0[a]
VW + 20% CW	0	0.0[a]	0.0[a]	0.0[a]	0.0[a]

In each column mean values followed by the same letter are not significantly different, as indicated by Tukey's test ($P = 0.05$)

1/2 MS, half-strength MS medium; 1/2 MS + 20% CW, half-strength MS medium supplemented with 20% CW; VW, VW medium; VW + 20% CW, VW medium supplemented with 20% CW

Data recorded every alternate day

Visible morphological changes associated with embryo germination were observed 20 days after inoculation (DAI) on culture medium which was marked by swelling of the embryos. On average, the best germination response for both Vs × Av and Av × Vs was observed on half-strength MS medium supplemented with 20% (*v*/*v*) CW. For the cross Vs × Av greening of the germinating embryos or development of chlorophyll took place at 42 DAI (Table 2; Fig. 1c). First leaf and first root simultaneously emerged at 63 DAI. For the reciprocal cross the germinating embryos turned green at 28 DAI, developed leaf primordia or ridge at 42 DAI, and first leaf and root developed simultaneously at 63 DAI (Table 3). The germination percentage on this medium was 100. On the media VW and VW supplemented with 20% (*v*/*v*) CW few morphological changes were observed; the embryos enlarged but further

Table 3 Effect of different media on asymbiotic seed germination of the hybrid *A. vandarum* × *V. stangeana*

Media	Germination %	Time taken for development (days)			
		Green globular	Leaf ridge	First leaf	First root
1/2 MS	95	42.2[b]	42.5[b]	77.8[b]	0.0[a]
1/2 MS + 20% CW	100	28.6[c]	42.2[b]	63.0[c]	63.9[b]
VW	50	60.0[a]	0.0[a]	0.0[a]	0.0[a]
VW + 20% CW	30	60.0[a]	0.0[a]	0.0[a]	0.0[a]

In each column mean values followed by the same letter are not significantly different, as indicated by Tukey's test ($P = 0.05$)

1/2 MS, half-strength MS medium; 1/2 MS + 20% CW, half-strength MS medium supplemented with 20% CW; VW, VW medium; VW + 20% CW, VW medium supplemented with 20% CW

Data recorded every alternate day

morphological changes associated with germination could not be observed.

PCR-RAPD for hybridity confirmation

Three sets of PCRs were carried out for RAPD fingerprinting of each sample. Only bands reproducible in all runs were considered for analysis. Out of 15 RAPD primers assessed only two primers OPA1 and OPA4 showed the desired polymorphic amplification pattern between the two parents. Both the primers showed reproducible major polymorphic banding patterns. The primer OPA1 generated four markers for *V. stangeana*, out of which two amplicons of approximately 710 and 800 bp were monomorphic with that of *A. vandarum*, Vs × Av, and Av × Vs (Fig. 2). The two other polymorphic amplicons of 1,020 and 1,750 bp approx. were detected in the hybrids. For *A. vandarum* OPA1 amplified six amplicons, out of which only two, 710 and 800 bp approx., were monomorphic with that of *V. stangeana*, Vs × Av, and Av × Vs whereas the polymorphic amplicons, viz., 550, 1,350, 1,400 and 2,440 bp, were also generated in the hybrids. Similar banding patterns were generated by OPA1 for both Vs × Av and Av × Vs.

The second primer OPA4 generated three amplicons for *V. stangeana*; one of about 700 bp was monomorphic with that of *A. vandarum* while the rest, viz., 450 and 1,500 bp approx., were polymorphic (Fig. 3). The same primer also amplified three bands for *A. vandarum* out of which two were polymorphic (630 and 1,450 bp approx.). All the parental monomorphic and polymorphic bands were generated in the hybrids. Two polymorphic bands, 1,500 bp approx. of *V. stangeana* and 1,400 bp approx of *A. vandarum*, were amplified together as a single band for both Vs × Av and Av × Vs by the primer OPA4.

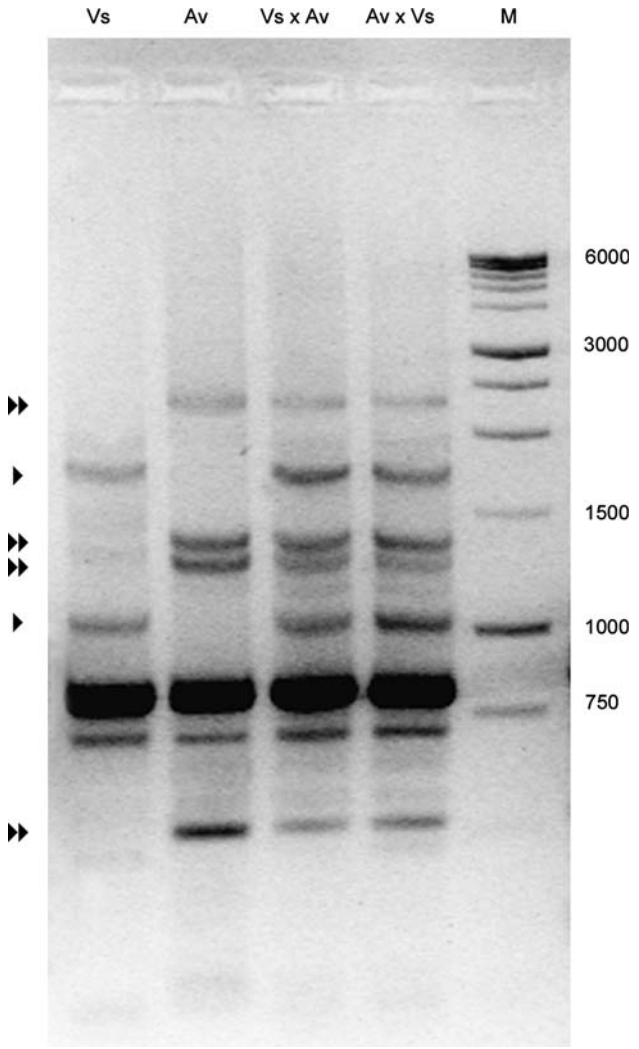

Fig. 2 Randomly amplified polymorphic DNA (RAPD) amplification profiles of *V. stangeana* (*Vs*), *A. vandarum* (*Av*), Vs × Av, and Av × Vs using primers OPA1. *M*, 1-kb ladder; *single triangles* indicate bands specific to *V. stangeana* and the reciprocal crosses; *double triangles* indicate bands specific to *A. vandarum* and the reciprocal crosses

PCR amplification of *trn*L-F non-coding region of chloroplast DNA

Amplification of the *trn*L-F non-coding region of chloroplast DNA of *V. stangeana*, *A. vandarum*, and their hybrids was carried out to detect polymorphism of the non-coding region in them, and their inheritance pattern. The *trn*L-F non-coding regions of *V. stangeana* and *A. vandarum* had length polymorphism as indicated by a difference of 100 bp approximately. The amplified *trn*L-F region of *V. stangeana* was observed to be 1,245 bp approx. and the same band was amplified for Vs × Av. For *A. vandaraum* the region was 1,347 bp and the same was also observed for Av × Vs (Fig. 4).

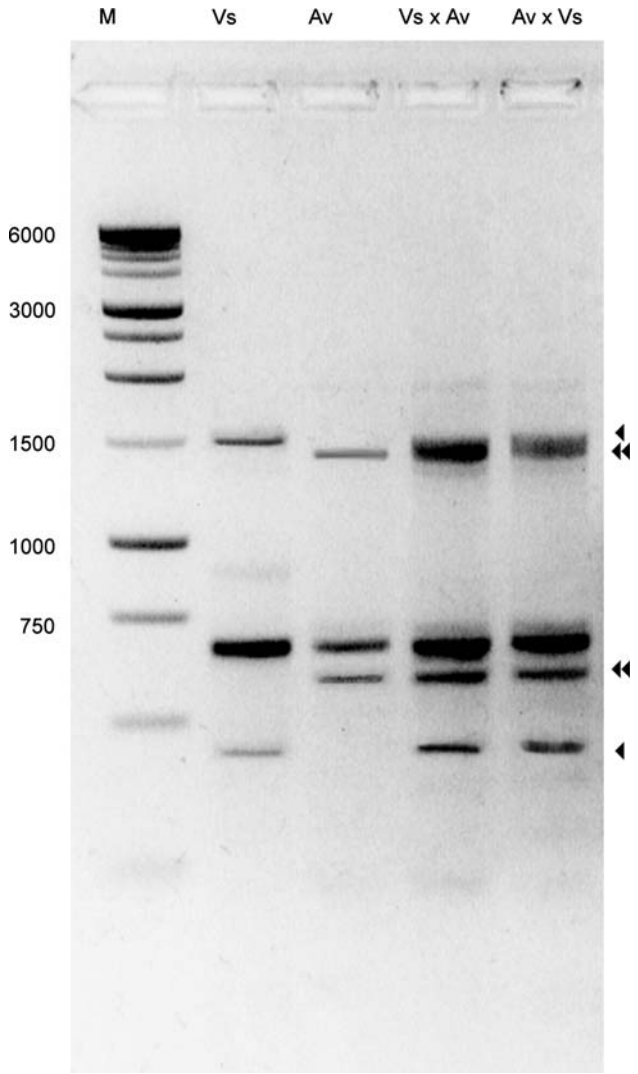

Fig. 3 Randomly amplified polymorphic DNA (RAPD) amplification profiles of *V. stangeana* (*Vs*), *A. vandarum* (*Av*), Vs × Av, and Av × Vs using primers OPA4. *M*, 1-kb ladder; *single triangles* indicate bands specific to *V. stangeana* and the reciprocal crosses; *double triangles* indicate bands specific to *A. vandarum* and the reciprocal crosses

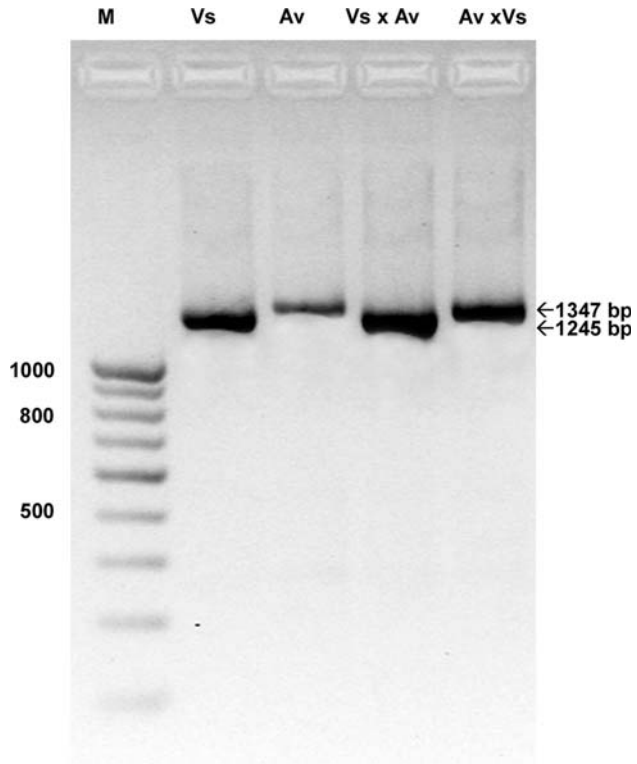

Fig. 4 Polymerase chain reaction (PCR) amplification of *trn*L-F non-coding region of chloroplast DNA profiles of *V. stangeana* (*Vs*), *A. vandarum* (*Av*), Vs × Av, and Av × Vs. *M*, 100-bp ladder

RFLP of the amplified *trn*L-F non-coding regions

RFLP was observed when the PCR amplified *trn*L-F non-coding region of chloroplast DNA of *V. stangeana*, *A. vandarum* and their hybrids (Vs × Av and Av × Vs) were digested by the restriction endonuclease *Eco*R1. The 1,245 bp long *trn*L-F region of *V. stangeana* was digested giving two fragments of 480 and 628 bp (Fig. 5). The same RFLP pattern was observed for the hybrid Vs × Av. *Eco*R1 also digested the 1,347 bp long *trn*L-F region of *A. vandarum* giving two fragments of 350 and 628 bp and this same restriction pattern was observed for Av × Vs.

Discussion

Intergeneric hybrids are possible in the family Orchidaceae because of an ill-defined genetic barrier (Arditti 1992). The modern commercial varieties are complex hybrids involving two or more genera. Such outcrossings result in synthesis of new form, texture, colour, shapes, sizes, fragrances, etc. Even though the two orchid species chosen in the present hybridization programme belonged to two different genera and had different distinct floral and vegetative features they showed 100% compatibility. Hence, there is no reproduction block between *A. vandarum* and *V. stangeana*.

Despite poor organization and limited food reserves, the microscopic orchid seeds can germinate in vitro if specific nutritional and environmental conditions are provided (Knudson 1922). In our study, we found that half-strength MS medium was more suitable than VW for in vitro seed germination. This might be because of the rich macro and micro-nutrient regime rather than the VW medium. We added CW to the germination medium because its beneficial effect on seed germination has been reported for endangered orchids like *Rhynchostylis retusa* and *V. coerulea* (Nath et al. 1991). Results obtained from our

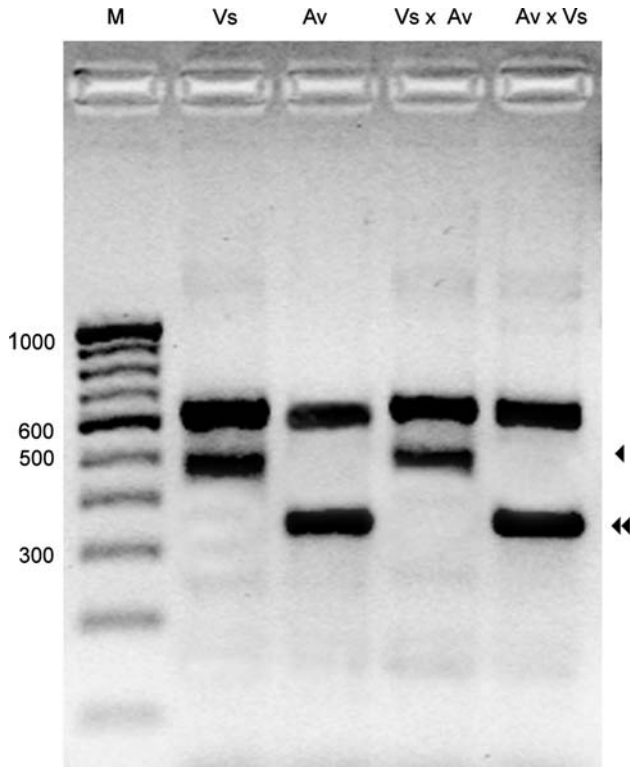

Fig. 5 Restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) amplified *trn*L-F non-coding region of chloroplast DNA profiles of *V. stangeana* (*Vs*), *A. vandarum* (*Av*), Vs × Av, and Av × Vs. *M*, 100-bp ladder; *single triangle* indicates fragments specific to *V. stangeana* and Vs × Av; *double triangle* indicates fragments specific to *A. vandarum* and Av × Vs

study also showed that the seed germination response was enhanced by addition of CW.

It is evident from our experiment that RAPD markers provided enough data to confirm the success of synthesis of the intergeneric hybrids involving the parents, viz., *A. vandarum* and *V. stangeana*, and using the RAPD technique the hybridity could be confirmed as soon as the immature embryos started germination. In our case, approximately 28-day-old germinating embryos/protocorms were chosen as the plant material for DNA extraction. The growing protocorms were coarse, granular, and very convenient in DNA extraction process as only slight crushing was required to break the cell wall. Harvested DNA quality and quantity were also good.

The random decamer primers OPA1 and OPA4 could generate the desired banding patterns exclusive of the two parental orchid species. Simultaneously they also generated the same parental bands in the hybrids, indicating F_1 status. This was irrespective of the cytoplasmic origin of the hybrids. Thus, PCR-RAPD which is a simple, fast, and reliable technique can effectively be used for the characterization of the intergeneric hybrids without resorting to complex processes.

In an RAPD assay using 10-mer primers, relatively low annealing temperatures are usually used (Williams et al. 1990). However, in our experiment annealing temperature lower than 40°C produced one or two bands (result not shown). When we used a high annealing temperature of 53°C with 3 mM $MgCl_2$ the number of amplified fragments increased with more clarity. All the major bands could be reproduced in all three experiments. Yamagishi (1995) also reported the effectiveness of a high annealing temperature of 54°C for developing RAPD markers in *Lilium*.

In our experiment PCR-RAPD generated specific markers that characterized *A. vandarum* and *V. stangeana*; it could, however, not provide a decisive profile that could be used to identify the reciprocal crosses. RAPD markers specific to either of the reciprocal crosses could have been generated had we tried a large number of random primers. Such an exhaustive search would require more time and labour. Hence, we resorted to the inheritance pattern of the parental chloroplast DNA in their hybrids. Maternal inheritance was observed in the hybrids Vs × Av and Av × Vs. In our case we found that the *trn*L-F non-coding regions of *V. stangeana* and *A. vandarum* were differed by about 100 bp. This difference in size of the region aided easy characterization of the two parent species and identification of the hybrids resulting from their reciprocal crosses. Generally, the *trn*L-F non-coding region of the chloroplast is used to infer plant phylogenies at different taxonomic levels, because this non-coding region evolves faster than the coding regions such as *rbc*L (Gielly and Taberlet 1994). Again, RFLP of the amplified fragments convincingly showed polymorphism in the restriction sites of *Eco*R1. Barbara et al. (2001) tried restriction digestion of the *trn*L intron and *trn*L-F spacer using 19 restriction enzymes. They reported that the *trn*L-F spacer was uninformative regarding the restriction sites of the tried enzymes whereas the *trn*L intron sequence was digested by three enzymes viz., *Dra*1, *Eco*R1 and *Eco*RV. Therefore, we tried with *Eco*R1 to see the restriction pattern and it was found effective. Even though the primers *trn*L-Fc and *trn*L-Ff amplified the *trn*L intron and the *trn*L-F spacer together we assumed that the restriction site was present in the *trn*L intron region. Use of chloroplast DNA in our experiment, thus, aided easy, fast, and reliable identification of the reciprocal crosses.

In conclusion, these experiments have shown that the hybridity of the reciprocal crosses could be established by employing PCR-RAPD at the protocorm stage. However, chloroplast DNA markers are more appropriate for identification of the reciprocal crosses. Such PCR based molecular markers will also be useful in determination of the genetic background of the plant materials the breeders use to ensure greater success in achieving the specific aims of hybridization rapidly and reliably.

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