IN VITRO MULTIPLICATION AND MOLECULAR CHARACTERIZATION OF SELECTED *DENDROBIUM* HYBRIDS

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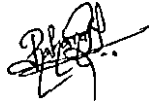
DECLARATION

I hereby declare that this thesis entitled "***In vitro* multiplication and molecular characterization of selected *Dendrobium* hybrids**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.



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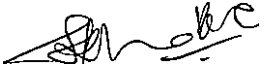
Certified that this thesis entitled "***In vitro* multiplication and molecular characterization of selected *Dendrobium* hybrids**" is a record of research work done independently by Ms. Rahana, S.N. (2004-11-21) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.



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Dedicated to

My Beloved Father

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Rahana, S.N.

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LIST OF ABBREVIATIONS

°C	–	Degree Celsius
µg	–	microgram
µl	–	micro litre
µM	–	micro molar
2,4-D	–	2,4-dichlorophenoxy acetic acid
ABA	–	Abscissic acid
AC	–	Activated charcoal
AFLP	–	Amplified Fragment Length Polymorphism
BA	–	Benzyl adenine
BAP	–	Benzyl amino purine
bp	–	Base pair
CD	–	Critical difference
CH	–	Casein Hydrolysate
cm	–	Centimeter
CRD	–	Completely Randomised Design
CSTD	–	*Candy Stripe* x *Tomie Drake*
CW	–	Coconut water
DBT	–	Department of Biotechnology
DNA	–	Deoxyribonucleic acid
dNTPs	–	Deoxy nucleotide triphosphates
EDTA	–	Ethylene diamino tetra acetic acid disodium salt

LIST OF ABBREVIATIONS CONTINUED

et al.	–	And others
Fig.	–	Figure
g	–	Gram
GA_3	–	Gibberellic Acid
HCl	–	Hydrochloric Acid
IAA	–	Indole-3-acetic acid
IBA	–	Indole-3-butyric acid
KC	–	Knudson C medium
kg	–	kilogram
KN	–	Kinetin (6-furfurylamino purine)
l	–	Litre
l^{-1}	–	Per litre
M	–	Molar
mg	–	milligram
$MgCl_2$	–	Magnesium Chloride
ml	–	milli litre
mM	–	*milli molar*
MS	–	Murashige and Skoog medium
N	–	Normality
NAA	–	α-Naphthalene acetic acid
NaCl	–	Sodium Chloride
NaOH	–	Sodium Hydroxide
ng	–	nanogram

nm	–	nanometer
No.	–	Number
OPA	–	Operon primer of A series
OPB	–	Operon primer of B series
PCR	–	Polymerase chain reaction
PLB	–	Protocorm Like Body(ies)
pM	–	Pico mole
RAPD	–	Random amplified polymorphic DNA
RFLP	–	Restriction Fragment Length Polymorphism
SDS	–	Sodium dodecyl sulphate
SE	–	Standard Error
TAE	–	Tris acetic acid EDTA
TDZ	–	1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea
Tris HCl	–	Tris (hydroxy methyl) amino methane hydrochloride
UPGMA	–	Unweighted pair group method for arithmetic average
viz.	–	Namely
VNTR	–	Variable number of tandem repeats
VW	–	Vacin and Went (1949) medium
UV	–	Ultra violet

INTRODUCTION

1. INTRODUCTION

Orchids are the most fascinating and advanced group among flowering plants. They are highly specialized in many ways. They belong to the family Orchidaceae. It is among the largest families of flowering plants having about 800 genera and 35,000 species (Singh, 1986) with innumerable hybrids and inexhaustible varieties. The family (Orchidaceae) has not only outnumbered other families in species wealth but also evolved ingenuity and higher levels of specialization to surpass all other plant families.

The orchids are distinctive plants and are highly priced in the international florists' trade due to their intricately designed spectacular flowers, brilliant colours, delightful appearance, myriad sizes, shapes and forms and long lasting qualities. Cut flowers have been the commonest form of commercial utilization of orchids. It has emerged as a lucrative profession with a much higher potential for returns compared to other agri-horticultural crops. The orchid cut flower industry is growing at the rate of 10-20 per cent annually (Pradhan, 2001) and their ornamental value accounts for multimillion dollars. It is a fast emerging and highly competitive industry with thousands of hybrids being added on every year. The commercial applications of tissue culture in mass propagating the hybrids have helped in revolutionizing orchid industry.

Although India is bestowed with orchid resources, our country has made only marginal progress in exploiting its orchid wealth in the international market. The major constraints in this direction are shortage of good quality planting material and lack of their continuous supply. Therefore, in order to meet our demand we have to import them at a very high price. Moreover traditional methods of propagation are all slow processes and help in providing only a few additional orchid plants in a year. All these factors necessitate the production and popularisation of our own varieties and hybrids in the international market. This

can be achieved by the integration of tissue culture techniques with the development of hybrids.

The range of variations in orchids that can be brought about by new gene combinations is tremendous. The compatibility between the genomes of different species and genera of orchids is mainly due to the absence of an effective hybridization barrier which is usually present in other crops (Mercy and Dale, 1997).

Based on the plant structure orchids can be divided into monopodial and sympodial. The genus *Dendrobium* with commercial cut flower qualities is a renowned sympodial epiphytic orchid currently enjoying very high popularity among the commercial orchids in Kerala (Lekha Rani, 2002). *Dendrobium* hybrids are the most popular of the sympodial orchids grown commercially at present. Flower spikes are medium sized with flowers numbering from five to twenty, in colours such as white, mauve, pink, red, blue, purple and yellow (Mercy and Dale, 1997).

Most of the popular hybrids such as *Dendrobium* Sonia, *Dendrobium* Nagoya Pink, *Dendrobium* Pramot etc. grown in Kerala are exotic. Moreover, very few centers are involved in Orchid breeding in India. So we have to develop our own hybrid material adaptable to our agro-climatic conditions that can meet the international standards.

As an initial step to tackle this problem, several indigenous *Dendrobium* hybrids have been developed under the DBT project entitled "Breeding for commercial orchid hybrids" in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani (Lekha Rani, 2002). From this hybrid population about 40 hybrids belonging to different grex combinations have been observed to posses novelty, distinctiveness and uniformity in floral characters for promotion as hybrid varieties, which have to be urgently multiplied. Among these, 9 grexes have been registered with the Royal Horticultural Society, England. Molecular characterization is also

important in the present scenario to investigate the genetic diversity and relatedness among the hybrids prior to release.

In this view, the present study was undertaken with the following objectives.

(i) To undertake initial culture establishment of the 40 selected *Dendrobium* hybrids in the identified best culture medium (Sivamani, 2004).

(ii) To refine the protocol for rapid multiplication using *in vitro* leaf explants.

(iii) To carryout molecular characterization of the 40 selected *Dendrobium* hybrids using RAPD

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Kerala has recently become a very active state in terms of orchid cultivation. The sympodial orchid *Dendrobium* as well as the monopodial orchid *Aranthera* are of great demand in Kerala. Demand of orchids for domestic consumption and export is steadily increasing. In India a good share of the orchids consumed in metros are supplied by the growers of Kerala. But the availability of planting material is insufficient to meet the market demands. So there is a need to develop our own varieties and hybrids that can be made available to the growers at an affordable price.

Tissue culture techniques have opened new possibilities in the conservation and commercialization of plants. They ensure rapid and round the year multiplication of desired genotypes, economize on time and space and are much more advantageous than the conventional means of propagation. Their efficacy is well appreciated in floriculture crops like orchids where the commercial demands exceed the frequency of natural regeneration.

Regeneration can follow different pathways in orchids, depending on the nutritional regime in the culture medium and also the species. According to Murashige (1974) there are three possible routes available for *in vitro* propagule multiplication : (a) enhanced release of axillary buds (b) production of adventitious shoots through organogenesis and (c) somatic embryogenesis.

Orchids are the first horticultural plants cloned by tissue culture methods on a commercial scale. The major break through in the propagation of orchids is the *in vitro* germination of the orchid seed by Knudson (1922). The technique of mericloning orchids was successfully demonstrated for the first time by Morel (Morel, 1960). The technique was further strengthened by subsequent work of Wimber (Wimber, 1965). Since then several workers have successfully utilized tissue culture for mass multiplication of a number of desired orchid clones. Clonal propagation in *in vitro* is called micropropagation.

Continuous release of new varieties lead to the development of new genetic purity diagnostic techniques. Recently various molecular markers have been developed into powerful tools for diversity analysis, cultivar identification and genetic purity testing. Among these, PCR based dominant markers like RAPD have gained wide acceptance and are employed to analyse genome fingerprinting, mapping, gene localization etc. In addition, the RAPD technique is technically the simplest, is less expensive, fast and does not require huge infrastructure to start with.

The available reports on the *in vitro* propagation and molecular characterization of orchids are briefly reviewed here.

2.1 FACTORS INFLUENCING SUCCESS IN MICROPROPAGATION

2.1.1 Explant

The type, size and position of the explant and age of the mother plant as source of explant has an important role in the success of micropropagation (Devi and Deka, 2001).

The non-dividing, differentiated, quiescent cells of the explant (a piece of tissue used to initiate tissue culture) when grown on a nutrient medium undergoes dedifferentiation and redifferentiation to active a whole plant or a plant organ (Chawla, 2002).

In vitro sourced explants were reported to regenerate rather slowly (Arditti and Ernst, 1993). Kaur and Vij (2000) reported that *in vitro* sourced leaf tissues responded far more frequently to a wide range of stimuli in comparison to greenhouse sourced tissue in *Saccolabium papillosum*. Sivamani (2004) reported regeneration of plantlets through PLB formation in *Dendrobium*.

Regeneration in orchids through tissue culture can be either through direct differentiation of PLBs from cultured explants and their subsequent development into plantlets, or through indirect differentiation of PLBs from explant tissues through an intermediary callus phase (Das and Bhattacharjee, 2006).

Martin and Madassery (2006) reported direct organogenesis from *in vitro* derived foliar explants of *Dendrobium* hybrids Sonia 17 and 28, by the induction of PLBs and its conversion to shoots. No significant difference were observed in the induction of direct shoots, shoot multiplication, PLB formation and subsequent shoot development and rooting of shoots between the two cultivars.

2.1.1.1 Size of the Explant

Size of the explant greatly influences the establishment and survival of the culture. Tanaka *et al.* (1975) found earlier formation of PLBs from the leaf segments of 0.8 – 1.2 cm size in *Phalaenopsis* and *Vanda.*

Vij and Pathak (1989) observed maximum number of shoots using pseudobulb segments of *Dendrobium* with a length of 0.5 – 1.0 cm.

Scapes of *Oncidium, Dendrobium* and *Phalaenopsis* cut into single nodes 0.4 – 0.5 cm away from the nodes were used by Nuraini and Shaib (1992).

Chen and Chang (2003) observed that one cm long leaf tip segments of *Oncidium* Gower Ramsey produced clusters of somatic embryos.

Indhumati *et al.* (2004) observed that in *Dendrobium* hybrid Sonia-17 leaf tip and leaf bits of size 1 cm^2 when inoculated on MS, VW and KC media supplemented with different concentrations of BA and NAA remained green and did not show any shoot proliferation.

Shoot tips measuring 0.3 – 0.5 cm size excised from six months old *in vitro* established *Dendrobium* var. Betty Ho could produce multiple shoots in a period of 25-35 days (Kurup, 2005). Stem nodal segment of 1.0 – 1.5 cm length, with one node each from keikis were used for culture initiation in *Dendrobium* hybrids (Sivamani, 2004).

Kishore *et al.* (2005) reported that 0.3 – 0.5 cm long segments of *in vitro* maintained pseudobulbs of the *Dendrobium* hybrids responded well in half MS medium.

2.1.1.2 Position of the Explant

Regenerative potential of leaf base in *Cattleya* was recorded by Champagnat *et al.* (1970). In *Laeliocattleya* and *Epidendrum* leaf tip shows greater regeneration potential (Churchil *et al.*, 1973).

In *Phalaenopsis* and *Vanda*, the proximal tissue of leaves showed better PLB formation than distal ones (Tanaka *et al.*, 1975). Allenberg (1976) reported the regeneration ability of leaf tip in *Paphiopedilum.*

In the studies with leaf explants of *Aranda, Cattleya, Dendrobium* and *Ascocenda* cultivars and hybrids leaf tip did not survive but the leaf parts including the base proliferated and formed calli, which differentiated into plantlets depending on the cultivar (Lay, 1978).

Seeni (1988) reported the regeneration ability of leaf base in *Vanda coerulea.*

Greater regenerative potential of leaf base than leaf tip was reported in *Coelogyne, Dendrobium* and *Oncidium* by Abdulkarim and Hairani (1990). Seeni and Latha (1992) observed that cultured leaf base produced more uniform plantlets in *Renanthera.* Pindel and Miczynski (1996) observed production of plantlets from basal parts of leaves of *Cymbidium* hybrids.

2.1.1.3 Age of the Explant

Young seedling tissue in *Phalaenopsis* was reported to yield better PLBs than that formed by mature tissue (Tanaka *et al.*, 1975).

Yam and Weatherhead (1991) reported that younger leaf tip produced more number of plantlets in *Pholidota chinensis, Liparis virdifiora* and *Acampe rigida.*

Nutritional regimes *in vitro* and physiological age of the donor tissues greatly influence the regeneration potential of explants in orchid tissue culture (Arditti and Ernst, 1993).

Juvenility of explants is another important factor that influences the regeneration response in orchid tissue culture. This is because juvenile cells do not have rigid cell walls and are more active physiologically and biochemically (Kaur and Vij, 2000).

Available literature suggests that use of pre-existing meristems as explant is of great importance in ensuring genetic stability of the micropropagated plants (Das and Bhattacharjee, 2006).

2.1.1.4 Type of Explant

Time required for PLB or callus formation was reported to be greatly influenced by the orchid species, type of explant used and nutritional regime in the culture medium (Arditti and Ernst, 1993). Culture of shoot tip has become a well-established technique for orchid micropropagation since it is useful for maintaining uniformity of genotype. But it requires the sacrifice of the entire new growth or the only growing point (Devi and Deka, 2001).

Meristem or shoot tip remains the first choice as explant for commercial orchid micropropagation. However, a number of other explants were also used successfully by different workers. These include leaf or leaf segments, roots, inflorescence and floral parts, pseudobulbs, stem or nodal segments etc. (Das and Bhattacharjee, 2006).

2.1.1.4.1 Stem Node

Micropropagation of orchids using stem nodal segments as explants were reported by several workers.

When stem segments with a node were cultured for the *in vitro* propagation of *Phalaenopsis*, buds enlarged in two weeks followed by leaf and root development in 6-20 weeks and plantlets were ready for transfer in 30 weeks (Sagawa, 1961).

Sagawa and Sehgal (1967) reported development of plantlets in *Vanda* Miss Joaquim in two to three months by using stem node segments.

Mosich *et al.* (1974) used entire nodes of *Dendrobium* and obtained growth of bud after four weeks and development of plantlets in 45 days.

Yam and Weatherhead (1990) successfully micropropagated *Dendrobium aduncum, Dendrobium lodigesii* and *Dendrobium transparens* using stem node as explants.

Regeneration potential of uninodal explants *via* PLB formation in *Cymbidium pendulum* was examined by Vij *et al.* (1994).

The top nodes of *Phalaenopsis* are used for cyclic propagation of new explants and middle nodes for producing shoots or adventitious buds (Duan *et al.*, 1996).

Jimenez and Guevara (1996) reported regeneration of complete rooted plants from the stem nodal cuttings of commercial hybrids of *Phalaenopsis,* which were ready for transfer within six months of culturing *in vitro.*

Nayak *et al.* (1997) induced high frequency shoot proliferation from shoot segments of *Dendrobium aphyllum* and *Dendrobium moschatum* on MS medium containing thidiazuron.

Regeneration to the extent of 98.5 per cent from nodal explants dissected from eight months old pot grown seedlings of *Spathaglottis plicata* was reported by Teng *et al.* (1997).

Pathania *et al.* (1998) used stem explants procured from shoots emerging from pseudobulbs for successful micropropagation of *Dendrobium* cv. Sonia.

Kanjilal *et al.* (1999) reported PLB formation from stem discs of *Dendrobium moschatum* from third week of culturing onwards. Plantlets were formed in 10-12 weeks after subculturing of the PLBs. The same technique was reported to be successful in *Geodorum densiflorum* (Kanjilal and Datta, 2000).

Isolated nodes of *Anoectochilus sikkimensis* and *A. regalis* cultured for 12 weeks produced maximum number of shoots (Gangaprasad *et al.*, 2000).

Genera like *Cymbidium, Dendrobium, Epidendrum, Phalaenopsis* etc. have been successfully cultured through stem nodal segments. When the stem segments with node are used to start cultures, dormant buds enlarge followed by leaf and root development (Devi and Deka, 2001).

Stem nodal segment with dormant buds cultured *in vitro* showed PLB formation and development of plantlet in *Dendrobium* (Sivamani, 2004).

Keikis are produced from the nodal region of the stem or inflorescence axis. These tiny plantlets are not desirable on an orchid, as keikis indicate that the orchid is becoming old or it is not healthy and many times formation of keikis suppress flower production. However, they can become effective means for multiplication (Das and Bhattacharjee, 2006).

2.1.1.4.2 Leaf

Meristem culture, though widely practiced has its limitation in the fact that it requires sacrifice of an entire new growth. Attempts were therefore made to use other plant parts like leaf, root, inflorescence etc. as explants in orchid micropropgation. Regeneration potential of leaf tissues was demonstrated for the first time by Wimber (1965), who was able to produce PLBs from leaf tissues in *Cymbidum*.

Utility of leaf segments or entire leaf in producing a large number of identical clones through direct or callus mediated organogenesis was emphasized by Arditti (1967).

Regenerative potential of leaf base in *Cattleya* was recorded by Champagnat *et al.* (1970).

Regeneration potential of young leaf tips in *Laeliocattleya* and *Epidendrum* was reported by Churchill *et al.* (1973).

Loh *et al.* (1975) traced the regeneration ability of orchid leaves to the dermal layers. They also reported the regeneration potential of leaf tips in *Aranda.*

Tanaka *et al.* (1975) reported better PLB development from leaf segments of *Vanda* and *Phalaenopsis* when young seedlings was used. Mature tissues required etiolating to induce primordia, which subsequently develop into PLBs.

Allenberg (1976) reported the regeneration ability of leaf tip in *Paphiopedilum.*

In the studies with leaf explants of *Aranda, Cattleya, Dendrobium* and *Ascocenda* cultivars and hybrids leaf tips did not survive but the leaf parts including the base proliferated and formed calli, which differentiated into plantlets depending on the cultivar (Lay, 1978).

Lay (1979) observed that the entire young leaves were better than the leaf section in respect to plantlet production.

Kukulczanka and Wojciechowska (1983) tried leaf explants of *Dendrobium antennathum* and *D. phalaenopsis.* The leaves of *D. antennathum* dried in two to three weeks. In *D. phalaenopsis* discolouration and drying of leaves were observed in basal medium but remained green in medium enriched with peptone.

Monorama *et al.* (1984) traced the regeneration ability of orchid leaves to the dermal layers.

Mathews and Rao (1985) reported greater response from intact leaves in *Vanda,* whereas response from leaf tip were infrequent. A decisive role of leaf base in regeneration of leaf explants was suggested. Seeni (1988) reported the regeneration ability of leaf base in *Vanda coerulea.*

Segments from dorsal parts of PLBs produced plantlets while segments from basal parts produced no plantlets (Amaki and Higuchi, 1989). So far, regeneration ability of orchid leaves has been reported from about 60 species (Wang, 1989).

Greater regenerative potential of leaf base than leaf tip was reported in *Coelogyne, Dendrobium* and *Oncidium* by Abdulkarim and Hairani (1990).

Vij and Pathak (1990) suggested that leaf explants could be induced to become regenerative by manipulating the chemical composition of the culture media *in vitro*. They recorded that leaf explants proliferated along both abaxial and adaxial surfaces and the proliferations were traced to the epidermal or sub-epidermal cells.

Latha and Seeni (1991) reported the initiation of PLBs near the tip region and its quick spread in a sporadic fashion all over the sides of the leaves in *Phalaenopsis* and suggested that the entire surface of the organ was potentially meristematic and regenerative.

Seeni and Latha (1992) cultured young leaf explants of *Renanthera* and observed that cultured leaf bases produced large number of uniform plantlets.

Yam and Weatherhead (1991) reported that younger leaf tip produced more number of plantlets in *Pholidota chinensis, Liparis viridiflora* and *Acampe rigida.*

Segments of young leaves of *Oncidium* cv. Gower Ramsey produced clusters of somatic embryos without intervening callus within one month. Subculturing of these embryos produced more embryos followed by plantlets formation (Paek *et al.*, 1996).

Direct regeneration of shoots were better from leaf explants than from root explants in *Cymbidium* orchids (Pindel and Miczynski, 1996).

Teng *et al.* (1997) obtained regeneration of PLBs and subsequent plantlet development to the extent of 6.5 per cent by using leaf explants in *Spathoglottis plicata.*

Kaur and Vij (2000) reported that culture initiation in *Saccolabium papillosum* leaf segments was markedly influenced by the source and juvenility of the tissues. *In vitro* sourced tissues responded for more frequently to a wide range of stimuli than greenhouse sourced tissues.

Ramsunder *et al.* (2000) reported poor shootlet formation from leaf bits in *Dendrobium* Sonia.

Chen and Chang (2003) observed that one cm long leaf tip segments of *Oncidium* Gower Ramsey cultured *in vitro* on a modified half MS medium with paclobutrazol at 10 mg l^{-1} gave doubling of embryo numbers (193.2) per dish compared to the control treatment (89.4) after three weeks in culture.

Martin and Madassery (2006) reported rapid *in vitro* propagation of *Dendrobium* hybrids Sonia 17 and 18 using in vitro derived leaf explants. Plantlets derived exhibited more than 80% *ex vitro* establishment

2.1.2 Composition of the Culture Medium

In vitro response of an explant largely depends on the composition of the culture medium. The nutritional medium generally consists of inorganic nutrients, energy sources, vitamins, growth regulators and organic supplements.

2.1.2.1 Nutrient Medium

A number of chemically defined media and their modifications have been used successfully for orchid micropropagation. Commonly used media are Knudson C (1946), Vacin and Went (1949), Murashige and Skoog (1962), Heller (1953), Ichihashi (1979) and Nitsch (1969). Some workers have also made use of other media like Hyponex, Knop, Whites etc. However, Vacin and

Went (1949), Knudson C (1946) and Murashige and Skoog (1962) are the most commonly used media (Das and Bhattacharjee, 2006).

Sagawa and Shoji (1967) used modified VW medium for efficient protocorm development and vigorous plant growth in *Dendrobium.*

Churchill *et al.* (1973) found that *Epidendrum* leaf tip formed calli on MS medium.

Teo *et al.* (1973) reported that liquid VW medium supplemented with CW and sucrose could be employed for the initiation and multiplication of PLBs in *Vanda.*

Irawati *et al.* (1977) reported that the best growth and survival rates were obtained in *Dendrobium* when cultured in modified KC medium based on studies with *Aranda, Cattelya, Dendrobium* and *Ascocenda* cv.and hybrids in three different media.

Lay (1978) reported a media based on Murashige and Skoog inorganic salts and CW to be the most successful for leaf tissue culture of *Aranda, Ascocenda* and *Cattleya.*

All *Dendrobium* explants cultured on modified KC, VW or modified VW appeared expanded, but only the ones on modified VW medium continued to grow (Fernando, 1979).

Lim-Ho (1981) used liquid VW medium supplemented with CW for shoot tip culture of *Arachnis.* A solidified VW was used for PLB multiplication and the multiplied PLBs were then grown on a VW based formulation.

Soediono (1983) found that VW medium containing 15 per cent (by volume) coconut water was suitable for initial culture of *Dendrobium.*

Regeneration of several orchid species using leaf explants on three media namely Mitra, MS and modified KC with various combinations of growth regulators was reported by Vij and Pathak (1990).

PLB initiation in *Aranda* Deborah was achieved in Liquid Knudson C medium, but liquid VW medium was the best for further proliferation and solidified VW medium for faster plantlet development (Goh and Wong, 1990).

Shimasaki and Uemoto (1991) reported that MS medium with a concentration of 1/8 gave best results in axillary bud culture.

Seeni and Latha (1992) cultured young leaf explants of *Renanthera* on Mitra *et al.* (1976) medium.

MS basal medium supported rapid proliferation of multiple shoots from stem node segments in *Vanilla walkeriae* (Agarwal *et al.*, 1992).

Half strength MS and VW media were found best for bud initiation in *Dendrobium* (Sudeep, 1994). He also obtained more shoots in half MS medium than with VW medium.

Chan and Lee (1996) reported that high percentage of PLBs could be obtained in *Aranda, Mokara* and *Dendrobium* when cultured in VW medium.

Devi *et al.* (1997) reported that Nitsch medium was the best for formation and proliferation of PLBs.

Kuriakose (1997) observed that minimum number of days for bud initiation was recorded in VW basal medium.

Nayak *et al.* (1997) reported high frequency of shoot proliferation of *Dendrobium* in MS medium containing thidiazuron.

Both VW and KC media favoured formation of PLBs and subsequent development of plantlets of *Dendrobium* Sonia. Knudson C medium was the best for multiplication of PLBs (Pathania *et al.*, 1998).

Park *et al.* (1998) observed the highest ratio of PLB multiplication in VW medium while shoot regeneration was the most effective on hyponex medium.

Chen *et al.* (1999) reported direct somatic embryogenesis from leaf explants of *Oncidium* cv. Gower Ramsey in half MS medium.

Among the different strengths of MS media compared, full MS medium proved to be the best for multiple shoot induction in terms of number of shoots per culture, number of leaves per microshoot and shoot length in *Dendrobium* (Ganga *et al.*, 1999).

Santana and Chapparro (1999) used Knudson C medium to induce PLB formation and liquid MS for PLB multiplication and plantlet formation of *Oncidium* cv. Gower Ramsey.

In *Dendrobium* Sonia the per cent explants established, number of PLBs produced and number of shootlets per explant were high when cultured in KC medium (Ramsunder *et al.*, 2000).

Lekha Rani (2002) stated that the basal medium MS half strength was found to be the best for early germination and rapid *in vitro* development in *Dendrobium* as compared to MS quarter strength and MS full strength, KC and VW full strength.

Sivamani (2004) reported that among the various basal media tried with stem nodal segment explants, VW medium showed early culture establishment and rapid growth recording minimum number of days for PLB initiation, greening of PLB's and for first leaf initiation.

Shylaraj *et al.* (2005) reported that agitated liquid Vacin and Went medium could induce a large number of protocorms within a period of two weeks, whereas agitated liquid MS medium was identified as the rapid multiplication medium of the protocorms.

Kishore *et al.* (2005) studied the regeneration capacity of pseudobulb segments of a *Dendrobium* hybrid (*D. nobile* L. x *D. chrysotoxum* L.) *in vitro* on full strength (MS) and half strength MS (½ MS) medium. They found that in ½ MS medium the explant responded well.

Martin and Madassery (2006) reportd that *in vitro* derived leaf explants from *Dendrobium* hybrids Sonia 17 and 28 when cultured on half strength MS medium supplemented with 44.4 microM BA developed more than seven shoots per explant. The isolated shoots transferred on to the same medium induced PLBs from the base within 60 days, which upon transferred to fresh medium having the same level of BA facilitated rapid proliferation.

Jain and Babbar (2005) used Mitra's medium supplemented with 2% sucrose, peptone $1gl^{-1}$, BA 1 mg^{-1}, 3% guargum or 3% isabgol for the successful in vitro multiplication of *Dendrobium chrysotoxum* from leaf explants.

Chung *et al.* (2005) observed that the best media for direct embryo induction from leaf explants of *Dendrobium* cv. Chiangmai Pink was half strength MS with 18-16 microM TDZ (1-Phenyl-3-(1,2,3-thiadiazol-5-yl)-urea).

Kumaria *et al.* (2005) reported that in *Dendrobium wardianum* leaf explants cultured on MS medium supplemented with 2.5 microM NAA and 10.0 microM BA gave the optimum PLB formation.The nodal buds started developing in approximately 3 weeks of culture in MS medium supplemented with BA alone or in combination with IAA or NAA.

2.1.2.2 Growth Regulators

Plant growth substances are messenger substances which influence growth and development. The two main classes of growth regulators are the auxins and cytokinins, while the others *viz.*, gibberellins, abscisic acid, ethylene etc. are of minor importance.

Most commonly used growth regulators in orchid tissue culture are Indole-3-bytyric acid (IBA), Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), 2,4-Dichlorphenoxyacetic acid (2,4-D), Benzylamino purine (BAP), Kinetin (KN) and Benzyl adnine (BA). Growth promoting effect of these growth adjuncts was reported to be orchid specific and therefore, cannot be generalized; rather they

need to be empirically determined for each species and hybrid (Prakash *et al.*, 1996).

2.1.2.2.1 Auxin

A common feature of auxin is their property to induce cell division, cell elongation, swelling of tissues, formation of callus and the formation of adventitious roots.

Fonnesbech (1972) observed that IAA had no effect on *Cymbidium* PLB formation when used alone. NAA results in optimal fresh weight at 10 µm and the protocorms were vigorous but lighter green.

Kukulczanka and Wojciechowska (1983) reported more number of roots in medium supplemented with 1.75 mg l^{-1} NAA and 1.75 mg l^{-1} IBA in *Dendrobium antennathum* and *D. phalaenopsis*.

Soediono (1983) found that addition of 10 ppm NAA to VW medium led to rapid proliferation of PLBs in *Dendrobium*.

Beneficial effect of NAA when used with BAP in leaf tissue culture of *Vanda* was reported by Vij *et al.* (1986).

Acceleration in growth of PLBs of *Mormodes histrio* by potassium salt of NAA was recorded by Holters and Zimmer (1990).

Exogenous supply of IAA was reported to increase peroxidase activity, which is associated with plant defence against pathogens (Kumaria *et al.*,2005).

Rooting in *Dendrobium* was reported to be favoured by NAA (Sharon and Vasundhara, 1990).

Devi and Deka (1992) observed that the growth of hybrid seedlings of the cross *Dendrobium moschatum* x *D. amoenum* was enhanced by IAA and NAA at 1 mg l^{-1} each.

The addition of 2.0 mg l^{-1} IBA to the medium was necessary for root formation of *Phalaenopsis* hybrid and *Dendrobium* Miss Hawai (Nuraini and Shaib, 1992).

IBA at 0.1 mg l^{-1} was the best for producing many long rooted shoots in *Dendrobium* (Lim *et al.*, 1993).

Mujib and Jana (1994) observed induction of roots in *Dendrobium* Madame Pompadour in the presence of NAA at 0.1 mg l^{-1}.

Vij *et al.* (1994) suggested that for shoot multiplication in *Cymbidium pendulum* BA was required in the basal medium.

Nayak *et al.* (1997) found that rooting of *Dendrobium* was favoured by medium supplemented with IBA.

Pathania *et al.* (1998) found that rooting of *Dendrobium* cv. Sonia was favoured by medium supplemented with IBA 1.0 mg l^{-1} or NAA 1.8 mg l^{-1}.

Growth and plant differentiation were the best in medium containing 0.5 mg l^{-1} IBA from *Cymbidium longifolium* protocorms *in vitro* (Siddique and Paswan, 1998).

Fang *et al.* (1999) reported effective rooting of *Anoectochilus formosanus* with the addition of NAA 0.5 mg l^{-1}.

Kanjilal *et al.* (1999) reported significant increase in PLB production of *Dendrobium moschatum* with 2,4-D at 1 mg l^{-1} and IAA at 2.0 mg l^{-1}.

Roots induced by NAA were shorter and thicker than those by IAA at 1.0 mg l^{-1} in *Ipsea malabarica* (Gangaprasad *et al.*, 1999).

Vij *et al.* (2000) found IBA to be more beneficial as compared to IAA; replacement of IAA in the medium with IBA resulted in 100 per cent shoot bud regeneration.

At low auxin concentration adventitious root formation predominates, whereas at high auxin concentration, root formation fails to occur and callus formation takes place (Chawla, 2002).

2.1.2.2.2 Cytokinin

Cytokinins in general favours cell division, enhanced release of axillary buds, induction of multiple shoot and development of adventious buds. Along with auxins cytokinins are used for the initiation and maintenance of callus.

Kinetin at 100 μm induced growth of shoots from protocorms of *Cymbidium*. BA had similar effects but at lower concentrations (Fonnesbech, 1972).

Kim and Kako (1982) found that addition of BA encouraged PLB formation and shoot development.

The greatest number of shoots in *Dendrobium antennathum* and *D. phalaenopsis* was obtained by enriching the medium with BA at 5.0 mg l^{-1} (Kukulczanka and Wojciechowska, 1983).

Lin (1986) reported the effect of BAP or kinetin in inducing callus or multiple shoots in *Phalaenopsis*.

Shoot growth of *Cymbidium* hybrid was the greatest when explants were cultured on a medium containing BA at 10 mg l^{-1} for 10 days and then transferred to a medium containing 0.5 mg l^{-1} BA (Paek *et al.*, 1989).

The leaves of *Laelio cattleya* cultured in medium with 0.5 – 1.0 mg l^{-1} BA gave higher yield of shoots (67 per explant) than medium without BA (Matos and Garcia, 1991).

Yam and Weatherhead (1991) reported that more number of plantlets could be obtained from leaf tip culture of *Pholidota chinensis* and *Acampe rigida* on medium containing 1.0 mg l^{-1} BA and 0.2 mg l^{-1} NAA. They also reported that

Liparis viridiflora leaf tips produced shoots more rapidly on medium containing BA 1.0 mg l^{-1}.

BA caused marked acceleration in formation of PLB in *Catasetum fimbriatum* (Colli and Kerbauy, 1993).

BAP at the rate of 1.0 mg l^{-1} was reported to promote callus mediated PLB whereas, a higher dose (2.0 mg l^{-1}) BAP promoted regeneration through multiple shoot bud formation in uninodal segment culture of *Bletilla striata* (Vij and Dhiman, 1997).

The pseudobulb segments of *Dendrobium* hybrid cultured in ½ MS medium with 2.0 mg l^{-1} KN induced shoot bud formation (Kishore *et al.*, 2005).

Kurup *et al.* (2005) reported that MS basal medium containing 5.0 mg l^{-1} BA and 1000 mg l^{-1} CH could initiate multiple shoots in *Dendrobium* var. Betty Ho using *in vitro* sourced shoot tip as explant.

Half strength MS medium containing 6.97 micro M KN facilitated conversion of more than 90 per cent PLBs to shoots in leaf explant culture of *Dendrobium* hybrids Sonia 17 and 28 (Martin and Madassery,2006).

2.1.2.2.3 Auxins and Cytokinins

The effects of combinations of auxins and cytokinins vary from orchid to orchid, growth regulators used, their concentration and ratios.

Fonnesbech (1972) observed optimal growth and best development of protocorms in *Cymbidium* at a growth regulator concentration of 10 µm NAA + 1 µm KN.

A high cocentration of NAA and BA was reported to induce maximum proliferation of shoots in *Cattleya* (Kusumoto, 1980).

High level of cytokinin had deleterious effects on shoot growth. Auxin added to the medium nullified the suppressive effects of high cytokinin contents on axillarry shoot growth (Ludergan and Janick, 1980).

Vij and Sood (1982) reported that a combined treatment of BAP and NAA was essential for root development in pseudobulb derived shoots of *Dendrobium moschatum.*

Latha and Seeni (1991) reported that highest number of PLBs were formed in *Phalaenopsis* when a combination of BA 10.0 mg l^{-1} and NAA 1.0 mg l^{-1} was employed.

For production of plantlets with shoots as well as roots, optimal dose for BA and NAA were 0.1 mg l^{-1} and 1.0 mg l^{-1} respectively (Shimasaki and Uemoto, 1991).

Seeni and Latha (1992) cultured young leaf explants of *Renanthera* in medium supplemented with BA, NAA and peptone.

Lakshmidevi (1992) observed that shoots of *Dendrobium* on 2.0 mg l^{-1} NAA and 3.0 mg l^{-1} BA had rapid shoot growth and well expanded leaves. With 5.0 mg l^{-1} BA, shoot production was found to increase but short shoots with small leaves were produced.

Influence of IBA and Kinetin in good shoot production of *Dendrobium moniliforme* was reported by Lim *et al.* (1993).

Tokuhara and Mii (1993) reported the highest rate of PLB formation (60 per cent) in *Phalaenopsis* and *Doritaenopsis* with 20 mg l^{-1} BA and 0.1 mg l^{-1} NAA.

Mujib and Jana (1994) reported that supplementation of 2,4-D and BA or NAA and BA in media resulted in callus induction from apical meristem of *Dendrobium.*

Sounderrajan and Lokeswari (1994) observed better shoot multiplication of *Dendrobium* Madame Pompadour when the medium was supplemented with BA 0.5 mg l^{-1} and NAA 0.1 mg l^{-1}.

NAA 1.0 mg l^{-1} together with BA 3.0 mg l^{-1} was found to be the best for bud initiation in both half strength MS and VW media. Application of BA resulted in increased shoot proliferation (Sudeep, 1994).

Leaf explants of *Cymbidium* hybrid produced plantlets in the presence of BA and NAA at 22 μm each (Pindel and Miczynski, 1996).

The NAA to BA ratio for PLB induction and plantlet development were 12.2 and 0.3 – 1.2 respectively. Maximum shoot proliferation was observed with Kinetin 7.5 mg l^{-1} and NAA 2.0 mg l^{-1} (Kuriakose, 1997).

A combination of 0.44 M BA and 5.37 M NAA was reported to induce the greatest regeneration of *Spathoglottis plicata* from leaf and stem nodal explants (Teng *et al.*, 1997).

Nayak *et al.* (1997) reported that high frequency shoot proliferation from shoot segments of *Cymbidium* and *Dendrobium* was observed on medium supplemented with BA or TDZ; NAA and BA were suggested to influence shoot elongation, whereas rooting of the shoots was obtained with supplementation of IBA.

Presence of NAA 0.1 mg l^{-1} and BAP 3-4 mg l^{-1} in the media was reported to assist multiple shoot formation in *Dendrobium* (Devi and Laishram, 1998).

Multiplication of PLB was reported in *Dendrobium* cv Sonia with BA at 1.5 mgl^{-1} and NAA at 0.4 mg l^{-1} (Pathania *et al.*, 1998).

PLB multiplication and plantlet formation of *Oncidium* Gower Ramsey was the best in the presence of NAA at 0.5 mg l^{-1} and BA at 5.0 mg l^{-1} (Santana and Chapparro, 1999).

PLB production was increased in *Geodorum densiflorum* by the addition of BA at 3.0 mg l^{-1} and NAA at 0.5 mg l^{-1}. Regeneration from PLBs of *Cymbidium aloifolium* showed well developed roots and a large number of shoot buds in the medium supplemented with Kinetin, NAA and IBA at 5.0 mg l^{-1} each (Buzarbarua, 1999).

The establishment of the explants, number of PLBs and shootlets per explant were higher in the combination of BAP 2.0 mg l^{-1} and NAA 1.0 mg l^{-1} in *Dendrobium* Sonia (Ramsundar *et al.*, 2000).

Differentiation of shoots from the PLBs of orchid *Cymbidium* hybrid was the best in a medium supplemented with NAA at 4.0 mg l^{-1} and kinetin 0.05 mg l^{-1} (Prasad and Verma, 2001).

Combination of IAA 1.0 mg l^{-1} and BA 1.0 mg l^{-1} with CW 150 ml l^{-1} produced the highest frequency of germination and protocorm development in *Cymbidium pendulum* (Talukdar *et al.*, 2002).

Optimum callusing was recorded in the presence of 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP of *Dendrobium fimbriatum* when cultured on modified nutrient solution of KC (Roy and Banerjee, 2003).

Sivamani (2004) reported that the best *in vitro* culture medium for the rapid micropropagation of *Dendrobium* hybrids is VW with KN 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} + CW 200 ml l^{-1}, using stem nodal explant.

Shylaraj *et al.* (2005) reported that when the shoot tip explants of *Dendrobium* var Sonia were cultured in agitated liquid VW medium with BA 2.0 – 2.5 mg l^{-1} and NAA 0.5 – 1.0 mg l^{-1} could induce a large number of protocorms within two weeks. Agitated liquid MS medium supplemented with IBA 0.5 mg l^{-1} and 2.0 mg l^{-1} BA or 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} GA_3 and 3.0 mg l^{-1} BA was identified as the rapid multiplication medium of the protocorms.

A combination of 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BAP was reported to induce the greatest regeneration from pseudobulb segments of a *Dendrobium* hybrid (*D. nobile* L. x *D. chrysotoxum* L.) (Kishore *et al.*, 2005).

Indhumati *et al.* (2004) reported that stem nodal segments of *Dendrobium* hybrid Sonia-17 performed best in the KC medium supplemented with BAP at 5 mg l^{-1} and NAA at 3 mg l^{-1}

2.1.2.3 Complex Additives

Most commonly used complex additives in orchid tissue culture include coconut water, casein hydrolysate, banana homogenate, potato homogenate, tomato juice and peptone. With the exception of protein hydrolysate and coconut water, most of the others are used as a last resort (Chawla, 2002).

2.1.2.3.1 Casein Hydrolysate (CH)

Cultured cells are normally capable of synthesizing all the required amino acids, but it is often beneficial to supplement the medium with complex substances rich in amino acids like casein hydrolysate (Chawla, 2002).

In cases where nutritional requirements have not been established mixtures of amino acids such as casein hydrolysate (CH) may be added between 0.05 and 0.10 per cent. Incorporation of CH enhance the frequency of adventitious shoot formation (Huang and Murashige, 1977).

Beneficial effect of CW, inositol and casein hydrolysate in profuse PLB formation in *Phalaenopsis* was recorded by Hass-Von Schumude (1983).

Chaturvedi and Sharma (1986) reported that root tip explants of *Vanda* form PLBs in the optimum treatment containing BAP 1.0 mg l^{-1}, IAA 1.0 mg l^{-1} and CH 200 mg l^{-1}. The root explants with differentiated PLBs when subcultured in treatment containing BAP 0.5 mg l^{-1}, IAA 1.0 mg l^{-1}, 2,4-D 0.1 mg l^{-1} and CH 200 mg l^{-1} showed enormous proliferation of PLBs.

Latha and Seeni (1991) induced PLB formation from root explants of *Phalaenopsis* on KC medium supplemented with 500 mg l^{-1} casein hydrolysate.

For cell culture it is good to add 0.2 to 1.0 g l^{-1} of casein hydrolysate or vitamin free casamino acids (Chawla, 2002).

Protein digests like casein hydrolysate (0.02 to 0.1 per cent) are used sometimes, as a non-specific source of amino acids. It is beneficial for the culture of small sized embryos and for somatic embryogenesis in many species. It prevents precocious germination and abnormal development of somatic embryoids (Rajmohan *et al.*, 2004).

Kurup *et al.* (2005) observed that multiple shoots were initiated in a period of 25-35 days from shoot tips excised from *in vitro* established plant when the MS basal medium contains 5.0 mg l^{-1} BA and 1000 mg l^{-1} CH.

2.2 MOLECULAR CHARACTERISATION

2.2.1 Molecular Markers

Molecular markers that reveal polymorphism at the DNA level are known as DNA markers. They provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983). Various types of molecular markers are utilized to evaluate DNA polymorphism and among them, the most important is polymerase chain reaction (PCR) based markers.

2.2.1.1 Polymerase Chain Reaction based DNA Markers

Molecular characterization is possible using a wide range of characters and molecular markers in orchids. Among the PCR based markers techniques, the important ones used in orchids are Random Amplified Polymorphic DNA (RAPD), AFLP, minisatellite and microsatellite.

2.2.1.1.1 Random Amplified Polymorphic DNA (RAPD)

This method was first developed by Welsh and McClelland (1990) and Williams *et al.* (1990). The advantage of RAPD when compared to RFLP is that RAPD is less labour intensive, requires smaller quantities of genome DNA, is less costly and quicker than RFLP. It can be used to detect even single gene mutations (Williams *et al.*, 1990).

RAPD was reported to be used for population studies by Astley (1992). RAPD was used for identification of genome specific markers and other uses by Williams *et al.* (1990) and Erlich *et al.* (1991).

Several authors have applied the RAPD technique to investigate genetic variability and found the technique very efficient and reliable (Brown *et al.*, 1993).

RAPD technique have been used for the identification of hybrids and their parent determination as well. Wang *et al.* (2003) proposed RAPD fingerprinting as a convenient tool for the identification, protection and parentage determination of plant hybrids.

RAPD markers are commonly used for molecular characterization studies despite discrepancies in reliability (Peteira *et al.*, 1999).

RAPD analysis in particular has proven to be a rapid and efficient means for genome mapping (Williams *et al.*, 1990).

RAPD is well suited for genetic resource characterization (Anderson and Fairbanks, 1990).

RAPD relies on the repeatable amplification of DNA sequence amplified using arbitrary primers to provide DNA fingerprints (Rout *et al.*, 2006).

Advances in the use of molecular genetic markers have enabled research on genetic variation at the DNA level (Ben-Meir *et al.*, 1997).

DNA marker polymorphism analyses are advantageous for genetic resource characterization, since DNA markers represent only genetic variation and are not subject to environmental influence (Anderson and Fairbanks, 1990). The DNA markers are extremely useful for testing the clonal fidelity as well as identification (Paden *et al.*, 1996).

2.2.1.1.1.1 RAPD in Orchids

Fu *et al.* (1994) reported that RAPD technique was used to identify wild *Phalaenopsis* species and to study their relationship.

DNA polymorphism within the genus *Cattleya* was studied using RAPD (Benner *et al.*, 1995). A high level of molecular variability was detected among the eight species, with each of them exhibiting an unique DNA fingerprint with 9 out of 10 arbitrary primers used in single primer RAPD reactions.

Choi et al.(1998) used RAPD analysis for studying the compatability of Korean native *Cymbidium goeringii* with other *Cymbidium* species. RAPD analysis indicated that the taxonomic relationship between *Cymbidium goeringii* and either *Cymbidium faberi* and *Cymbidium aloifolium* was distant, with no compatability and even more distant in the case of *Dendrobium chrysotoxum* or *Phalaenopsis* which have different chromosome numbers from the *Cymbidium* species.

Grunanger *et al.* (1998) characterized the genetic material of seven Italian populations of the *Ophrys bertolonii* using RAPD markers. They reported a high genetic variability occurring within the same population.

Okeyo and Kako (1998) reported that RAPD technique was used to study genetic diversity and to identify *Cymbidium* cultivars. A total of 132 RAPD markers, 78 per cent of which were polymorphic were produced from 15, 10-mer arbitrary primers. All cultivars were distinguishable when a number of primers were considered.

Lim *et al.* (1999) used RAPD markers to study the genetic closeness of various species of *Vanda.* They reported that five strap-leaved *Vanda* species (including *Vanda sandariana* and *Ascocentrum miniatum*) were more closely related to each other than to the terete-leaved *Vanda* species. On the basis of RAPD analysis, terete-leaved *Vanda* species and *Vanda hookeriana* could be grouped in a separate genus, *Papilionanthe,* while *Vanda sanderiana* remains in the genus *Vanda.*

Molecular (RAPD) analysis of some taxa of the *Ophrys bertolonii* aggregate (Orchidaceae) was done by Caporali *et al.* (2001). The RAPD methodology was utilized for analyzing the genetic material for four allopatric populations of the *Ophrys bertolonii formis* type, as well as of two populations of *Ophrys bertolonii* together with *Ophrys fuciflora* as out group. Significant genetic diversity was observed for six taxa under examination, thus suggesting separation at specific (or subspecific) level.

Phenetic relationship and identification of subtribe *Oncidiinae* genotype by RAPD markers was done by Taai *et al.* (2002). The study was conducted to generate random amplified polymorphic DNA markers for 24 accessions of subtribe *Oncidiinae* (one of the diverse groups in the orchid family) and to determine the phenetic relationship among them. Eighty decamer primers were screened and 14 primers producing clear and reproducible DNA patterns were selected. In total, 263 bands were scored of which 257 revealed polymorphism. Cluster analysis based on molecular data of band distribution in all the samples showed six major clusters and one independent cluster.

Fujii *et al.* (2001) studied the effects of explants and plant hormones on putative variability in cloned *Cymbidium* plantlets using random amplified polymorphic DNA (RAPD) analysis. A total of 113 distinct major RAPD bands were consistently generated from 18 primers. The appearance of polymorphic bands in cloned plantlets clearly demonstrates somaclonal variation and suggest

that the apperance of putative variability may relate to the type of explants used and the plant hormone added for plant tissue culture.

Screening for genetic markers in a *Dendrobium* hybrid, White Angel x (Lucian Pink x Black Spider), a potential cutflower variety was done by Hong *et al.* (2003). Results of the analysis yielded more than 24 markers that could be linked to such phenotypes as overall yield, inflorescence length, vase life, days to flowering and flower colour.

Pillai (2003) reported molecular characterization of fifteen *Dendrobium* varieties using RAPD technique. The primers OPA-19, OPB-02, OPB-04 and OPB-10 yielded good resolution bands out of 40 decamer primers tested. These primers amplify 44 RAPD markers of which 39 were polymorphic and five were monomorphic. The 15 varieties were divided into six clusters in the dendrogram.

RAPD analysis was used to characterize genetic variability and relationships among 12 cultivars of *Dendrobium* at molecular level. The primers OPB-11, OPB-12 and OPB-17 were identified for RAPD analysis based on their performance in DNA amplification, reproducibility and production of highest number of polymorphic bands as well as intense bands. The three primers together produced 27 bands. Among these, 24 were polymorphic (Krishnapriya, 2005).

Chakrabarti (2005) used RAPD technique to study the genetic variability and relation of 15 species of three morphologically distinct groups of orchids belonging to the subfamily Epidendroideae and tribe *Dendrtebieae* and Cymbideae. A total of 227 distinct major RAPD bands of which 97 per cent were polymorphic were generated from 15 arbitrary primers. The molecular analysis grouped all the species into five groups. The polymorphic pattern generated by RAPD profiles showed different degrees of genetic relationship among the species studied and the RAPD markers were found to be an useful tool for detecting genetic variation within the species of three important genera of orchids.

Ding *et al.* (2005) used RAPD markers for molecular authentication of 8 wild populations of *Dendrobium officinale*. A total of 104 amplified bands were generated, 95 were polymorphic, corresponding to 91.35% genetic polymorphism. Primer S412 was used to authenticate 8 wild populations completely.

The genetic diversity of an epiphytic orchid, *Dendrobium moniliforme* (L) Sw. were investigated using RAPD. UPGMA dendrogram based on RAPD data showed two principal clusters differing from each other with genetic variability (Kobayashi and Iwami, 2005).

2.2.1.1.1.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP is based on PCR amplification of restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Vos *et al.*, 1995). Limited application of AFLP in orchids are reported.

The AFLP fingerprinting was used to determine the level of genetic diversity in the population of *Orchis simian* (Faridah *et al.*, 1998). Hedren *et al.* (1998) reported that AFLP proved to be a powerful tool in elucidating detailed relationship in *Dactylorhiza* polyploidy complex.

2.2.1.1.1.3 Minisatellites [Variable number of tandem repeats (VNTR)]

Minisatellites are repeat sequences having repeat units ranging from 11 to 60 bp in length. The repeat sequences comprises upto 90 per cent of total DNA in certain plant genome. The conserved sequence flanking minisatellites can be amplified using a suitable primer to reveal the polymorphism. The polymorphism are attributed to the variation in the length of minisatellites.

Cafesso *et al.* (2001) reported the occurrence of a tandem repeat in the chloroplast genome of the marsh orchid *Orchis palustris*. The repeat unit is an 'AT' rich, 16 bp sequence located in the chloroplast tRNA. The 16 bp repeat unit was found to be present in all *Orchis palustris* accessions studied, as well as in closely related *Orchis laxiflora*.

In contrast to RAPD, the banding pattern yielded by minisatellite probes is highly reproducible but it involves high cost and complexity.

2.2.1.1.1.4 Microsatellite

Microsatellite markers, also known as simple sequence repeats or SSRs and are cluster of short (usually 2 to 6) tandemly repeated nucleotide bases distributed throughout the genome (Litt and Luty, 1989).

Solvia *et al.* (2000) isolated and characterized a microsatellite loci from O*phrys araneola* to study the influence of pollinating system on population genetic structure and on gene flow between similar co-flowering *Ophrys* species.

The microsatellites are highly polymorphic and thus highly informative. Being shorter in length, they are easy to be cloned, sequenced and amplified through PCR. But the identification of informative microsatellite loci and consequence of suitable primer sequence is more cumbersome and expensive.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present investigations were carried out in the Department of Plant Breeding and Genetics and the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2005-2006. The study formed a part of the DBT project entitled "Breeding for commercial orchid hybrids".

The experiment consisted of the following major studies:

1. To undertake initial culture establishment of the selected *Dendrobium* hybrids in the identified best culture medium.
2. To refine the protocol for rapid multiplication using *in vitro* leaf explants.
3. To carry out molecular characterization of the 40 selected *Dendrobium* hybrids.

3.1 INITIAL CULTURE ESTABLISHMENT OF THE 40 SELECTED *DENDROBIUM* HYBRIDS

3.1.1 Varieties

The base material for the study consisted of *Dendrobium* hybrids already developed under the DBT project, "Breeding for commercial orchid hybrids" and maintained in the greenhouse. From among the 500 flowering hybrids, 40 were selected based on novelty, distinctiveness and uniformity in floral characters and were used for the study. These 40 hybrids belong to 9 grex (hybrid) combinations (Table.1) and have been registered with the Royal Horticultural Society, England.

3.1.2 Explant

3.1.2.1 Stem Nodal Segment

Stem nodal segments of 1.0 – 1.5 cm length each, with one node were excised from keikis. Keikis are produced from the nodal region of the stem.

Plate 1. Flowers of some of the selected *Dendrobium* hybrids

Table 1. The description of the 40 selected *Dendrobium* hybrids

Hybrids	Parents	Description
20	Pramot × CSTD	Long arching inflorescences with 10-12 flowers per inflorescence. Flowers large, deep pink and striped with broad, rounded appearance. Sepals and petals thick and glossy, labellum attractively rounded.
33	Pramot × CSTD	Medium long arching inflorescences with 10-14 flowers per inflorescence. Flowers medium large, pink and striped with prominent shading on sepals and petals. Sepals and petals thick and glossy
59	Nagoya Pink × CSTD	Medium long arching inflorescences with 5-8 flowers per inflorescence. Flowers large, deep pink and striped with full appearance. Sepals and petals thick and glossy.
60	Nagoya Pink × CSTD	Long arching inflorescences with 8-12 flowers per inflorescence. Flowers large, light pink with prominent dark pink stripes and full appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy.
61	Nagoya Pink × CSTD	Short arching inflorescences with 5-8 flowers per inflorescence. Flowers large, purplish magenta coloured, striped and with full appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy.
63	Nagoya Pink × CSTD	Medium long arching inflorescences with 7-12 flowers per inflorescence. Flowers large, light pink and striped with full appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy.

Table 1. Continued

Hybrids	Parents	Description
93	Chiangmai Pink × CSTD	Medium long arching inflorescences with 7-12 flowers per inflorescence. Flowers medium large, striped and light magenta with a green tinge, having rounded, full appearance. Sepals and petals medium thick and glossy.
100D	Nagoya Pink × CSTD	Medium long arching inflorescences with 6-10 flowers per inflorescence. Flowers large, deep pink and striped with rounded, full appearance. Sepals and petals thick and glossy.
102	Sakura × White Fairy	Medium long arching inflorescences with 8-12 flowers per inflorescence. Flowers medium large, very light pink and stellar appearance. Sepals and petals medium thick and glossy.
111	Pramot × CSTD	Long arching inflorescences with 8-12 flowers per inflorescence. Flowers large, dark magenta coloured and lightly striped with full appearance. Sepals and petals thick and glossy.
122	Pramot × CSTD	Long arching inflorescences with 8-12 flowers per inflorescence. Flowers large, deep pink and striped with a full appearance. Sepals and petals thick and glossy.
124	Pramot × CSTD	Medium long erect inflorescences with 6-8 flowers per inflorescence. Flowers large, pink and striped with novel shading on the prominently spatula petals. Sepals and petals thick and glossy.
127	Pramot × CSTD	Long erect inflorescences with 10-14 flowers per inflorescence. Flowers medium large, pink and striped with novel shading on the prominently spatula petals. Sepals and petals thick and glossy.

Table 1. Continued

Hybrids	Parents	Description
132	Nagoya Pink × CSTD	Long arching inflorescences with 9-12 flowers per inflorescence. Flowers large, magenta coloured and striped with full appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy.
147	Sonia 16 × CSTD	Medium long arching inflorescences with 6-10 flowers per inflorescence. Flowers large and dark magenta coloured with rounded, full appearance. Sepals and petals thick and glossy.
176	Rungnapa × CSTD	Long slightly arching inflorescences with 10-15 flowers per inflorescence. Flowers large, purplish magenta coloured and striped with full appearance. Sepals and petals thick and glossy.
177	Sonia 16 × CSTD	Long arching inflorescences with 8-12 flowers per inflorescence. Flowers large and dark purple coloured with stellar appearance. Sepals and petals thick and glossy.
210	Rungnapa × CSTD	Medium large slightly arching inflorescences with 7-10 flowers per inflorescence. Flowers large and faintly striped, magenta coloured with rounded, full appearance. Sepals and petals thick and glossy.
223	Chiangmai Pink × CSTD	Long arching inflorescences with 10-14 flowers per inflorescence. Flowers medium large and flat, with a squarish appearance, light pink and greenish white with stripes. Sepals and petals moderately thick and glossy.

Table 1. Continued

Hybrids	Parents	Description
231	Rungnapa × CSTD	Medium long arching inflorescences with 6-10 flowers per inflorescence. Flowers medium large and dark magenta coloured with rounded, full appearance. Sepals and petals thick and glossy.
234	Pramot × CSTD	Long arching inflorescences with 6-10 flowers per inflorescence. Flowers large and dark magenta coloured, lightly striped with rounded, full appearance. Sepals and petals thick and glossy.
247	Chiangmai Pink × CSTD	Long erect inflorescences with 5-9 flowers per inflorescence. Flowers large , squarish, dark pink stripes, having full appearance. Sepals and petals thick and glossy.
253	Pramot × CSTD	Short arching inflorescences with 5-8 flowers per inflorescence. Flowers large, pink and striped with full appearance. Sepals and petals thick and glossy.
281	White Fairy × CSTD	Long arching inflorescences with 6-10 flowers per inflorescence. Flowers large, white with light purple tinges at tips with rounded, full appearance. Sepals and petals thick and glossy.
283	Sakura × White Fairy	Short erect inflorescences with 4-8 flowers per inflorescence. Flowers medium large, very light pink and white with narrow, spatulate sepals and petals and a stellar appearance. Sepals and petals thick and glossy.
285	Sakura × White Fairy	Short erect inflorescences with 6-10 flowers per inflorescence. Flowers medium large, white with pinkish tinge, narrow sepals and stellar appearance. Sepals and petals thick and glossy

Table 1. Continued

Hybrids	Parents	Description
291	Rungnapa × White Fairy	Long erect inflorescences with 12-18 flowers per inflorescence. Flowers medium large and solid deep magenta pink with narrow sepals, spatulate petals and narrow labellum. Sepals and petals thick and glossy.
298	Rungnapa × CSTD	Long slightly arching inflorescences with 8-12 flowers per inflorescence. Flowers large, magenta coloured and striped with full appearance. Sepals and petals thick and glossy.
299	Rungnapa × CSTD	Long arching inflorescences with 12-15 flowers per inflorescence. Flowers large and solid deep magenta coloured with stellar appearance. Sepals and petals thick and glossy.
316	Rungnapa × CSTD	Long slightly arching inflorescences with 10-12 flowers per inflorescence. Flowers large dark solid purple coloured with rounded, full appearance. Sepals and petals thick and glossy.
330	Sakura × White Fairy	Medium long arching inflorescences with 8-10 flowers per inflorescence. Flowers large, white with pinkish tinge, spatulate petals and stellar appearance. Sepals and petals thick and glossy.
342	White Fairy × CSTD	Long slanting inflorescences with 8-12 flowers per inflorescence. Flowers large, white with light purple (lilac) tinges at tips and semi full appearance. Sepals and petals thick and glossy
372	Rungnapa × CSTD	Short inflorescences with 5-8 flowers per inflorescence. Flowers large and faintly striped, magenta coloured with rounded full appearance. Sepals and petals thick and glossy.

Table 1. Continued

Hybrids	Parents	Description
376	Nagoya Pink × CSTD	Short arching inflorescences with 4-8 flowers per inflorescence. Flowers large, light pink and striped with full appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy.
377	Rungnapa × Nagoya Pink	Medium long arching inflorescences with 5-10 flowers per inflorescence. Flowers large, pink and white with rounded, full appearance. Sepals and petals thick and glossy.
380	Sakura × White Fairy	Short erect inflorescences with 5-8 flowers per inflorescence. Flowers medium large, white with pink tinge and stellar appearance. Sepals and petals medium thick and glossy
381	Sakura × White Fairy	Short erect inflorescences with 6-10 flowers per inflorescence. Flowers medium large, with very light pink, sepals narrow and stellar appearance. Sepals and petals thick and glossy
383	Pramot × CSTD	Medium long arching inflorescences with 6-10 flowers per inflorescence. Flowers large, pink striped with full appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy.
388	Rungnapa × CSTD	Medium long arching inflorescences with 7-13 flowers per inflorescence. Flowers large and deep velvety magenta coloured with rounded, full appearance. Sepals and petals thick and glossy.
391	Rungnapa × CSTD	Medium long arching inflorescences with 6-10 flowers per inflorescence. Flowers large, deep magenta, flat pointed sepals with stellar appearance. Petals very broad overlapping the sepals. Sepals and petals thick and glossy

They are effective means of multiplication. The leaf sheath surrounding the node was removed to expose the dormant bud.

3.1.3 Collection and Preparation

The explants were collected from the forty selected *Dendrobium* hybrids maintained in the greenhouse of the DBT project "Breeding for commercial orchid hybrids". The explants were washed thoroughly with tap water followed by a washing with Tween 20 emulsifier for 30 minutes. Then they were washed two to three times with double distilled water.

3.1.4 Surface Sterilization

Surface sterilization of the explant was carried out inside a laminar airflow chamber (Klenzaids; Horizontal model-1104) just before inoculation. The explants were transferred to a sterilized beaker and surface sterilized by soaking in freshly prepared 0.1 per cent mercuric chloride solution for 10 minutes with intermittent shaking. The solution was drained off and the explant was washed 4 to 5 times with sterile double distilled water to remove all traces of the chemical. The explant was carefully transferred into a sterile petriplate.

3.1.5 Inoculation and Incubation

All inoculation operations were carried out inside a laminar air flow chamber. The vessels and tools (beakers, petriplates, blades, forceps etc.) required for inoculation were washed thoroughly, rinsed with glass distilled water, covered air tight with aluminium foil and autoclaved at 121°C temperature and 1.06 kg cm^{-2} pressure for 45 minutes. They were further flame sterilized just before inoculation using a spirit lamp inside the laminar air flow chamber.

To inoculate the explants into the culture medium the cotton plug of the culture vessel was removed and the mouth was flamed. The explant was inoculated into the medium using sterile forceps. The mouth of the culture vessel was flamed again and cotton plug was replaced.

The cultures were then incubated in a culture room with controlled conditions of light, temperature and humidity. A photoperiod of 15 hours light and nine hours darkness with a light intensity of 3000 lux under fluorescent tube light was provided. A uniform temperature of 26 ± 2°C and a relative humidity of 75 per cent was maintained in the culture room.

3.1.6 Culture Media

The basal media used for the study were VW (Vacin and Went, 1949) medium for initial culture establishment and MS (Murashige and Skoog, 1962) medium for subculturing. The chemicals used for the preparation of the culture media were of analytical grade obtained from British Drug House (Mumbai), SISCO Research Laboratory (Mumbai) and Merck (Mumbai).

Standard procedures were followed for the preparation of media (Thorpe, 1980). Stock solution of major and minor nutrients were prepared by dissolving the required quantity of chemicals in specified volume of double distilled water. Plant growth substances were first dissolved in dilute acid/alcohol and volume made up with double distilled water. The stock solutions were stored under refrigerated condition (+4°C).

The culture vessels used were 'Borosil' brand test tubes (25 × 150 mm) and Erlenmeyer flasks (100 ml). They were washed with 1000 times diluted Labolene and tap water, rinsed with glass distilled water and autoclaved at 121°C temperature and 1.06 kg cm^{-2} pressure for 45 minutes.

All items of glasswares and vessels used for the preparation of culture media were washed thoroughly in 1000 times dilute Labolene and tap water and rinsed with glass distilled water. Specific quantities of stock solutions were pipetted out into a 1000 ml beaker. Growth regulators, sucrose and myo-inositol were added fresh and dissolved. Coconut water (CW) when used was collected from freshly harvested tender coconuts (8 months old) of the local West Coast Tall variety, filtered and added to the medium. The volume was made up to 1000 ml using glass distilled water. The pH of the

medium was adjusted between 5.6 – 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter (Global Electronic, Model DPH 500).

The medium was heated by placing the vessels on a heating mantle. Agar was then added to the medium. The medium was constantly stirred using a glass rod till the agar melted. Activated Charcoal (AC) when used in the medium was added at this stage and stirred well for uniform distribution. The medium was then poured into pre-sterilized culture vessels at the rate of 15 ml for test tubes and 40 ml for Erlenmeyer flasks.

The mouth of the culture vessels were plugged tightly with sterilized cotton, covered with aluminium foil or paper, labeled and autoclaved at 121°C temperature and 1.06 kg cm^2 pressure for 20 minutes. After sterilization, the culture vessels were transferred to the culture room.

3.1.7 Experimental Details

3.1.7.1 Experiment (Ia)

The protocol standardized by Sivamani (2004) for the rapid micropropagation of *Dendrobium* hybrids using the best explant *viz.*, stem nodal segment in the best medium *viz.*, VW + kinetin 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} along with 200 ml l^{-1} of coconut water was employed for initial culture establishment of the 40 selected hybrids.

The medium employed for the subculturing of established culture was ½ MS + BA 4.0 mg l^{-1} + NAA 2.0 mg l^{-1} + IBA 2.0 mg l^{-1} along with 200 ml l^{-1} of coconut water and 1.0 g l^{-1} of AC.

3.1.7.2 Observations

1. Number of days for development of PLB's

Number of days from inoculation to the development of protocorm like bodies was observed.

2. Number of days for initiation of first leaf

Number of days from inoculation to the visible emergence of leaf was recorded.

3. Number of days for initiation of shoot

Number of days from inoculation to visible differentiation of shoot was computed.

4. Number of days at subculturing

Number of days from inoculation to the subculturing of the established explant/culture.

5. Number of days for initiation of first root

Number of days from inoculation to development of first root initial was recorded.

3.1.7.3 Statistical analysis

Design : CRD

Replication : 6

The collected data were subjected to the analysis of variance for drawing meaningful conclusions from the experiment following Panse and Sukhatme (1967).

3.2 REFINING THE PROTOCOL FOR RAPID MULTIPLICATION USING *IN VITRO* LEAF EXPLANTS

3.2.1 Explant

In vitro sourced leaf segments from small, fully opened, light green immature leaves with the mid vein were taken.

3.2.2 Collection and Preparation

The explants were collected from the *in vitro* established *Dendrobium* hybrids developed as a part of the DBT project "Breeding for commercial orchid hybrids". As the explants were *in vitro* sourced no surface sterilization method was adopted.

3.2.3 Inoculation and Incubation

All inoculation operations were carried out inside a laminar air flow chamber. The autoclaved petriplates, blades, forceps etc. were further flame sterilized just before inoculation using a spirit lamp inside the laminar air flow chamber.

Small immature leaf from already established cultures were taken in a sterile petriplate. Leaf segments of 1.5 to 2.0 mm^2 size were cut out using sterile forceps and blade. The explant was inoculated into the medium using sterile forceps. The mouth of the culture vessel was flamed and cotton plug was replaced.

The cultures were then incubated in a culture room with controlled conditions of light, temperature and humidity. A photoperiod of 15 hours light and nine hours darkness with a light intensity of 3000 lux under fluorescent tube light was provided. A uniform temperature of 26 ± 2°C and a relative humidity of 75 per cent was maintained in the culture room.

3.2.4 Culture Medium

The basal medium used for the study was half strength MS (Murashige and Skoog, 1962) medium. Standard procedures were followed for the preparation of media (Thorpe, 1980).

All items of glasswares and vessels used for the preparation of culture media were washed thoroughly in 1000 times dilute Labolene and tap water and rinsed with glass distilled water. Specific quantities of stock solutions were pipetted out into a 1000 ml beaker. Growth regulators, sucrose and inositol were added fresh and dissolved. Coconut water used was collected from freshly harvested tender coconuts (eight months old) of the local West Coast Tall variety, filtered and added to the medium. The volume was made up to 1000 ml using glass distilled water. The pH of the media was adjusted between 5.6 – 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter (Global Electronic, Model DPH 500).

The medium was heated by placing the vessels on a heating mantle. Agar was then added to the medium. The medium was constantly stirred

using a glass rod till the agar melted. Activated charcoal (AC) was added at this stage and stirred well for uniform distribution. The medium was then poured into the pre-sterilized culture vessels at the rate of 15 ml for test tubes and 40 ml for Erlenmeyer flasks.

The mouth of the culture vessels were plugged tightly with sterilized cotton, covered with aluminium foil or paper, labeled and autoclaved at 121°C temperature and 1.06 kg cm^2 pressure for 20 minutes. After sterilization, the culture vessels were transferred to the culture room.

3.2.5 Experimental Details

The experiment consisted of standardization of protocol for rapid multiplication using *in vitro* leaf explant. The protocol for regeneration from *in vitro* leaf segments (Kurup *et al.*, 2005) was employed, with necessary modifications. The objective was to arrive at the best treatment combination for the rapid micropropagation of the selected *Dendrobium* hybrids.

The description of each experiment is given below.

3.2.5.1 Experiment (Ib – i)

Table 2. Different levels of CH tried for the refinement of basal inoculation medium using *in vitro* leaf explants

Medium : ½ MS + 5.0 mg l^{-1} BAP + 5.0 mg l^{-1} KN + CW 200 ml l^{-1} + 1.0 g l^{-1} AC

Treatment number	Casein hydrolysate (mg l^{-1})
T_1	500 mg l^{-1}
T_2	250 mg l^{-1}

3.2.5.2 Experiment (Ib – ii)

Table 3. Different levels of CH tried for the refinement of the medium for first subculture

Medium : ½ MS + 5.0 mg l^{-1} BAP + 5.0 mg l^{-1} KN + CW 200 ml l^{-1} + 1.0 g l^{-1} AC

Treatment number	Casein hydrolysate (mg l^{-1})
T_1	250 mg l^{-1}
T_2	0

3.2.5.3 Experiment (Ib – iii)

Table 4. Different strengths of basal medium tried for the refinement of second subculture

Treatment	Basal medium
T_1	MS (full strength)+0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA +1 g l^{-1} AC
T_2	½MS (half strength)+0.5 mg l^{-1} BAP + 0.1 mg l^{-1} NAA +1 g l^{-1} AC

3.2.5.4 Observations

1. Number of days for swelling of cut leaf edges

Number of days from inoculation to the development of swelling of cut leaf edges was observed.

2. Number of days for development of callus

Number of days from inoculation to the development of callus was recorded.

3. Number of days for first subculture

Number of days from inoculation to the first subculture of the established explant was recorded.

4. Number of days for initiation of shoot

Number of days from inoculation to visible differentiation of shoot was computed.

5. Number of shoots at second subculture

Number of shoots developed at the time of second subculture was observed.

6. Number of days for deflasking

Number of days taken from inoculation of explant to hardening of the plantlet was recorded.

3.2.5.5 Statistical Analysis

Design : CRD

Replication : 6

The collected data were subjected to the analysis of variance for drawing meaningful conclusions from the experiment following Panse and Sukhatme (1967).

3.3 MOLECULAR CHARACTERIZATION

3.3.1 Materials

In the present study, RAPD markers were used for the molecular characterization of the forty selected *Dendrobium* hybrids.

3.3.2 Methods

3.3.2.1 Isolation of Genomic DNA

For the isolation of genomic DNA, leaf samples were collected from young new leaves of selected *Dendrobium* hybrids. The leaves were chopped into small pieces after removing the tip and midrib. 0.5 g of leaf material was first washed in running tap water and later in distilled water. Using tissue paper, the chopped leaves were pulverized by rapid grinding in liquid nitrogen with a pinch of PVP (Polyvinyl pyrrollidone) in a pre-cooled mortar

to a fine powder. Dry powder of plant material was transferred to a 2.0 ml centrifuge tube. 1 ml of extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2 M NaCl, 2 % SDS) and 5 µl B-mercaptoethanol were added to the centrifuge tube and incubated in water bath at 65°C for 45 minutes with occasional gentle shaking. The mixture was then subjected to centrifugation at 10,000 rpm for 10 minutes. The supernatant obtained was filtered using two layers of sterile muslin cloth to a 2 ml centrifuge tube and the remaining extraneous matter was discarded. 200 µl of chloroform : isoamylalcohol (24 : 1) was added to the centrifuge tube and the two phases were mixed gently and centrifuged at 10,000 rpm for 10 minute at 4°C. Then the supernatant was collected and to this was added 100 µl 3.0 M sodium acetate followed by double volume of chilled absolute isopropyl alcohol. It was mixed gently and centrifuged at 10,000 rpm for 10 minutes at 4°C for pelleting the DNA. The supernatant was discarded and the pellet was washed in 70 per cent ethanol. The pellet was air dried and then dissolved in 100 µl of 1 x Tris EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 8) and stored at 4°C.

All the materials used in the preparation and storage of reagents including reagent bottles, conical flasks, centrifuge tubes, spatula, glass rods and tips of micropipettes were washed with Labolene solution, rinsed with distilled water and autoclaved.

3.3.2.2 Quantification of DNA

The quantification of DNA is necessary before it is subjected to amplification by PCR. DNA quantification was carried out with the help of UV-visible spectrophotometer (Spectronic Genesys 5).

The buffer in which the DNA was already dissolved was taken in a cuvette to calibrate the Spectrophotometer at 260 and 280 nm wavelength. The optical density (O.D.) of the samples dissolved in the buffer was recorded at both 260 and 280 nm.

The quantity of DNA in the sample was estimated by employing the following formula:

$$\text{Amount of DNA } (\mu g\ \mu l^{-1}) = \frac{A_{260} \times 50 \times \text{dilution factor}}{1000}$$

where, A_{260} – absorbance at 260 nm

The quantity of DNA could be judged from the ratio of the O.D. values recorded at 260 and 280 nm. The A_{260}/A_{280} ratio between 1.8 and 2.0 indicates good quality of DNA, where A_{280} is the absorbance at 280 nm.

3.3.2.3 Agarose Gel Electrophoresis

Bands were analysed by Agarose gel electrophoresis carried out in a horizontal gel electrophoresis unit supplied by the Bangalore Genei. The required amount of agarose was weighed out (0.8 per cent for visualizing the genomic DNA and 1.2 per cent for visualizing the amplified products) and melted in I x TAE buffer (0.04 mM Tris acetate, 0.001 mM EDTA, pH 8) by boiling. After boiling to about 50° C, ethidium bromide was added to a final concentration of 0.5 μg ml^{-1}. The mixture was then poured to a preset template with appropriate comb. After solidification, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank filled with I x TAE buffer. The gel was completely immersed in the buffer. The DNA sample was mixed with the required volume of gel loading buffer (6x loading dye *viz.*, 30 per cent glycerol, 0.25 per cent Bromophenol blue). Each well was loaded with 20 μl of sample. One of the wells were loaded with 5.0 μl of molecular weight marker along with the required volume of gel loading buffer. Electrophoresis was performed at 75 volts until the loading dye reached ¾th of the length of the gel. The gel was visualized and documented using the gel documentation system.

3.3.2.4 Random Amplified Polymorphic DNA (RAPD) Analysis

Forty random decamer primers (Operon Technologies, Inc., USA) were screened on template DNA from the hybrids so as to identify those

giving good and scorable amplification products. Each reaction mixture (25 µl) for PCR amplification consisted of 2.5 µl 10 x PCR buffer, 2.5 mM $MgCl_2$, 200 µM each of dNTPs, 0.6 units of Taq polymerase (GENEI, Bangalore), 10 pM of primer (Operon Technologies, USA) and 35 ng genomic DNA template. Amplification was performed in a Programmable Thermal Controller (PTC-100, MJ Research Inc.).

PCR amplification conditions were as follows:

Initial denaturation at 95°C for one minute followed by 45 cycles of denaturation at 95°C for one minute, primer annealing at 35°C for two minutes and extension at 72°C for 10 minutes. The amplification products were cooled at 4°C after the reaction. A negative control containing sterile water instead of template was included in each reaction set.

The PCR product was size fractioned on a 1.2 per cent agarose gel prepared in I x TAE buffer and stained with ethidium bromide. The bands were visualized and documented using gel documentation system (BIO RAD, USA). The RAPD bands were represented as '+' for presence and '-' for absence and recorded. Eight oligonucleotide primers were finally selected for further analysis.

3.3.2.5 Data Analysis

The banding pattern from RAPD analysis for each primer was scored by visual observation. Reproducible bands were scored for their presence (+) or absence (-) for the 40 selected *Dendrobium* hybrids. From this RAPD marker data, Jaccard's Similarity coefficient values were calculated, for each pair-wise comparison between hybrids and a similarity coefficient matrix was constructed. The similarity matrix was subjected to cluster analysis using unweighted pair-group method for arithmetic averages analysis (UPGMA) to generate a dendrogram for the hybrids. All the computations were carried out using NTSYS-PC software.

The reproducible bands were scored for their presence (+) or absence (-) for all the genotypes studied. A genetic similarity matrix was constructed using Jaccard's coefficient method (Jaccard, 1908).

$S_j = a / (a+b+c)$

Where, a = number of bands present in both the genotypes in a pair

b = number of bands present in the first genotype, but not in the other

c = number of bands present in the second genotype, but not in the other

Based on the similarity coefficient a dendrogram was constructed with the help of the software package 'NTSYS' (Version 2.02). Association between the genotypes was found out from the dendrogram.

RESULTS

4. RESULTS

Investigations were carried out for the *in vitro* multiplication and molecular characterisation of the selected *Dendrobium* hybrids in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani, during 2004-2006. The *in vitro* experiments and molecular characterisation were carried at the Department of Plant Biotechnology, College of Agriculture, Vellayani. Data obtained were subjected to statistical analysis. The results are presented in this chapter.

4.1 INITIAL CULTURE ESTABLISHMENT OF THE 40 SELECTED *DENDROBIUM* HYBRIDS USING STEM NODAL EXPLANTS

The stem nodal explants of the 40 selected *Dendrobium* hybrids were micropropagated in the initial culture medium *viz.*, VW medium containing KN 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} + CW 200 ml l^{-1} . The analysis of variance revealed significant differences among the 40 selected hybrids for the various *in vitro* responses. The observations include number of days for development of PLBs, number of days for initiation of first leaf, number of days for shoot initiation, number of days for first subculturing and number of days for initiation of first root.

4.1.1 Mean Performance of the 40 selected hybrids during initial culture establishment

4.1.1.1 Number of Days for Development of PLBs

The mean performance of each of the 40 selected *Dendrobium* hybrids during initial culture establishment is given in Table 5. Wide variation was noticed between the hybrids in the case of number of days for development of PLBs ranging from 16.33 to 83.33 days. H-281 showed early PLB development which was statistically on par with H-33, H-93, H-122, H-210, H-223, H-234, H-253, H-291, H-299, H-316, H330, H-342, H-368 and H-381 (ranging from 16.83 to 23.67 days). H-102 took the maximum number of

Table 5. Initial culture establishment of the 40 selected *Dendrobium* hybrids using stem nodal explants

Hybrid Number	Number of days for development of PLBs	Number of days for initiation of first leaf	Number of days for shoot initiation	Number of days for first subculturing	Number of days for initiation of first root
20	31.00	71.33	52.67	48.67	42.00
33	22.33	42.83	57.50	46.33	34.50
59	58.00	39.00	22.67	42.00	43.33
60	64.25	48.75	30.75	49.50	38.50
61	67.75	51.75	26.75	43.50	44.75
63	26.00	52.33	36.67	42.67	46.33
93	17.83	31.50	44.67	35.00	28.83
100	77.00	37.33	24.00	38.33	54.33
102	83.33	57.00	36.67	42.00	61.17
111	52.00	37.40	19.60	34.60	41.20
122	23.67	51.83	43.33	43.67	52.00
127	26.25	41.00	63.25	32.50	58.50
132	78.17	46.67	32.83	40.33	66.67
176	32.33	72.17	57.67	46.33	44.83
177	39.25	28.25	13.25	42.00	34.75
210	22.33	59.67	41.33	42.67	63.17
223	22.83	44.50	83.00	42.67	65.83
231	29.33	53.83	79.33	45.83	47.5
234	21.67	67.33	45.83	42.83	45.50
247	49.17	64.67	85.17	43.67	65.17
253	21.75	49.25	58.25	40.50	35.50
281	16.33	75.33	60.00	46.67	41.67
283	40.00	66.33	57.50	44.17	50.67
285	80.67	61.00	36.00	45.33	64.33
291	17.33	63.33	46.50	40.50	59.00
298	76.00	42.67	26.00	35.00	35.00
299	20.83	48.17	63.50	38.17	37.17
316	23.67	38.17	66.00	43.00	35.50

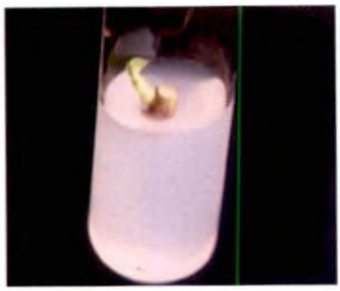
Table 5 Continued

Hybrid Number	Number of days for development of PLBs	Number of days for initiation of first leaf	Number of days for shoot initiation	Number of days for first subculturing	Number of days for initiation of first root
330	18.67	39.33	56.50	41.83	42.50
342	21.33	62.67	55.33	46.00	38.67
368	16.83	29:83	45.17	41.83	56.00
372	72.00	63.25	34.50	49.00	50.25
376	65.00	42.40	25.60	44.80	37.60
377	81.67	47.17	32.83	42.17	50.50
380	83.00	55.00	40.83	45.50	50.17
381	20.50	38.00	40.00	45.00	27.75
383	25.83	51.33	43.00	43.67	35.67
388	63.00	45.00	16.25	32.50	29.50
391	51.67	53.33	27.83	40.00	46.50
408	75.75	57.00	22.00	40.00	45.25
F	95.61**	15.09**	34.40**	4.491**	14.10**
CD	6.374	7.670	7.820	4.491	7.406
	7.127	8.044	8.744	4.710	8.280
	7.807	8.575	9.578	5.021	9.070
	8.063	9.394	9.892	5.501	9.367
	8.433	10.146	10.345	5.941	9.797
	9.015	10.847	11.060	6.351	10.473

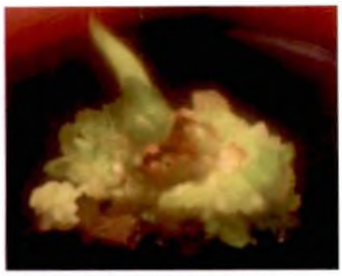
**Significant at 1 per cent level

- Since the number of replication of the treatments are different CD computed separately for each pair of treatment

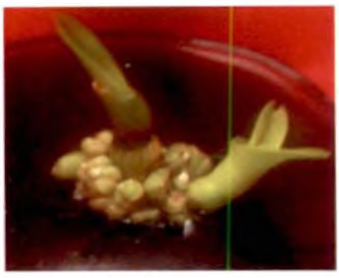
a) Stem nodal segment explant at inoculation



b) Bud initiation



c) PLB initiation



d) Leaf initiation

e) Regenerated plantlets at first subculture

f) Multiple shoot formation

g) Rooting of microshoots

h) Rooted plantlets *in vitro*

i) Plantlets ready for deflasking

j) Deflasked plantlets

Plate 2. Stages of initial culture establishment of 40 selected *Dendrobium* hybrids using stem nodal explants

days for development of PLBs. H-100-D, H-132, H-285, H-298, H-377 and H-380 were statistically on par with durations ranging from 76 to 83 days.

4.1.1.2 Number of Days for Initiation of First Leaf

For initiation of first leaf, H-177 took the lowest number of days being 28.25 days which was statistically on par with H-368(29.83 days), H-93 (31.50 days), H-111(37.40 days) and H-59 (39.00 days). H-281 took the highest number of 75.33 days for the initiation of first leaf. H-176 (72.17 days), H-20 (71.33 days) and H-234 (67.33 days) were statistically on par with it and significantly different from all the other hybrids.

4.1.1.3 Number of Days for Shoot Initiation

Regarding the number of days for shoot initiation, H-177 recorded the minimum duration of 13.25 days, which was statistically on par with H-388(16.25 days), H-111(19.60 days) and H-59 (22.67 days). H-247 recorded the maximum duration of 85.17 days for shoot initiation which was statistically on par with H-223(83.00 days) and H-231(79.33 days). They were statistically different from all the other hybrids.

4.1.1.4 Number of Days for First Subculture

The number of days for first subculturing ranged from 32.5 to 49.5 days. H-388 took the minimum number of days for subculturing which was statistically on par with H-127(32.50 days), H-111(34.60 days), H-93 (35.00 days) and H-298 (35.00 days). H-60 took the maximum days for subculturing which was statistically on par with H-20 (48.67 days), H-33 (46.33 days) and H-61 (43.50 days).

4.1.1.5 Number of Days for Initiation of First Root

With respect to the number of days for initiation of first root, early rooting was observed in H-381 with 27.75 days. H-33, H-93, H-177, H-253, H-298, H-316, H-383 and H-388 were statistically on par with duration ranging from 28.83 to 35.67 days. The maximum number of 66.67 days for rooting was taken by H-132 which was statistically on par with H-102, H-

210, H-223, H-247 and H-285 with duration ranging from 61.17 to 64.33 days.

4.2 REFINING THE PROTOCOL FOR RAPID MULTIPLICATION USING *IN VITRO* LEAF EXPLANTS IN SELECTED *DENDROBIUM* HYBRIDS

The response of *in vitro* sourced leaf explants was observed in different levels of CH and basal media.

4.2.1 Effect of Different Levels of CH on Inoculation Medium in *in vitro* Leaf Explants

Effect of two different levels of CH, *viz.* CH 500 mg l^{-1} (T_1) and CH 250 mg l^{-1} (T_2) on swelling of cut leaf edges and callus initiation was studied (Table 6).

Regarding the number of days for swelling of cut leaf edges, T_1 (CH 500 mg l^{-1}) took the minimum duration of 5.67 days, which differed significantly from T_2. T_2 (CH 250 mg l^{-1}) took the maximum number of days for swelling of cut leaf edges (19.5 days) which was significantly greater than T_1 (Table 6).

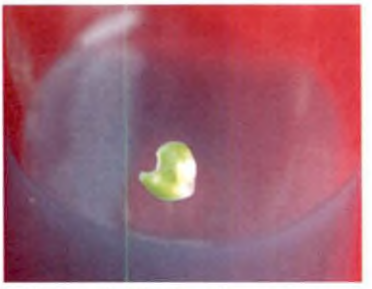
With respect to number of days for callus initiation, T_1 (CH 500 mg l^{-1}) recorded the minimum of 18.33 days for callus initiation while T_2 (CH 250 mg l^{-1}) recorded a duration of 33.33 days (Table 6).

Table 6. Effect of CH on inoculation medium for swelling of cut leaf edges and callus initiation of *in vitro* leaf explant

Treatment	Number of days for swelling of cut leaf edges	Number of days for callus initiation
(T_1) CH 500 mg l^{-1}	5.67	18.33
(T_2) CH 250 mg l^{-1}	19.5	33.33
t	2.49*	1.78*

* Significant at 5 per cent level

a) Initial swelling of *in vitro* sourced leaf explants



b) Callus initiation from cut ends

c) Callus proliferation

d) Regeneration from callus

e) Root development in regenerated plantlet

f) Shoot multiplication

g) Plantlets at first subculture

h) In vitro developed rooted plantlets

i) Deflasked plantlets

Plate 3. Stages of micropropagation of selected *Dendrobium* hybrids using *in vitro* sourced leaf explants

4.2.2 Effect of CH on first Subculturing Medium in *in vitro* Leaf Explant

Effect of CH on shoot initiation and number of shoots developed were studied in medium with CH 250 mg/l (T_1) and without CH (T_2) (Table 7).

In the case of number of days for shoot initiation, significantly early shooting was observed in T_1 (CH 250 mg l^{-1}) taking 51.67 days. The maximum number of 85.17 days for shooting was taken by the T_2 (without CH), which was found to be significantly different from T_1. No significant difference was observed between the treatments in the case of number of shoots developed (Table 7).

Table 7. Effect of CH on first subculturing medium for shoot initiation and number of shoots developed

Treatment	Number of days for initiation of shoot	Number of shoots at second subculture
(T_1) CH 250 mg l^{-1}	51.67	1.83
(T_2) CH 0	85.17	1.17
t	3.20**	1.91

** Significant at one per cent level

4.2.3 Effect of Different Strengths of MS Medium on Second Subculturing of *in vitro* Leaf Explants

The plantlets obtained from the first subculture were grouped into two based on the presence and absence of CH in the media. The plantlets obtained from the first subculturing media containing CH (250 mg l^{-1}) were grown on both half (T_1) and full strength (T_2) MS media. Likewise plantlets obtained from the treatment without CH in the first subculturing media were also grown on both half and full strength MS media. The effect of different

strengths of MS medium on number of days for deflasking was studied (Table 8).

For number of days for deflasking T_1 (MS half strength) took the minimum duration of 182.92 days which was significantly different from T_2. T_2 (MS full strength) took a higher period of 204.67 days for deflasking, which showed significant difference between the treatments.

An attempt was made to detect any interaction component between the strength of MS in second subculturing medium and presence or absence of CH on first subculturing medium, but no significant difference in their interaction was observed (Table 8).

Table 8. Effect of different strengths of MS medium and CH on number of days for deflasking

	MS half strength	MS full strength	Mean of CH
CH 250 mg l^{-1} in first subculture media	176.17	190.67	183.42
No CH	189.67	218.67	204.17
Mean of medium	182.92	204.67	

CD – A, B = 9.83 SE – A, B = 3.33

AB = 13.90 AB = 4.71

4.3 MOLECULAR CHARACTERISATION (RAPD)

In the present study, RAPD (Random Amplified Polymorphic DNA) analysis was used for the molecular characterization of 40 selected *Dendrobium* hybrids.

4.3.1 Isolation of Genomic DNA

Genomic DNA was extracted based on the method of Mondal *et al.* (2000) with slight modifications. The purity and yield of DNA were good when fresh tender leaves were used. The DNA yield for 40 selected *Dendrobium* hybrids ranged from 630 to 960 ng μl^{-1}. The purity of DNA A_{260}/A_{280} ratio) (Table 9) ranged from 1.60 to 1.85 $\mu g\ \mu l^{-1}$.

4.3.2 Testing the Quality of DNA

For RAPD profile analysis, the DNA should be free of RNA and protein. Moreover, it needs intact, unsheared DNA sample of sufficient quantity. To assess the quality, all the genomic DNA samples were run on 0.7 per cent agarose gel and the gel was stained with ethidium bromide and bands appearing in the gel were visualized, using ultraviolet transilluminator.

4.3.3 Polymerase Chain Reaction (PCR)

DNA amplification standardized by Lim *et al.* (1999) for *Vanda* was used. The same condition was found to be good for the *Dendrobium* hybrids studied. Forty decamer primer of series A and B were screened for their efficiency using the DNA isolated from H-298 as the representative sample. Out of these, eight decamer primers which showed high level of polymorphism were selected for final RAPD-PCR analysis.

The RAPD profile generated by eight selected primers *viz.* OPA-03, OPA-04, OPA-10, OPA-16, OPA-18, OPB-02, OPB-06 and OPB-10 are shown in Plates 4 to 11. A total of 69 scorable bands (average of 8.63 bands per primer) were generated of which three were monomorphic and the remaining 66 were polymorphic (95.65%). The number of amplification products ranged from 4 to 12 with an average of two per primer.

The highest number of scorable bands (12 bands) was given by OPA-18, followed by OPA-10 (11 bands), OPA-16 (11 bands), OPB-06 (9 bands) OPA-04 (8 bands), OPB-02 (7 bands), OPB-10 (7 bands) and OPA-03 (4 bands). The primer OPA-03 produced four scorable bands of which two

Table 9. Quality and yield of DNA from 40 selected *Dendrobium* hybrids

Sl. No.	Hybrid Number	A_{260}	A_{280}	A_{260}/A_{280}	DNA yield (ng / µl)
1	20	0.029	0.018	1.61	870
2	33	0.023	0.013	1.77	690
3	59	0.025	0.015	1.67	750
4	60	0.022	0.012	1.83	660
5	61	0.032	0.019	1.68	960
6	63	0.024	0.013	1.85	720
7	93	0.027	0.015	1.72	810
8	100.D	0.028	0.016	1.75	840
9	102	0.022	0.013	1.69	660
10	111	0.026	0.015	1.73	780
11	122	0.027	0.016	1.69	810
12	124	0.021	0.013	1.62	630
13	127	0.023	0.014	1.64	690
14	132	0.028	0.016	1.75	840
15	147	0.025	0.014	1.79	750
16	176	0.021	0.012	1.75	630
17	177	0.028	0.017	1.65	840
18	210	0.022	0.013	1.69	660
19	223	0.029	0.017	1.71	870
20	231	0.023	0.014	1.64	690

Table 9 Continued

Sl. No.	Hybrid Number	A_{260}	A_{280}	A_{260}/A_{280}	DNA yield (ng / µl)
21	234	0.024	0.015	1.60	720
22	247	0.023	0.013	1.77	690
23	253	0.028	0.017	1.65	840
24	281	0.026	0.016	1.63	780
25	283	0.028	0.016	1.75	840
26	285	0.031	0.018	1.72	930
27	291	0.025	0.015	1.67	750
28	298	0.028	0.016	1.75	840
29	299	0.029	0.018	1.61	870
30	316	0.022	0.012	1.83	660
31	330	0.027	0.016	1.68	810
32	342	0.024	0.014	1.72	720
33	372	0.024	0.013	1.85	720
34	376	0.029	0.016	1.81	870
35	377	0.026	0.015	1.73	780
36	380	0.021	0.013	1.62	630
37	381	0.028	0.017	1.65	840
38	383	0.029	0.017	1.71	870
39	388	0.025	0.014	1.78	750
40	391	0.026	0.015	1.73	780

Table10. Primers, their sequence and total number of amplified bands detected in the 40 selected *Dendrobium* hybrids

Sl. No.	Primer	Sequence (5^1-3^1)	Total number of bands amplified
1	OPA-03	AGTCAGCCAC	4
2	OPA-04	AATCGGGCTG	8
3	OPA-10	GTGATCGCAG	11
4	OPA-16	AGCCAGCGAA	11
5	OPA-18	AGGTGACCGT	12
6	OPB-02	TGATCCCTGG	7
7	OPB-06	TGCTCTGCCC	9
8	OPB-10	CTGCTGGGAC	7

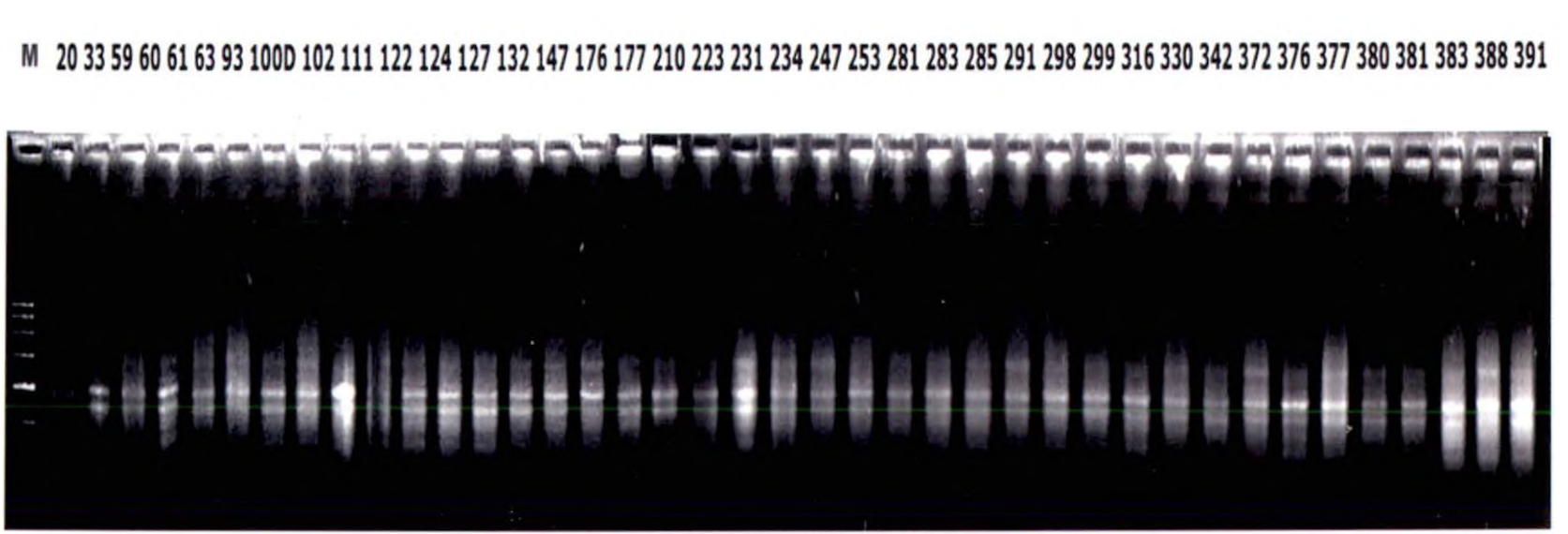


Plate 4. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPA - 03

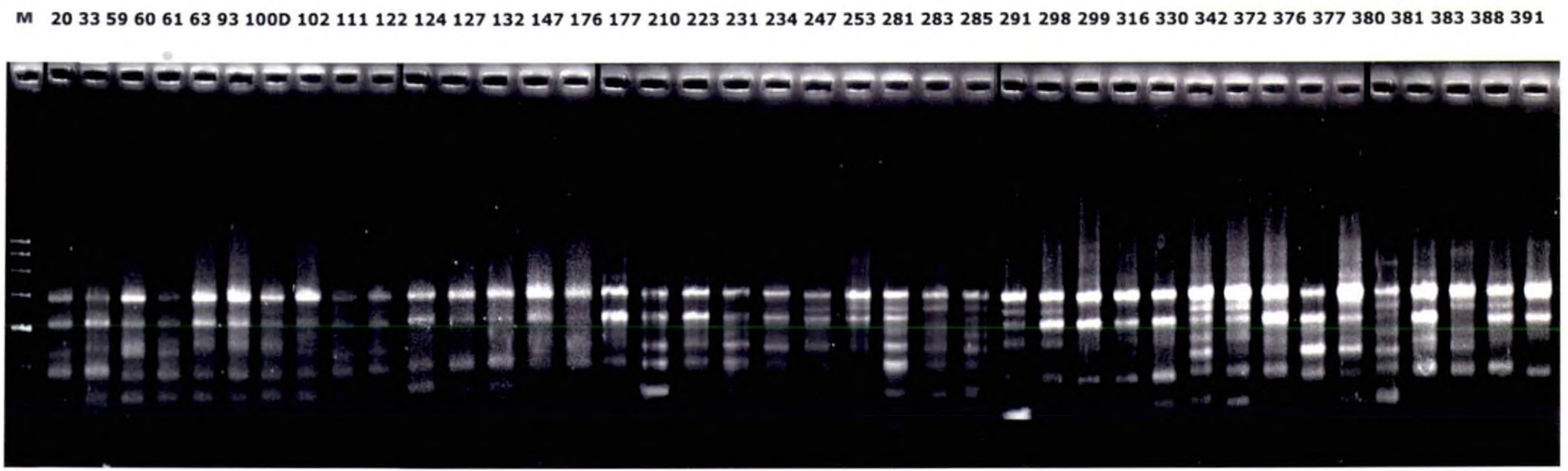


Plate 5. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPA - 04

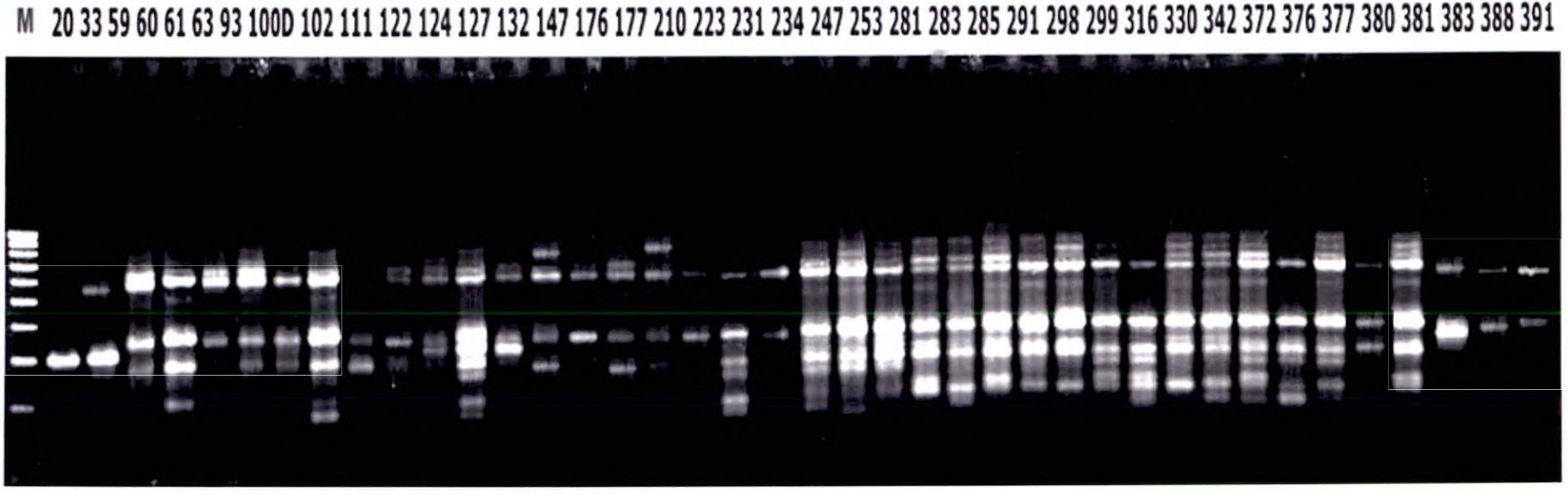


Plate 6. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPA - 10

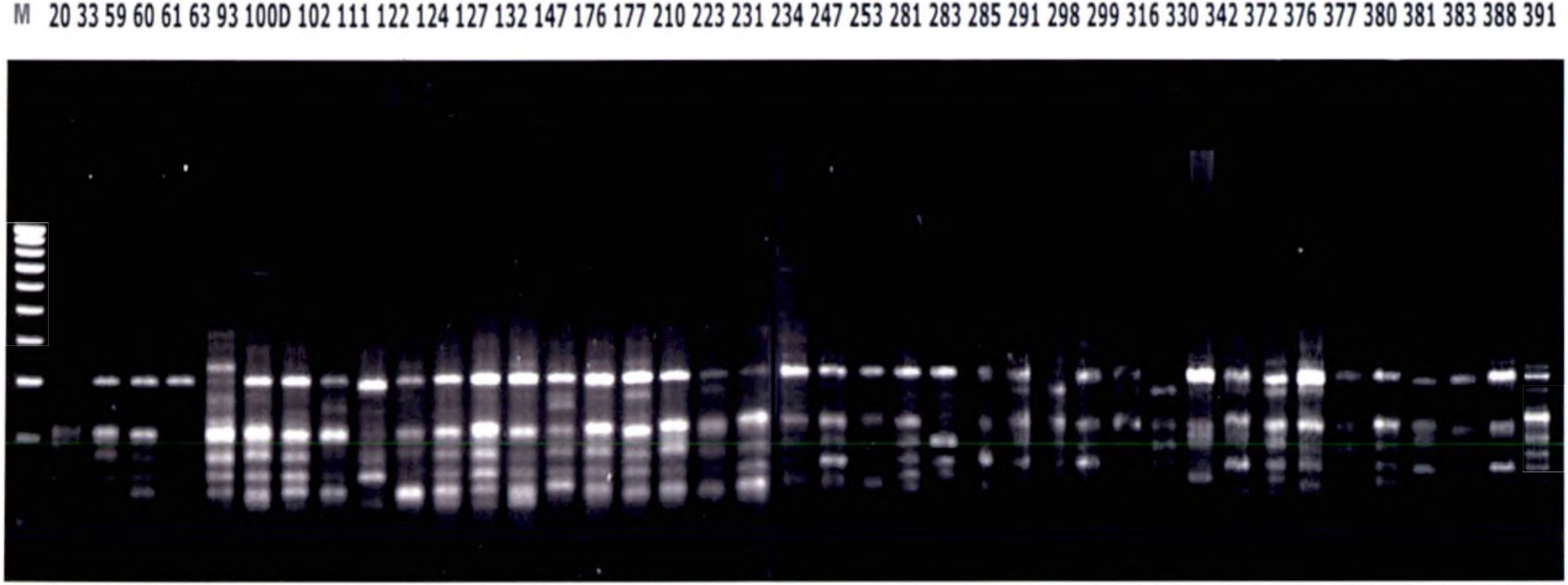


Plate 7. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPA - 16

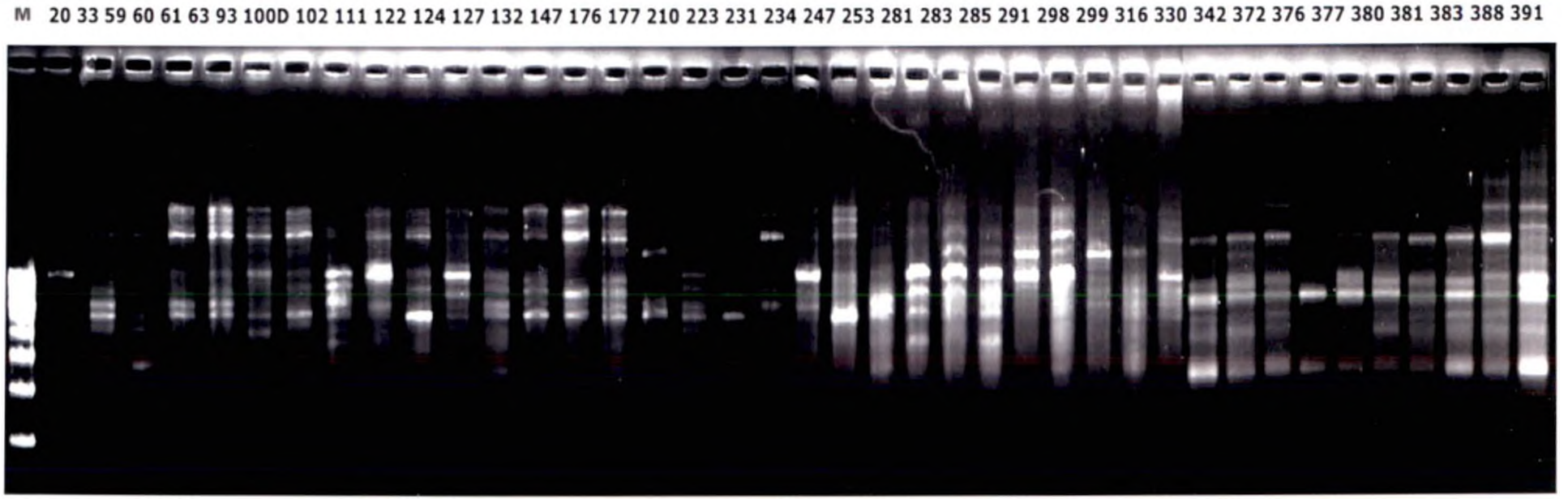


Plate 8. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPA - 18

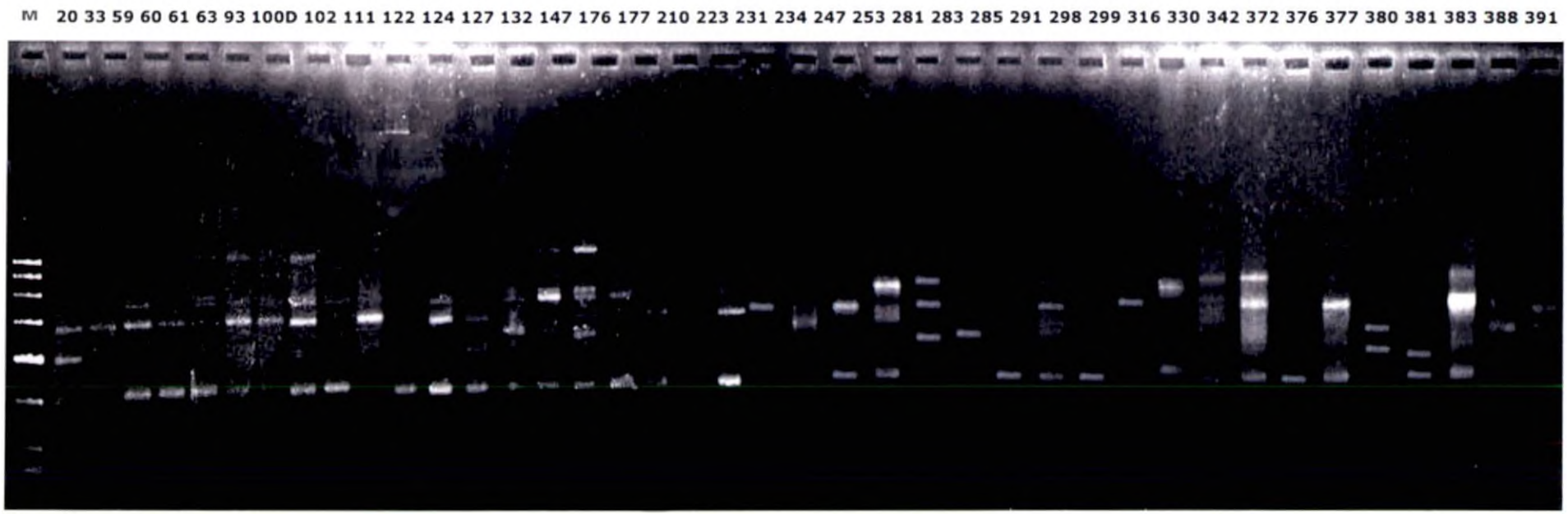


Plate 9. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPB - 02

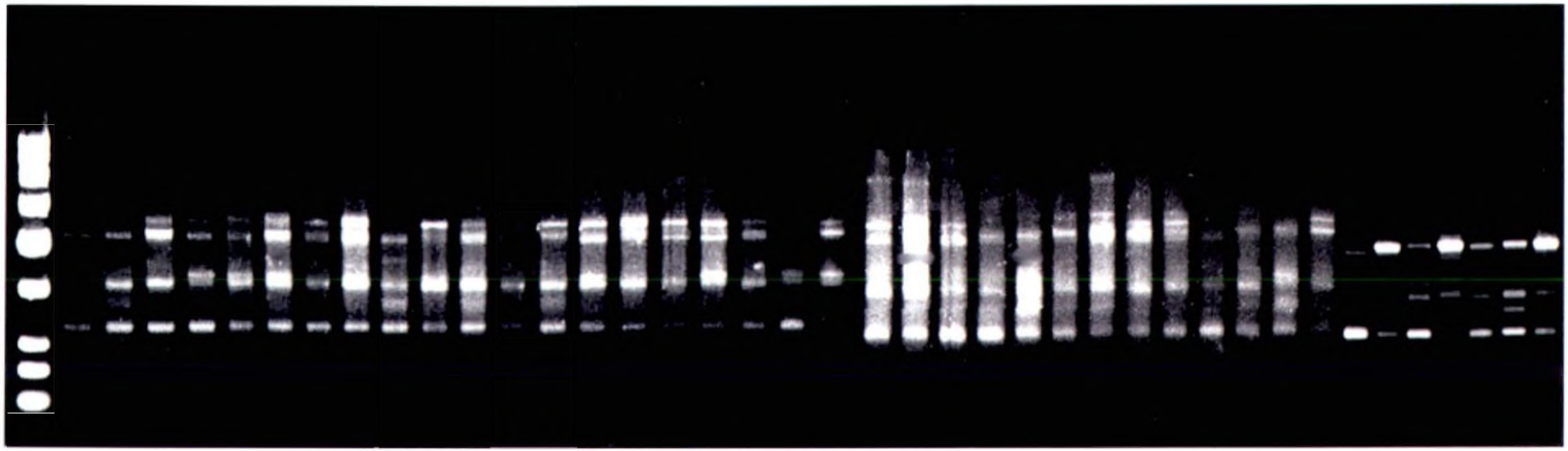


Plate 10. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPB - 06

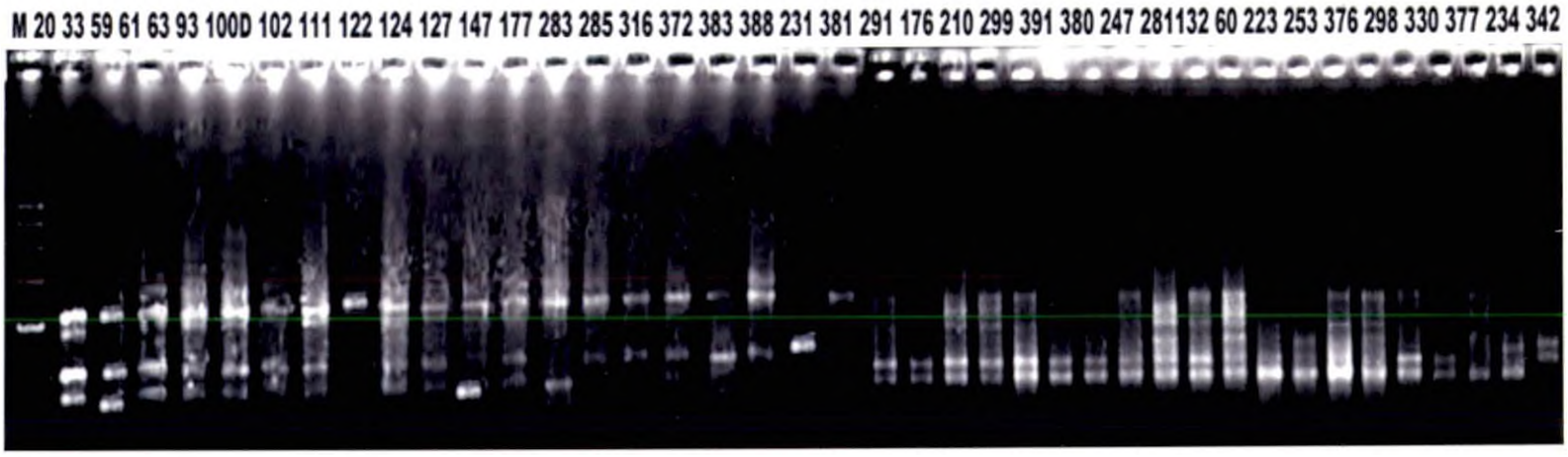


Plate 11. Amplification profile of the DNA of 40 selected *Dendrobium* hybrids using primer OPB - 10

Table 11. Similarity coefficient

	20	33	59	60	61	63	93	100D	102	111	122	124	127	132	147	176	177	210	223	231
20	1.000000																			
33	0.593750	1.000000																		
59	0.487805	0.571429	1.000000																	
60	0.444444	0.500000	0.634146	1.000000																
61	0.454546	0.533333	0.717391	0.555556	1.000000															
63	0.465116	0.581395	0.772727	0.533333	0.760870	1.000000														
93	0.527778	0.666667	0.707317	0.605263	0.659091	0.674419	1.000000													
100D	0.477273	0.489362	0.666667	0.613636	0.660000	0.640000	0.644444	1.000000												
102	0.441177	0.542857	0.488372	0.527778	0.488889	0.466667	0.611111	0.478261	1.000000											
111	0.432432	0.487180	0.658537	0.552632	0.690476	0.627907	0.675676	0.565217	0.400000	1.000000										
122	0.555556	0.605263	0.613636	0.512195	0.608696	0.622222	0.710526	0.595745	0.512821	0.657895	1.000000									
124	0.500000	0.585366	0.630435	0.571429	0.625000	0.604167	0.682927	0.645833	0.500000	0.595238	0.666667	1.000000								
127	0.583333	0.722222	0.636364	0.575000	0.666667	0.644444	0.736842	0.617021	0.538462	0.641026	0.763158	0.731707	1.000000							
132	0.512821	0.523810	0.681818	0.511628	0.638298	0.583333	0.581395	0.560000	0.409091	0.571429	0.604651	0.553192	0.590909	1.000000						
147	0.459460	0.552632	0.604651	0.500000	0.636364	0.577778	0.615385	0.553192	0.500000	0.564103	0.641026	0.700000	0.666667	0.675000	1.000000					
176	0.500000	0.585366	0.666667	0.571429	0.625000	0.571429	0.568182	0.612245	0.431818	0.522727	0.555556	0.574468	0.577778	0.780488	0.700000	1.000000				
177	0.473684	0.605263	0.651163	0.589744	0.681818	0.622222	0.710526	0.595745	0.594595	0.657895	0.650000	0.707317	0.675000	0.604651	0.684211	0.555556	1.000000			
210	0.617647	0.538462	0.627907	0.525000	0.659091	0.600000	0.729730	0.644444	0.487180	0.631579	0.666667	0.604651	0.609756	0.658537	0.575000	0.642857	0.666667	1.000000		
223	0.368421	0.540541	0.522727	0.567568	0.521739	0.500000	0.605263	0.510638	0.486487	0.552632	0.512195	0.609756	0.536585	0.511628	0.500000	0.466667	0.631579	0.564103	1.000000	
231	0.457143	0.435897	0.534884	0.583333	0.568182	0.478261	0.578947	0.555556	0.459460	0.526316	0.487805	0.477273	0.512195	0.488372	0.552632	0.547619	0.564103	0.578947	0.461539	1.000000
234	0.380952	0.465116	0.586957	0.560976	0.490196	0.595745	0.595238	0.540000	0.386364	0.547619	0.511111	0.600000	0.533333	0.510638	0.500000	0.565217	0.511111	0.522727	0.600000	0.431818
247	0.404762	0.391304	0.480000	0.511628	0.425926	0.433962	0.416667	0.500000	0.291667	0.434783	0.468085	0.489796	0.489362	0.565217	0.456522	0.586957	0.437500	0.478261	0.477273	0.488372
253	0.500000	0.448980	0.557692	0.531915	0.527273	0.509091	0.562500	0.574074	0.380000	0.553192	0.551020	0.600000	0.571429	0.612245	0.510204	0.568628	0.551020	0.666667	0.531915	0.510638
281	0.525000	0.534884	0.617021	0.595238	0.612245	0.560000	0.590909	0.666667	0.454546	0.581395	0.577778	0.666667	0.600000	0.574468	0.568182	0.630435	0.651163	0.666667	0.558140	0.500000
283	0.473684	0.487805	0.510638	0.550000	0.510204	0.489796	0.511628	0.630435	0.475000	0.500000	0.500000	0.590909	0.558140	0.468085	0.523810	0.489362	0.609756	0.547619	0.512195	0.564103
285	0.473684	0.487805	0.543478	0.550000	0.510204	0.553192	0.585366	0.595745	0.475000	0.500000	0.571429	0.590909	0.558140	0.500000	0.488372	0.555556	0.571429	0.585366	0.512195	0.452381
291	0.450000	0.431818	0.489796	0.488372	0.461539	0.500000	0.522727	0.540000	0.386364	0.444444	0.446809	0.469388	0.468085	0.510638	0.434783	0.636364	0.478261	0.558140	0.422222	0.431818
298	0.562500	0.527778	0.547619	0.555556	0.446809	0.488889	0.552632	0.533333	0.432432	0.425000	0.500000	0.560976	0.487805	0.500000	0.487180	0.600000	0.538462	0.594595	0.473684	0.486487
299	0.516129	0.485714	0.476191	0.558824	0.413044	0.488372	0.555556	0.466667	0.388889	0.421053	0.500000	0.525000	0.526316	0.428571	0.410256	0.487805	0.461539	0.600000	0.606061	0.485714
316	0.470588	0.486487	0.444444	0.435897	0.478261	0.488889	0.594595	0.533333	0.472222	0.461539	0.500000	0.600000	0.525000	0.465116	0.450000	0.454546	0.538462	0.638889	0.647059	0.410256
330	0.475000	0.454546	0.541667	0.585366	0.480769	0.490196	0.545455	0.591837	0.441861	0.466667	0.500000	0.622222	0.555556	0.469388	0.488889	0.460000	0.568182	0.511111	0.511628	0.422222
342	0.454546	0.533333	0.580000	0.555556	0.518519	0.500000	0.553192	0.627451	0.425532	0.479167	0.541667	0.695652	0.595745	0.509804	0.500000	0.529412	0.574468	0.520833	0.555556	0.437500
372	0.434783	0.510638	0.620000	0.565217	0.584906	0.566038	0.630435	0.700000	0.468085	0.520833	0.617021	0.818182	0.638298	0.519231	0.608696	0.600000	0.652174	0.562500	0.531915	0.479167
376	0.342105	0.435897	0.434783	0.425000	0.380000	0.416667	0.538462	0.458333	0.459460	0.380952	0.452381	0.547619	0.409091	0.422222	0.404762	0.444444	0.452381	0.500000	0.628571	0.435897
377	0.404762	0.488372	0.574468	0.585366	0.509804	0.520000	0.619048	0.625000	0.512195	0.500000	0.500000	0.697674	0.521739	0.440000	0.522727	0.520833	0.568182	0.511111	0.625000	0.560976
380	0.444444	0.500000	0.522727	0.450000	0.489362	0.500000	0.564103	0.479167	0.486487	0.512821	0.476191	0.466667	0.536585	0.511628	0.538462	0.466667	0.589744	0.525000	0.611111	0.540541
381	0.410256	0.463415	0.489362	0.525000	0.489796	0.469388	0.523810	0.574468	0.414634	0.476191	0.511628	0.604651	0.571429	0.511111	0.500000	0.468085	0.585366	0.488372	0.648649	0.463415
383	0.459460	0.512821	0.533333	0.500000	0.500000	0.479167	0.657895	0.553192	0.540541	0.452381	0.523810	0.619048	0.547619	0.425532	0.589744	0.478261	0.600000	0.575000	0.500000	0.512821
388	0.447368	0.578947	0.521739	0.487805	0.489796	0.500000	0.684211	0.541667	0.567568	0.476191	0.585366	0.604651	0.650000	0.478261	0.615385	0.533333	0.585366	0.523810	0.487805	0.538462
391	0.410256	0.578947	0.521739	0.487805	0.553192	0.531915	0.684211	0.541667	0.450000	0.512195	0.547619	0.568182	0.571429	0.511111	0.575000	0.568182	0.585366	0.560976	0.525000	0.578947

Table 11. Continued

	234	247	253	281	283	285	291	298	299	316	330	342	372	376	377	380	381	383	388	391
234	1.000000																			
247	0.613636	1.000000																		
253	0.591837	0.645833	1.000000																	
281	0.553192	0.541667	0.687500	1.000000																
283	0.511111	0.533333	0.617021	0.690476	1.000000															
285	0.581395	0.500000	0.617021	0.690476	0.534884	1.000000														
291	0.590909	0.543478	0.529412	0.586957	0.446809	0.700000	1.000000													
298	0.476191	0.465116	0.555556	0.585366	0.500000	0.714286	0.631579	1.000000												
299	0.512821	0.463415	0.522727	0.476191	0.461539	0.583333	0.512821	0.700000	1.000000											
316	0.512195	0.400000	0.521739	0.511628	0.500000	0.578947	0.512195	0.542857	0.645161	1.000000										
330	0.510638	0.500000	0.645833	0.681818	0.533333	0.682927	0.479167	0.536585	0.428571	0.465116	1.000000									
342	0.583333	0.540000	0.680000	0.717391	0.644444	0.608696	0.490196	0.545455	0.444444	0.511111	0.790698	1.000000								
372	0.591837	0.519231	0.592593	0.620000	0.551020	0.617021	0.529412	0.555556	0.456522	0.590909	0.645833	0.714286	1.000000							
376	0.500000	0.488372	0.479167	0.466667	0.452381	0.452381	0.400000	0.447368	0.485714	0.486487	0.422222	0.468085	0.479167	1.000000						
377	0.613636	0.565217	0.580000	0.644444	0.604651	0.642857	0.543478	0.575000	0.500000	0.575000	0.600000	0.673913	0.717391	0.641026	1.000000					
380	0.488372	0.477273	0.565217	0.595238	0.589744	0.550000	0.422222	0.435897	0.432432	0.473684	0.585366	0.555556	0.440000	0.500000	0.547619	1.000000				
381	0.522727	0.545455	0.595745	0.627907	0.547619	0.625000	0.456522	0.552632	0.473684	0.552632	0.700000	0.738095	0.595745	0.463415	0.700000	0.605263	1.000000			
383	0.500000	0.367347	0.510204	0.642857	0.560976	0.488372	0.404255	0.414634	0.410256	0.487180	0.634146	0.636364	0.644444	0.475000	0.595238	0.578947	0.465116	1.000000		
388	0.488889	0.446809	0.470588	0.521739	0.585366	0.444444	0.456522	0.404762	0.435897	0.512821	0.511111	0.586957	0.630435	0.463415	0.581395	0.564103	0.454546	0.750000	1.000000	
391	0.488889	0.387755	0.442308	0.489362	0.444444	0.444444	0.425532	0.404762	0.435897	0.512821	0.511111	0.553192	0.630435	0.428571	0.545455	0.525000	0.523810	0.657895	0.729730	1.000000

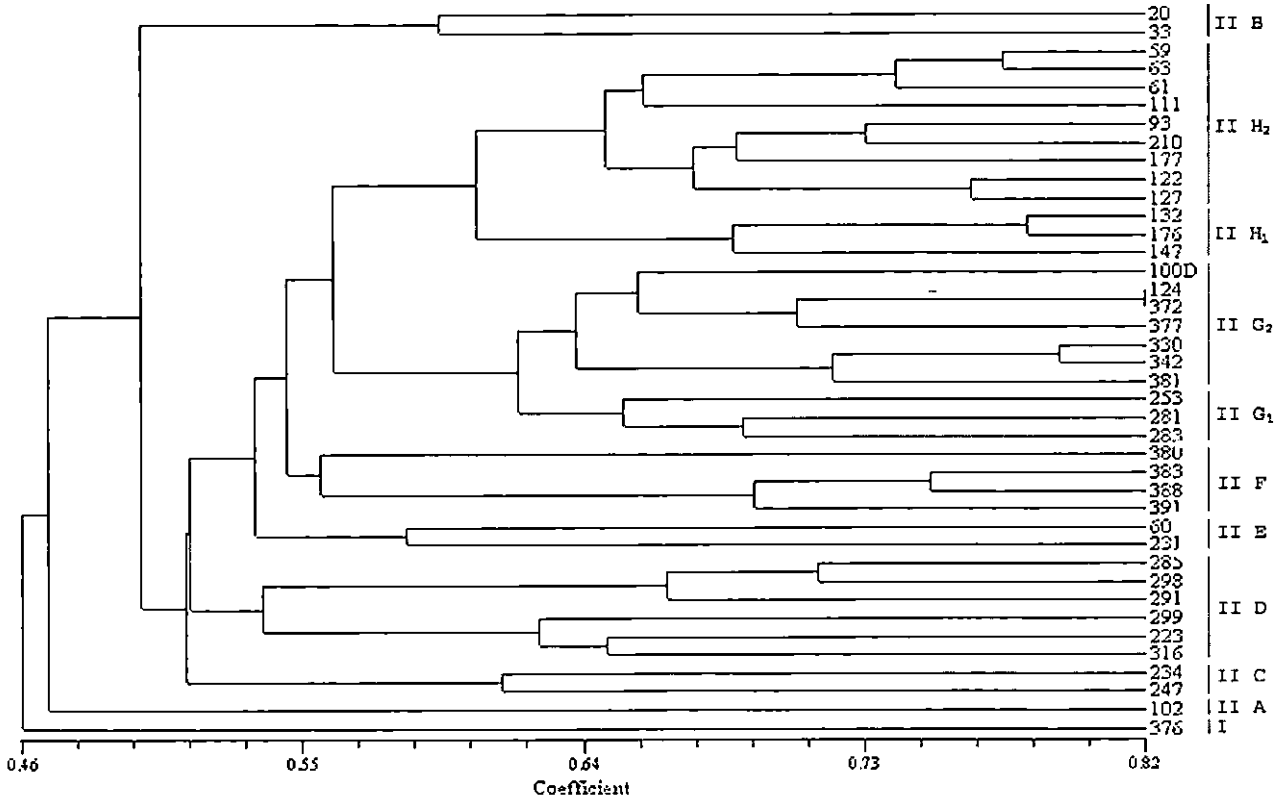


Fig. 1. Dendrogram for 40 selected *Dendrobium* hybrids based on the RAPD analysis

bands were monomorphic. Out of the eight scorable bands produced by primer OPA-04 one band was monomorphic.

Data obtained from the eight primers that gave reproducible bands were used for statistical analysis.

4.3.4 Data Analysis

Overall similarity indices ranged from 0.29 to 1.00. The genetic distance revealed the genetic variability that exists between the hybrids. The clustering of 40 selected *Dendrobium* hybrids using UPGMA is depicted in Fig.1. The similarity coefficient revealed from the dendrogram ranged from 0.46 to 0.82.

Cluster analysis revealed that at about 0.46 similarity coefficient, the 40 selected *Dendrobium* hybrids got divided into two group. Among the 40 hybrids, H-376 fell in the first group showing high degree of variability from the other hybrids.

At 0.47 similarity coefficient, the second group divided into two groups. Here H-102 fell into cluster IIA standing quite distinct from the rest of the 38 hybrids.

At 0.50 similarity coefficient, cluster II B bifurcated from group II. Cluster IIB consisted of two hybrids H-20 and H-33 which fell out into a separate group. H-20 and H-33 showed 59.3 per cent similarity with each other.

At 0.526 similarity coefficient, cluster IIC got separated from group II with H-247 showing 61.3 per cent similarity with H-234.

Group II at 0.52 similarity coefficient further divided into two subclusters D and E. Cluster II D included 6 hybrids with H-316, H-223 and H-299 falling in one group and H-291, H-298 and H-285 coming under the second group. H-316 showed 64.7 per cent similarity with H-223 and H-299 stood out from the group at 62.1 per cent similarity. H-298 and H-285

showed 71.4 per cent similarity with each other while H-291 stood out singly at 0.66 similarity coefficient.

Cluster II E contained H-231 and H-60 which stood out from the rest of the group at 0.54 similarity coefficient. H-231 showed 58.33 per cent similarity with H-60.

At 0.549 similarity coefficient cluster II F separated from the second group. Cluster II F comprised of four hybrids (H-391, H-388, H-383 and H-380). This cluster with four hybrids was further subdivided into two subgroups at 0.55 similarity coefficient. H-391, H-388 and H-383 formed one group while H-380 remained distinct. At 70.9 per cent similarity, H-391 got distinct from H-388 and H-383. H-388 showed 75 percent similarity with H-383.

At 0.56 similarity coefficient the second group again divided into two clusters G and H. Cluster II G further divided into two subclusters, cluster IIG_1, and cluster II G_2 at 0.63 similarity coefficient. Cluster II G_1 comprises of three hybrids H-283, H-281 and H-253. Among these H-253 stood distinct from H-281 and H-283 at 66.3 per cent similarity while H-283 showed 69.04 per cent similarity with H-281. Cluster II G_2 further divided into two subgroups at 0.63 similarity coefficient. H-381, H-342 and H-330 formed one subgroup while H-377, H-372, H-124 and 100 D remained in the second subgroup.

At 0.72 similarity coefficient in the first group H-381 got distinct from H-342 and H-330. H-342 showed 79.09 per cent similarity with H-330. In the second sub group, H-100 D got distinct from the other three hybrids at 0.66 similarity coefficient. H-377 showed 71.73 per cent similarity with H-372. The dissimilarity is found to be negligible between H-377 and H-124 showing 82 per cent similarity with each other.

At 0.61 similarity coefficient cluster II H further divided into two subclusters, cluster II H_1, and cluster II H_2. Cluster II H_1 consisted of three

hybrids of which H-147 stood singly at 0.70 similarity coefficient, while H-176 and H-132 showed 78.04 per cent similarity.

Rest of the hybrids fell under cluster II H_2, which further divided into two subgroups at 0.65 similarity coefficient. The first subgroup consisted of H-127, H-122, H-177, H-210 and H-93. Among these, H-127 showed 76.37 per cent similarity with H-122. H-177 fell out singly at 0.69 similarity coefficient, while H-210 and H-93 showed 72.97 per cent similarity.

The second subgroup of cluster II H_2 comprise of H-111, H-61, H-63 and H-59. H-111 and H-61 stood singly at 0.67 and 0.74 similarity coefficients respectively while H-63 showed 77.27 per cent similarity with H-59.

The 40 selected *Dendrobium* hybrids that were studied fell under 11 clusters in UPGMA cluster analysis. Cluster I and cluster II A contained one hybrid each, whereas cluster II B, cluster II C and cluster II F contained two hybrids each. Cluster II G_1 and cluster II H_1 included three hybrids each. Four hybrids fell under cluster II F_1 whereas cluster II D_1, II $G_2$and II H_2 included 6, 7 and 9 hybrids respectively

DISCUSSION

5. DISCUSSION

Dendrobium is the second largest genus in the family Orchidaceae; it possesses marvelously showy flowers exhibiting a wide range of forms and colours with a long vase life. Propagation of orchid through conventional means is a very slow process and tissue culture is the only alternative for its large scale propagation. The increasing demand of high quality planting material for export and marketing competition in the orchid industry has necessitated their true-to-type rapid clonal propagation through tissue culture. At present in Kerala, we have a dearth of good quality acceptable varieties of planting materials of orchids. This problem can be overcome by producing our own hybrids possessing high quality and novelty. As a solution to this dilemma several indigenous *Dendrobium* hybrids have been developed under the DBT project entitled "Breeding for commercial orchid hybrids" in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani (Lekha Rani, 2002). From this about 40 hybrids possessing novelty, distinctiveness and uniformity in floral characters have been selected for commercial release, which have to be urgently multiplied.

In this context, the present study deals with the initial culture establishment of the 40 selected *Dendrobium* hybrids in the identified best culture medium (Sivamani, 2004) and refining the protocol for rapid multiplication using *in vitro* leaf explants. The use of molecular markers has significantly contributed to our understanding of the species at the genetic level. Molecular characterization is possible using a wide range of molecular markers in orchids. Among them RAPD has been extensively used for characterization studies in orchids. In the present study, RAPD was employed for studying the genetic diversity and for the fingerprinting of the 40 selected *Dendrobium* hybrids. The results are discussed below.

5.1 INITIAL CULTURE ESTABLISHMENT

In vitro multiplication using stem nodal explants of keikis are shown to be ideal for clonal propagation of *Dendrobium* hybrids since the propagules are true to type and does not involve the sacrifice of the entire mother plant which is a precious selected single plant hybrid (Sivamani, 2004). Growth and morphogenesis *in vitro* are regulated by the interaction and balance between substances produced endogenously by cultured cells and those supplied exogenously in the medium. Size of the explant greatly influences the establishment and survival of the culture. In the present study, stem nodal segments of 1.0 to 1.5 cm length with one node each from keiki were used for culture initiation in VW medium supplemented with KN 4.0 mg l^{-1} + IAA 4.0 mg l^{-1} + CW 200 ml l^{-1}. The above mentioned explant and media composition was selected based on the findings of Sivamani (2004) in *Dendrobium* hybrids developed under the DBT project.

Devi and Deka (2001) observed that when stem segments with node are used to initiate culture, the dormant buds swell and enlarge followed by leaf and root development. Sivamani (2004) reported that the stem nodal segment of *Dendrobium* hybrids with dormant buds cultured *in vitro* showed PLB formation and development of plantlets. In the present study, the response of the stem nodal explant and mode of development of the PLBs or shoot buds therefrom, was found to be varied in different hybrids. In some hybrids (H-59, H-60, H-61, H-100D, H-102, H-111, H-132, H-177, H-285, H-298, H-376, H-377, H-380, H-388, H-391 and H-408) shoot buds developed directly and the PLBs developed thereafter whereas others (H-20, H-33, H-63, H-93, H-122, H-127, H-176, H-210, H-223, H-231, H-234, H-247, H-253, H-281, H-283, H-291, H-299, H-316, H-330, H-342, H-368, H-372, H-381 and H-383) showed regeneration of explant through PLB formation.

In the present study, the duration for the initiation of PLBs varied from 2 to 11 weeks in the different hybrids. This is in conformity with the

findings of Kim *et al.* (1970) that the buds excised from bulbs and leaf axils of *Dendrobium* produced PLBs in 4-5 weeks and plantlets in eight weeks. Significant and rapid production of PLBs with two weeks in *Dendrobium* var. Sonia was reported by Shylaraj *et al.*(2005). Kaur and Vij (2000) has pointed out that juvenility of tissue was an important factor in influencing the regeneration competence of stem nodal explant. In the present study, it was also observed that immature keikis with unopened leaves showed more response than mature ones. Initiation of bud or proliferation of PLBs was along the nodal and basal portion of keikis. Shoot tip explants of keikis gave less response.

In the present study, the number of days for initiation of shoot differed significantly between the hybrids. In many of the hybrids, single small buds showed initiation. During the course of development, the original explant gradually turned brown. Sivamani (2004) also observed significant difference in number of days for shoot initiation in *Dendrobium* hybrids varying from 39.67 to 57.17 days. In *Dendrobium wardianum*, the nodal buds started developing in approximately three weeks of culture (Kumaria *et al.*, 2005). Lakshmidevi (1992) observed that in *Dendrobium* cultures rapid shoot growth was obtained in the presence of BA 3.0 mg l^{-1} and NAA 2.0 mg l^{-1}, approximately in 1 : 1 ratio. Sivamani (2004) pointed out that KN 4.0 mg l^{-1} along with IAA 4.0 mg l^{-1} was the best for rapid bud initiation and shoot growth. These reports are in support of the present study.

Number of days for initiation of first leaf differed significantly between the hybrids. Paired leaves appeared from the shoot buds or PLBs after four to 10 weeks in culture. This is in agreement with the results reported by several workers. Time taken for the *in vitro* differentiation of first leaf was reported to be varying from 6 to 15 weeks in *Rhynchostylis retusa* by Vij and Kaur (1992). Sivamani (2004) also reported significant difference in the duration of *in vitro* initiation of first leaf in *Dendrobium* hybrids ranging from 26.50 to 43.17 days. Addition of CW significantly influenced the

development of protocorms into plantlets in terms of early formation of leaf and maximum number of leaves in *Dendrobium* var. Sonia (Ramsunder *et al.*, 2000).

In the present study, once the PLB or shoot bud had been differentiated, in about four to seven weeks the cultures were transferred to the first subculturing medium (1/2 MS + BA 4 mg l^{-1} + NAA 2 mg l^{-1} + IBA 2 mg l^{-1}) for faster growth and development. This procedure was followed based on the findings of several workers. Lakshmidevi (1992) observed that shoots of *Dendrobium* on 2.0 mg l^{-1} NAA and 2.0 mg l^{-1} BA had rapid shoot growth and well expanded leaves. A high concentration of NAA and BA was reported to induce maximum proliferation of shoot by Kusumoto (1980). Influence of IBA on good shoot production of *Dendrobium monoliforme* was reported by Lim *et al.* (1993).

In the present study, a wide variation was noticed in the duration of root initiation from the PLBs or shoot buds among the hybrids ranging from four to nine weeks. Cultures in the first subculturing media developed one to four thread like roots from the base of the shoots. Sivamani (2004) reported a minimum of 29.83 days for root initiation in the presence of IBA 2 mg l^{-1}. This is in agreement with the present study. Similar results were also obtained by several workers. Lakshmidevi (1992) reported that half strength MS with the auxin IBA was the best with respect to root initiation within the shortest time in maximum number of cultures in *Dendrobium.* Nuraini and Shaib (1992) reported that the addition of IBA in the medium was necessary for root initiation in *Dendrobium* Miss Hawai cultures.

5.2 *IN VITRO* MULTIPLICATION USING LEAF EXPLANTS

The present study was conducted for exploring the potential of *in vitro* leaves as source of explants for micropropagation. The main advantage of this technique is that in selected single plant hybrids once they are established *in vitro*, they can further be mass multiplied using the *in vitro* leaf as explant. The advantages of leaf segment culture are preferred for

more than one reason, the leaves are easy to obtain, easier to disinfect, their excision is simple and does not endanger the mother plant (Deb and Temjensangha, 2006). Kaur and Vij (2000) reported that *in vitro* sourced leaf tissues responded far more frequently to a wide range of stimuli in comparison to greenhouse sourced tissues. Rapid *in vitro* propagation of *Dendrobium* hybrids Sonia 17 and 18 using *in vitro* derived leaf explants was reported by Martin and Madassery (2006). Regeneration potential of leaf tissues was demonstrated for the first time by Wimber (1965), who was able to produce PLBs from leaf tissues in *Cymbidium*.

Use of pre-existing meristem as explant is of great importance in ensuring stability of the micropropagated plants (Das and Bhattacharjee, 2006). Nutritional regimes *in vitro* and physiological age of the donor tissues greatly influences the regeneration potential of explants in orchid tissue culture (Arditti and Ernst, 1993). Latha and Seeni (1991) suggested that the entire surface of the leaf was potentially meristematic and regenerative. In the present study, juvenile leaf segments excised from three to four months old *in vitro* established selected *Dendrobium* hybrids were used as explants. Culturing of young *in vitro* leaf segments of size 1.5 – 2.0 mm^2 produced best results with good regeneration of PLBs and subsequent plantlet development.

Time required for PLB or callus formation was reported to be greatly influenced by the orchid species, type of explant used and nutritional regime in the culture medium (Arditti and Ernst, 1993). In the present study, the first sign of activity namely the swollen cut leaf edges could be seen after one week of culture. It was observed that at higher level of CH (500 mg l^{-1}) the swollen explants produced significant and rapid callus development than at lower level of CH (250 mg l^{-1}). The reports of Latha and Seeni (1991) that *Phalaenopsis* cultures showed rapid PLB formation in the presence of CH 500 mg l^{-1} supports this finding. Deb and Temjensangha (2006) reported PLB development from *in vitro* sourced 20-25 week old leaf segment of *Arachnis labrosa* on MS medium containing 500 mg l^{-1} CH and 4 mg l^{-1} BA

which is also in accordance with the present findings. The beneficial effect of CH on *in vitro* culture of orchids have also been reported by Hass Von Schmude (1983) and Chaturvedi and Sharma (1986). Higher concentration of CH (1000 mg l^{-1}) was reported to induce callus formation in *Vanilla walkeria* by Agarwal *et al.* (1992). Kinetin containing medium was reported to be the best for PLB production from *in vitro* leaf explants by Abdulkarim *et al.* (1992).

Utility of leaf segments in producing identical clones through direct or callus mediated organogenesis was emphasized by Arditti (1967). According to Lay (1978) leaf tip explants of *Dendrobium* cultivars and hybrids did not survive but the leaf parts including the base proliferated and formed calli, which differentiated into plantlets. In the present study, *in vitro* leaf segments from basal parts of leaf including the midrib region had shown greater proliferation and development of callus than from the leaf tip region. This is in conformity with the findings of Champagnat *et al.* (1970), Abdulkarim and Hairani (1990) and Seeni and Latha (1992).

During the investigation, after 45 days of inoculation the *in vitro* leaf segments with differentiated calli were transferred on to the same medium with two different levels of CH, *viz.*, CH at 250 mg l^{-1} and no CH. Out of the two treatment combinations, the callus in the medium containing CH at 250 mg l^{-1} responded well by showing considerable vigour and rapid shoot growth with well expanded leaves, while absence of CH produced short shoots and small leaves. But no significant difference was observed between the treatments in the case of number of shoots developed. Several workers have pointed out similar results in support of the present findings. Incorporation of CH enhances the frequency of adventitious shoot formation (Huang and Murashige, 1977). Half strength MS medium containing 6.97 micro M KN facilitated conversion of more than 90 per cent PLBs to shoots in *in vitro* leaf explant culture of *Dendrobium* hybrids Sonia 17 and 28 (Martin and Madassery, 2006). Kurup *et al.* (2005) reported that MS basal

medium containing 5 mg l^{-1} BA and 1000 mg l^{-1} CH could initiate multiple shoots in *Dendrobium* var. Betty Ho. Fonnesbech (1972) reported an increase in the growth of *Cymbidium* Sw. and *Cattleya* tissues by inclusion of CH in the medium. Agarwal *et al.* (1992) reported that presence of CH at 1000 mg l^{-1} in the basal medium promoted shoot multiplication. Kononowicz and Janick (1984) obtained multiple shoots *in vitro* in MS medium supplemented with 1000 mg l^{-1} CH. Higher levels of kinetin (5 mg l^{-1}) and BAP (1.5 mg l^{-1}) was found suitable for maximum shoot development and shoot growth in *Dendrobium* cv. Sonia (Pathania *et al.*, 1998).

In the present study, the effect of different strengths of MS medium tested on number of days for deflasking have shown that half strength MS medium was the best. The tiny plantlets when subcultured on charcoal (1 g l^{-1}) and coconut water (200 ml l^{-1}) enriched half strength MS medium supplemented with combinations of NAA (0.1 mg l^{-1}) and BAP (0.5 mg l^{-1}) showed early establishment of plantlets which were ready for plant out in six months. Mature, well formed seedlings with three to four leaves and four to five roots after three to four sub-culture passages were deflasked and acclimatized in a humidity chamber. The report of Martin and Madassery (2006) that half strength MS medium with 2 g l^{-1} AC was the best for *in vitro* rooting of plants derived from *in vitro* leaf explant culture of *Dendrobium* hybrids Sonia 17 and 28 supports these findings. Sudeep (1994) obtained more shoots in half strength MS medium. Lekha Rani (2002) stated that half strength MS medium was found to be the best for early germination and rapid *in vitro* development in *Dendrobium* hybrids. Half strength MS medium was reported to be ideal for better establishment of certain species of orchids *in vitro* by many workers (Agarwal *et al.*, 1992; Chung *et al.*, 2005; Kishore *et al.*, 2005).

A combination of 0.1 mg l^{-1} NAA and 0.5 mg l^{-1} BAP was reported to induce greater regeneration in *Dendrobium* hybrids (Kishore *et al.*, 2005). The establishment of rooted plantlets were higher in the combination of BAP

2.0 mg l^{-1} and NAA 1.0 mg l^{-1} in *Dendrobium* Sonia (Ramsunder *et al.*, 2000). Vij and Sood (1982) reported that a combined treatment of BAP and NAA was essential for root development in *Dendrobium moschatum*. Optimum callusing and plant regeneration was recorded in the presence of 0.5 mg l^{-1} NAA and 1 mg l^{-1} BAP in *Dendrobium fimbriatum* (Roy and Banerjee, 2003).

The above discussion points out that the best *in vitro* culture medium for rapid multiplication using *in vitro* leaf explants in selected *Dendrobium* hybrids are as follows: For culture initiation the best media combination was half strength MS medium supplemented with BAP 5 mg l^{-1} + KN 5 mg l^{-1} + CH 500 mg l^{-1} + CW 200 ml l^{-1} + AC 1 g l^{-1}. For first subculturing after 45 days of inoculation, half strength MS medium with BAP 5 mg l^{-1} + KN 5 mg l^{-1} + CH 250 mg l^{-1} + CW 200 ml l^{-1} + AC 1 g l^{-1} was found to be ideal. For better *in vitro* rooting and early establishment of plantlets, the best media combination for second subculture was half strength MS supplemented with 0.1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP + 1 g l^{-1} AC + 200 ml^{-1} CW. The findings of this investigation opens new route for mass multiplication of *in vitro* established selected *Dendrobium* hybrids using *in vitro* leaf explants.

5.3 MOLECULAR CHARACTERIZATION

Various types of molecular markers are utilized to evaluate DNA polymorphism and among them, the most important is polymerase chain reaction (PCR) based markers. Now-a-days PCR based molecular markers have developed into powerful tools to analyse genetic relationships and genetic diversity. RAPD technique is one among them. RAPD techniques have been used for the identification of hybrids and their parent determination. Wang *et al.* (1994) proposed RAPD fingerprinting as a convenient tool for the identification, protection and parentage determination of plant hybrids.

In the present study RAPD analysis was done for the molecular characterization of 40 selected *Dendrobium* hybrids, making use of arbitrary primers to amplify random DNA sequence in the genome.

Isolation of genomic DNA of selected *Dendrobium* hybrids was done using Mondal *et al.* (2000) method with slight modification. Tissues from young tender leaves were found to yield good quality DNA. The DNA yield for 40 selected *Dendrobium* hybrids ranged from 630 to 960 ng/µl. The purity of DNA (A_{260}/A_{280} ratio) ranged from 1.60 to 1.85 µg μl^{-1}.

To identify the promising primers for RAPD analysis, 40 decamer primers of kit A and B were screened using the DNA of hybrid H-298. The procedure standardized by Lim *et al.* (1999) for *Vanda* was tried for amplification.

For further amplification of DNA eight decamer primers were identified for RAPD analysis based on the performance in DNA amplification and production of highest number of polymorphic bands as well as intense bands with reproducibility. They were OPA-03, OPA-04, OPA-10, OPA-16, OPA-18, OPB-02, OPB-06 and OPB-10. Pillai (2003) reported that four primers OPA-10, OPB-02, OPB-04 and OPB-10 were yielding good resolution bands when used for the molecular characterization (RAPD) of fifteen *Dendrobium* varieties. Krishnapriya (2005) used three primers OPB-11, OPB-12 and OPB-17 for RAPD analysis to characterize genetic variability and relationships among 12 cultivars of *Dendrobium* at molecular level. However, Bhat and Jarret (1995) suggested that the number of polymorphisms might be more important than the number of primers for the generation of stable phenogram and it would vary with plant material under investigation and the sequences that are amplified.

In the present study a total of 69 scorable bands (average of 8.63 bands per primer) were generated by the selected eight primers of which three were monomorphic and the remaining 66 were polymorphic (95.65 per cent). The number of amplification products ranged from four to twelve with an

average of two per primer. Pillai (2003) obtained 44 amplified RAPD markers in the molecular characterization of fifteen *Dendrobium* varieties, of which 39 were polymorphic and five were monomorphic. Chakrabarti (2005) obtained a total of 227 distinct major RAPD bands of which 97 per cent were polymorphic whereas out of the 27 bands amplified, 24 were found to be polymorphic by Krishnapriya (2005).

The highest number of scorable bands (12 bands) was given by OPA-18 followed by OPA-10 (11 bands), OPA-16 (11 bands), OPB-06 (9 bands), OPA-04 (8 bands), OPB-02 (7 bands), OPB-10 (7 bands) and OPA-03 (4 bands). The primer OPA-03 produced four scorable bands of which two bands were monomorphic while among the eight scorable bands produced by primer OPA-04 one band was monomorphic.

The estimation of Jaccard's coefficient's and construction of dendrogram by using UPGMA revealed the presence and extent of genetic similarities among the 40 selected *Dendrobium* hybrids. The overall similarity coefficients ranged from 0.29 to 1.00. The genetic distance revealed the genetic variability that exists between the hybrids. Cluster analysis revealed that at about 0.46 similarity coefficient, the 40 selected *Dendrobium* hybrids got divided into two groups. Among the 40 hybrids, H-376 and H-102 fell separately in cluster I and cluster II A respectively. This substantiates the moderately broad distribution of genetic variability, which can be attributed to the broad genetic base in their origin. All the forty hybrids included in the present study are higher order hybrids with three to four distinct species in varying dosages involved in their parentage (Appendix I). The grouping of the hybrids in cluster was largely consistent based on the knowledge about their breeding history.

Cluster II B contained two hybrids H-20 and H-33. This grouping supports their belonging to the same grex combination (*Dendrobium* Pramot x *Dendrobium* CSTD).

The two hybrids H-234 and H-247 which showed 61.3 per cent similarity fell in cluster II C. This can be substantiated by their pedigree relationship which reveals seven parents in common for both of them (*Appendix I*).

Based on similarity coefficient, cluster II D was found to include six hybrids (H-285, H-298, H-291, H-299, H-223 and H-316). The pedigree chart revealed that H-285 and H-298 were having three parents in common, whereas H-291 showed four and seven parents in common with H-285 and H-298 respectively. With respect to the three hybrids H-299, H-223 and H-316, *Dendrobium* CSTD was found to be in common as the male parent and three other parents were found to be common among them on the female side which supports their clustering in one group.

Among the two hybrids H-60 and H-281 in cluster II E, seven parents in their ancestry were found to be common which attributed to 58.33 per cent similarity between them.

The cluster II F comprises of four hybrids (H-391, H-388, H-383 and H-380) of which H-391, H-388 and H-383 formed one group while H-380 remained distinct. This can be substantiated by their pedigree relationship. H-380 had three parents in common with the rest of the three hybrids in the cluster which made it fall out separately at 0.55 similarity coefficient; whereas for H-391, H-388 and H-383 almost all the parents were common in their ancestry which made them come under the same group in cluster analysis.

At 0.63 similarity coefficient cluster II G further divided into cluster II G_1 and cluster II G_2. Of these, cluster II G_1 consisted of three hybrids (H-283, H-281 and H-253). Among these H-253 stood distinct from H-281 and H-283 at 66.3 per cent similarity which can be attributed to the two common parents in their ancestry whereas H-281 and H-283 was found to share four common parents in their pedigree which led to 69.04 per cent similarity between them in cluster analysis.

Cluster II G_2 formed two subgroups at 0.63 similarity coefficient with H-381, H-342 and H-330 forming one subgroup while H-377, H-372, H-124 and H-100 D remained in the second subgroup. In the first group, at 0.72 similarity coefficient, H-381 got distinct from H-342 and H-330 which might be attributed to their having four to eight common parents in their ancestry, whereas H-342 and H-330 shared four parents in common.

In the second group of cluster II G_2, at 0.66 similarity coefficient H-100 D got distinct from the other three hybrids (H-124, H-372 and H-377) which might be due to its commonness in seven parents with the rest of three hybrids. H-377 shared 10 parents in common with H-372 and H-124 showed 82 per cent similarity with H-377 which is attributed to the commonness in seven parents in their pedigree.

Cluster II H_1 consisted of three hybrids (H-147, H-176 and H-132). Among these H-147 stood singly at 0.70 similarity coefficient which might be due to the commonness in seven parents in their pedigree. H-176 and H-132 showed 78.04 per cent similarity with each other, which might be due to the eight common parents in their ancestry.

Cluster II H_2 consisted of two subgroups at 0.65 similarity coefficient. The first subgroup consisted of H-127, H-122, H-177, H-210 and H-93. Among these H-122 and H-127 showed 76.37 per cent similarity with each other, which may due to their belonging to the same grex. H-177 stood out separately sharing seven common parents in their ancestry with H-210 and H-93 showing 72.97 per cent similarity with one another.

The second subgroup of cluster II H_2 comprised of H-111, H-61, H-63 and H-59 at 0.67 similarity coefficient with the other three hybrids. H-59, H-63 and H-61 fall under one cluster due to their belonging to the same grex. H-111 stood out at 66.3 per cent similarity sharing 7 parents in common with H-59, H-63 and H-61

In the present study, the cluster based on RAPD analysis using eight primers clearly demonstrates the existence of genetic variation within the 40 selected *Dendrobium* hybrids. Polymorphism obtained in the present study will be useful in fingerprinting of these selected *Dendrobium* hybrids. Finally, the results support the idea that RAPD technique being relatively simpler, quicker, inexpensive and non-radioactive can detect sufficient polymorphisms in genetic distance studies. The clustering based on RAPD analysis also reveals the expression of ancestral characters and thereby the occurrence of recombination in the 40 selected *Dendrobium* hybrids.

SUMMARY

6. SUMMARY

Attempts were made in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2004-2006 to undertake initial culture establishment and molecular characterization of 40 selected *Dendrobium* hybrids developed here itself under a DBT funded orchid breeding project and to refine the protocol for rapid multiplication using *in vitro* leaf explants.

The results of the study are summarized below:

- Initial culture establishment of the 40 selected *Dendrobium* hybrids was carried out using stem nodal segments of 1.0 to 1.5 cm length with one node each from keiki's in the identified best medium, *viz.*, VW + KN 4 mg l^{-1} + IAA 4 mg l^{-1} + CW 200 ml l^{-1}.
- The response of the stem nodal explant and mode of development of the PLBs or shoot buds therefrom, were found to be varied among the hybrids. In some hybrids shoot buds developed directly and the PLBs developed thereafter, whereas others showed regeneration of explant through PLB formation.
- In the stem nodal culture, the duration for the initiation of PLBs, shoot, leaf and root were found to differ significantly between the hybrids.
- Once the PLBs or shoot bud had been differentiated, in about four to seven weeks the stem nodal cultures were transferred to the subculturing media.
- In the *in vitro* multiplication using leaf explants, culturing of young *in vitro* leaf segments of size 1.5 – 2.0 mm^2 obtained from *in vitro* established *Dendrobium* hybrids had proven to be a potential method for micropropagation.

- Among the different levels of CH tried with *in vitro* leaf explants, CH at 500 mg l^{-1} showed early swelling of cut leaf edges and recorded the minimum number of days for PLB initiation. For culture initiation the best media combination was half strength MS medium supplemented with BAP 5 mg l^{-1} + KN 5 mg l^{-1} + CH 500 mg l^{-1} + CW 200 ml l^{-1} + AC 1 gl^{-1}.

- *In vitro* leaf segments from basal part of leaf including the midrib region had shown greater proliferation and development of callus in comparison to the leaf tip region.

- Out of the two different levels of CH tried in the subculturing media of *in vitro* leaf culture, the medium containing CH at 250 mg l^{-1} responded well by showing considerable vigour and rapid shoot growth with well expanded leaves. But no significant difference was observed between the treatments in the case of number of shoots developed. For first subculturing, half strength MS medium with BAP 5 mg l^{-1} + KN 5 mg l^{-1} + CH 250 mg l^{-1} + CW 200 ml l^{-1} + AC 1 gl^{-1} was found to be ideal.

- Trial with different strengths of MS media for *in vitro* leaf culture tested on number of days for deflasking have shown that half strength MS medium was the best, recording early establishment of plantlet in six months. For better *in vitro* rooting and early establishment of plantlet, the best media combination for second subculture was half strength MS supplemented with 0.1 mg l^{-1} NAA + 0.5 mg l^{-1} BAP + 1 gl^{-1} AC + 200 ml l^{-1} CW.

- In the present study, molecular characterization of the forty selected *Dendrobium* hybrids have been carried out using RAPD. The DNA yield ranged from 630 to 960 ng/µl. The purity of DNA (A_{260}/A_{280} ratio) ranged from 1.60 to 1.85 µg/µl. The eight primers, selected from 40 initially screened primers, generated 69 scorable bands of

which three were monomorphic and the remaining 66 were polymorphic (95.65 per cent).

- The estimation of Jaccard's similarity coefficients and construction of dendrogram by using UPGMA revealed the presence and extent of genetic similarities among the forty selected *Dendrobium* hybrids examined. The overall similarity coefficient ranged from 0.29 to 1.00. The forty selected *Dendrobium* hybrids formed 11 clusters in UPGMA cluster analysis.

- The clustering based on RAPD analysis of the 40 selected *Dendrobium* hybrids clearly demonstrates the existence of genetic variation among them. It also reveals the expression of ancestral characters. Thereby the present study will be useful in the fingerprinting of these 40 selected *Dendrobium* hybrids.

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7. REFERENCES

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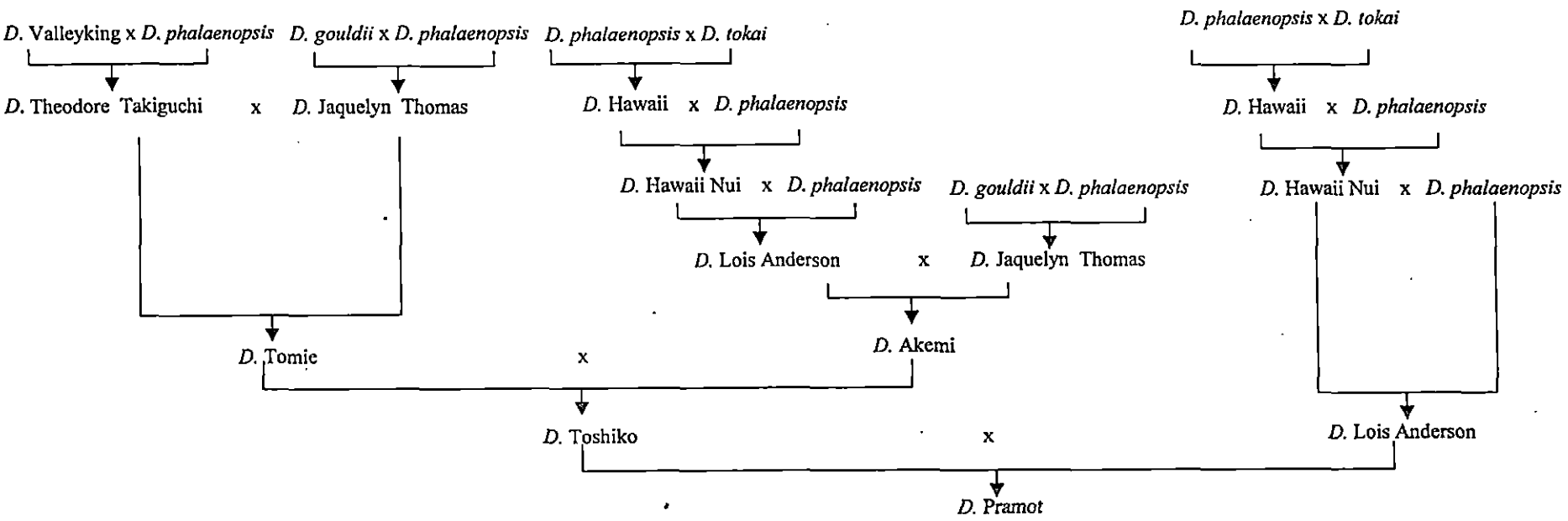
*Original not seen

APPENDICES

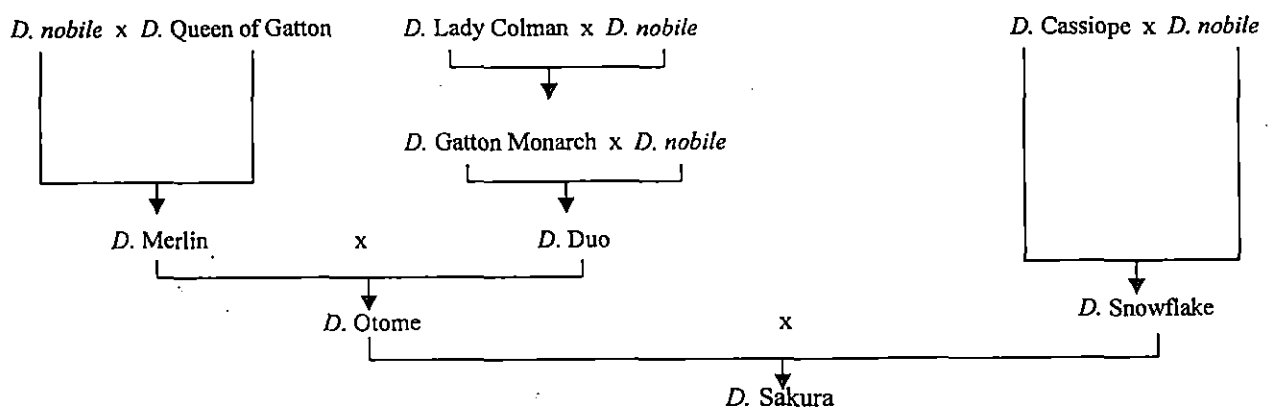
APPENDIX - I

Pedigree chart showing the ancestry of the nine parental varieties involved in the hybridisation programme

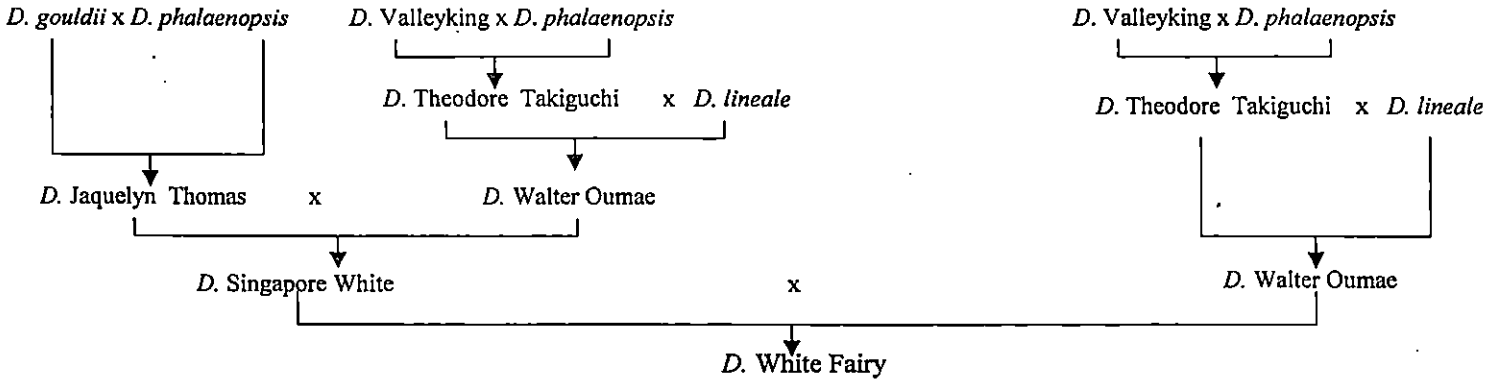
(1) *Dendrobium* Pramot



(2) *Dendrobium* Sakura



(3) *Dendrobium* White Fairy



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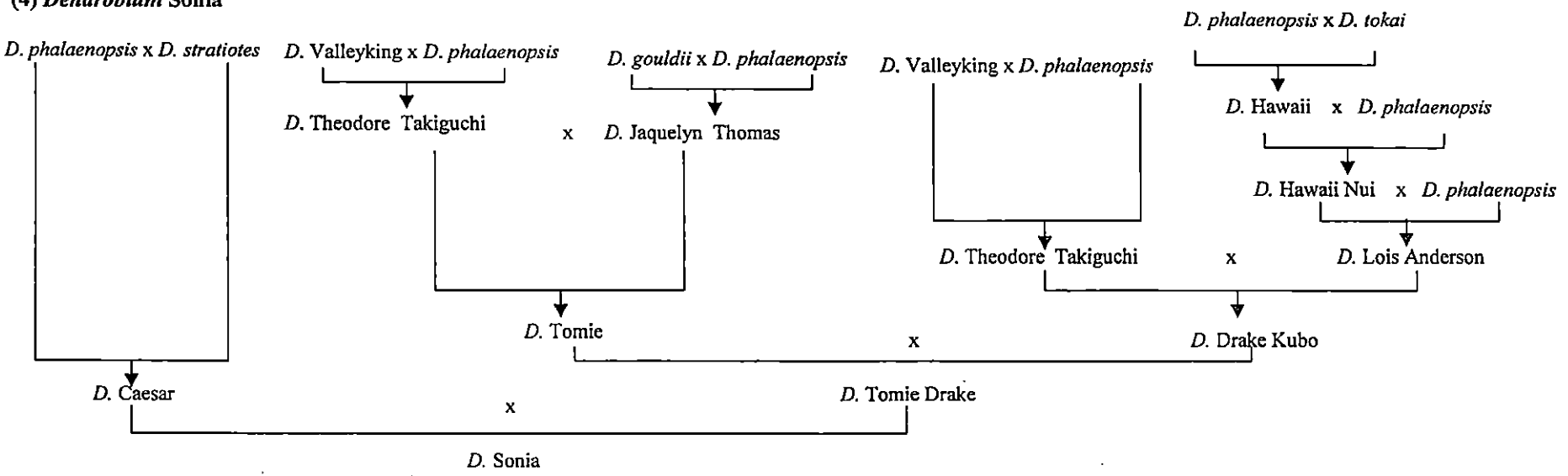

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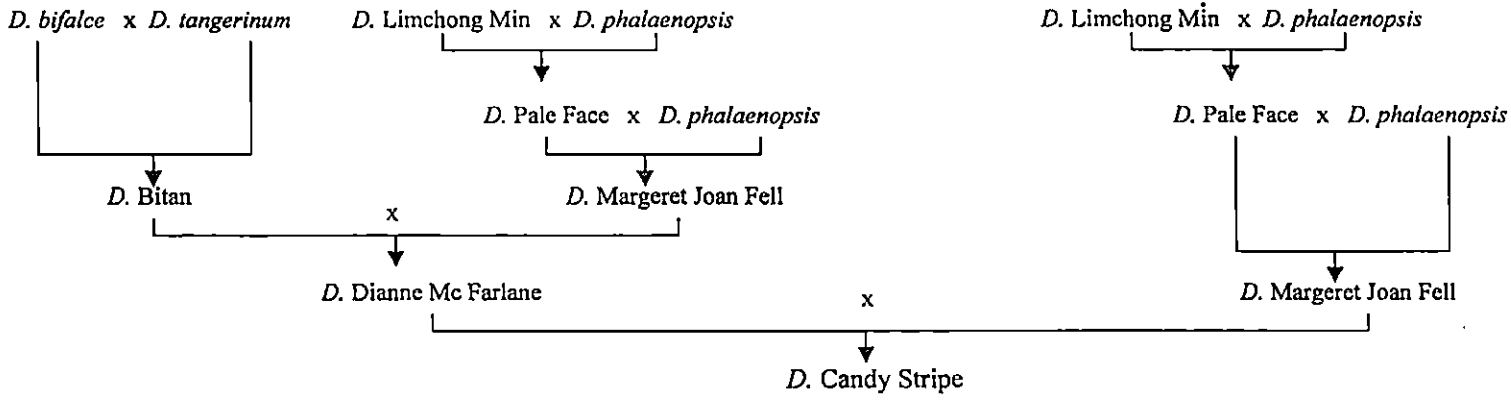
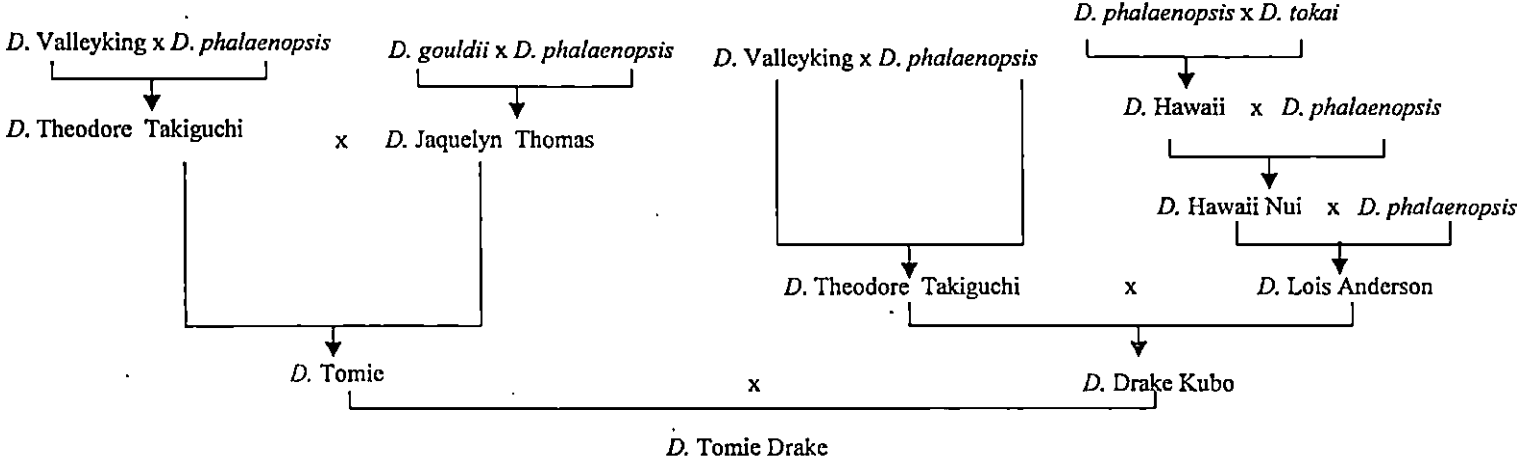
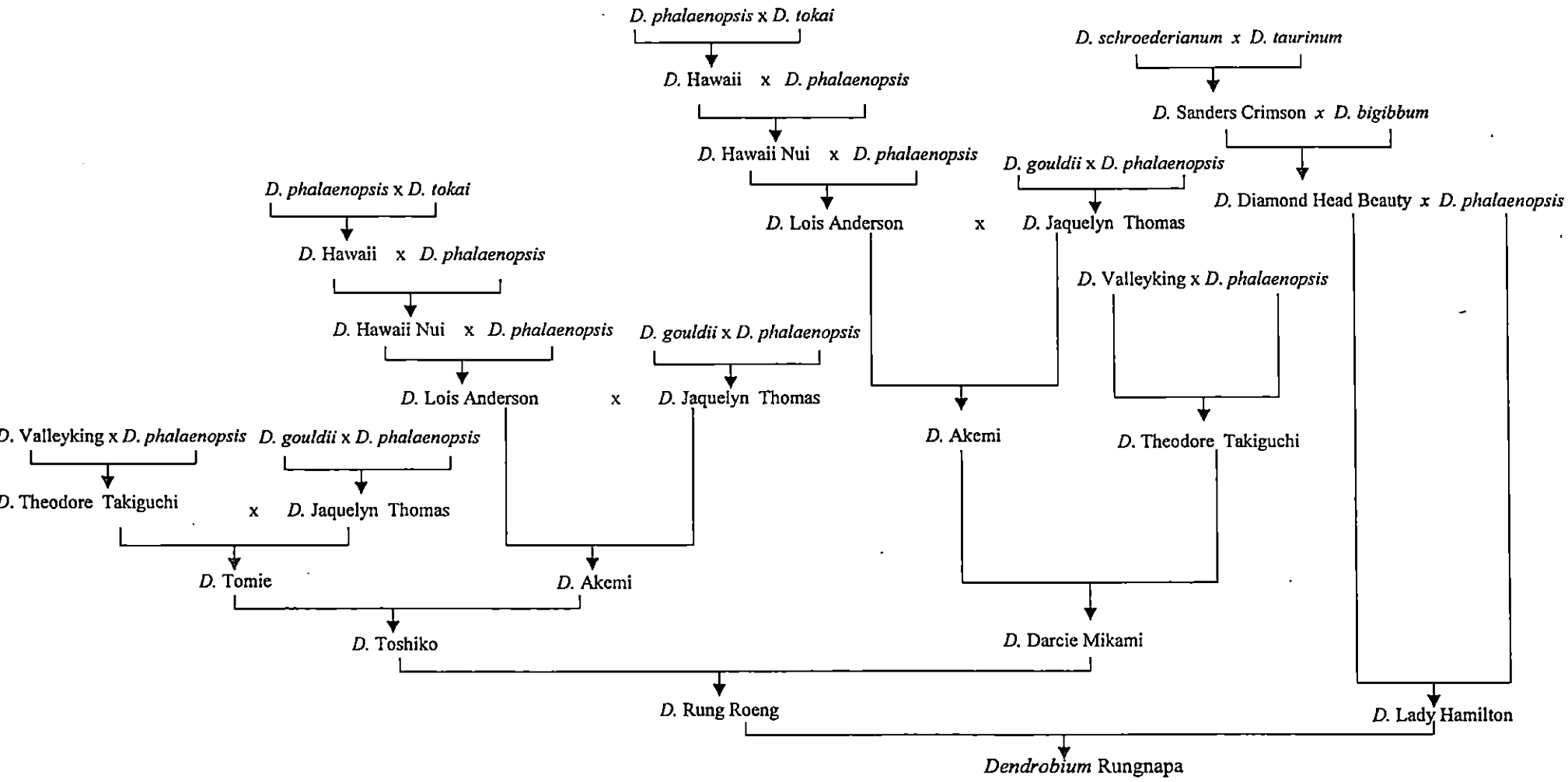


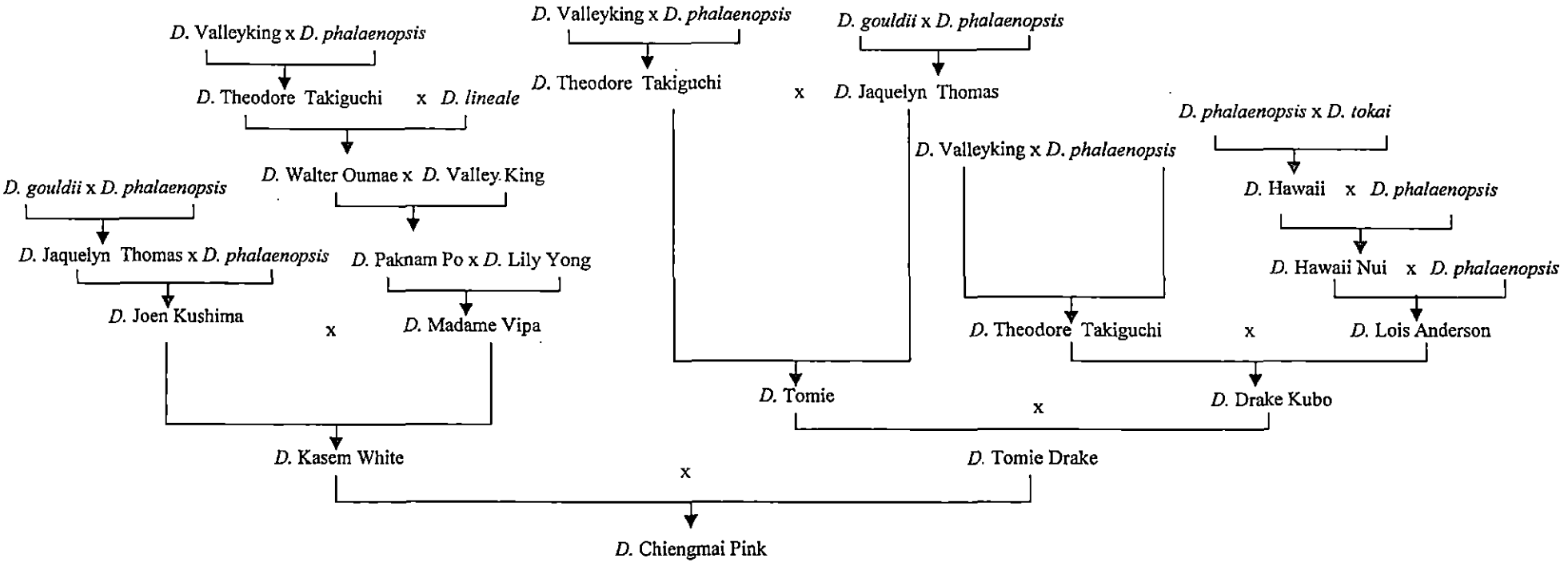
(6) *Dendrobium* Tomie Drake



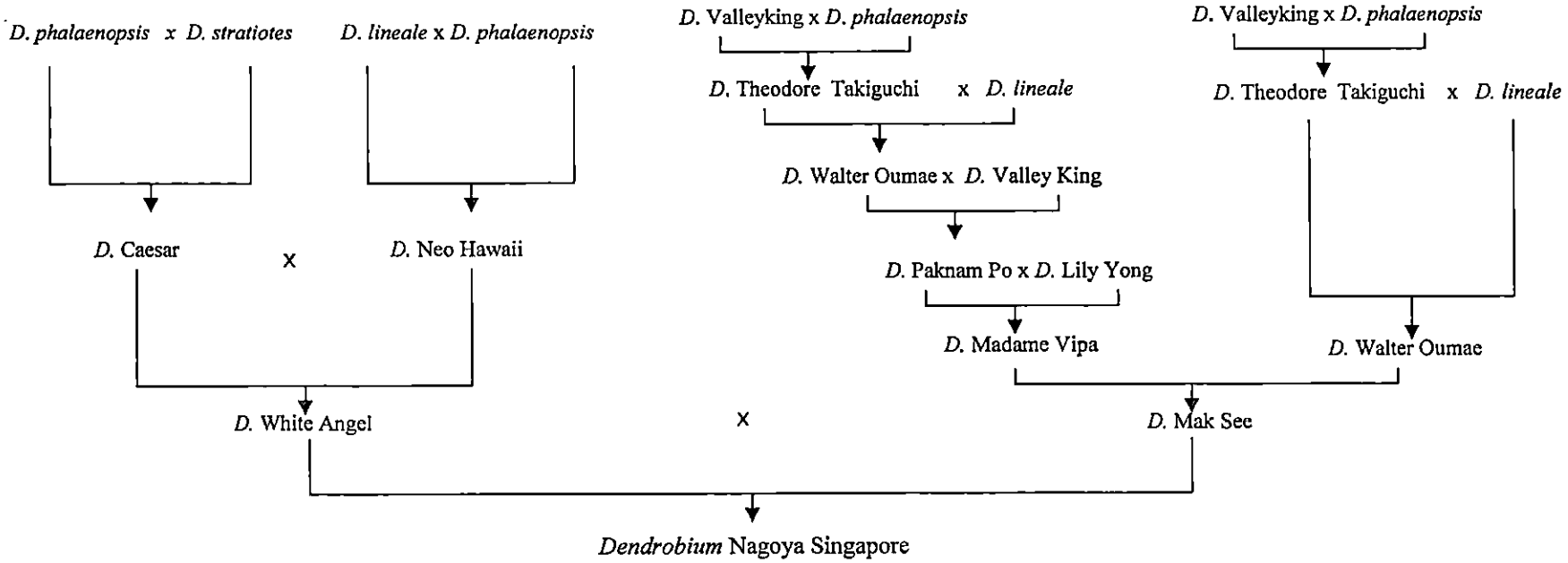
(7) ***Dendrobium*** Rungnapa



(8) *Dendrobium* Chiengmai Pink



(9) *Dendrobium* Nagoya Singapore



APPENDIX - II

Species and varieties involved in the parents

	Parental variety	Species and varieties involved
1)	*Dendrobium* Sonia	*D. phalaenopsis*, *D. stratiotes*, *D. gouldii*, *D. tokai*, *D.* Valley king
2)	*D.* CSTD	*D. phalaenopsis*, *D. gouldii*, *D. tokai*, *D.* Valley king, *D. bifalce*, *D. tangerinum*, *D.* Limchong Min
3)	*D.* Rungnapa	*D. phalaenopsis*, *D. gouldii*, *D. tokai*, *D.* Valley king, *D. bigibbum*, *D. schroederianum*, *D. taurinum*
4)	*D.* Chiangmai Pink	*D. phalaenopsis*, *D. gouldii*, *D. tokai*, *D.* Valley king, *D. lineale*, *D.* Lily Yang
5)	*D.* Nagoya	*D. phalaenopsis*, *D. stratiotes*, *D.* Valley king, *D. lineale*, *D.* Lily Yang
6)	*D.* Pramot	*D. phalaenopsis*, *D. gouldii*, *D. tokai*, *D.* Valley king
7)	*D.* Sakura	*D. nobile*, *D.* Queen of Gatton, *D.* Lady Colman, *D.* Cassiope
8)	*D.* White Fairy	*D. phalaenopsis*, *D. gouldii*, *D.* Valley king, *D. lineale*

IN VITRO MULTIPLICATION AND MOLECULAR CHARACTERIZATION OF SELECTED *DENDROBIUM* HYBRIDS

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Abstract of the
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ABSTRACT

Attempts were made to undertake initial culture establishment and molecular characterization of 40 selected *Dendrobium* hybrids developed in the Department of Plant Breeding and Genetics under a DBT funded orchid breeding project and to refine the protocol for rapid multiplication using *in vitro* leaf explants. The studies were carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani during 2004-2006.

Initial culture establishment of the 40 selected *Dendrobium* hybrids were carried out using stem nodal explants obtained from keikis and were inoculated in the identified best media, *viz.*, VW + KN 4 mg l^{-1} + IAA 4 mg l^{-1} + CW 200 ml l^{-1} (Sivamani, 2004). The nature of response of the stem nodal explant was found to be varied among the hybrids. The duration taken for the initiation of PLBs, shoot, leaf and root differed significantly among the hybrids. Once the PLBs or shoot buds had been differentiated, the cultures were transferred to the subculturing media for faster growth and development. Leaves and roots appeared from the shoot buds or PLBs after four to ten weeks in culture. The plantlets obtained were hardened and transferred to the greenhouse. Plantlets obtained through stem nodal cultures were more vigorous than that from *in vitro* leaf explant cultures.

The regeneration potential of *in vitro* sourced leaf explants appeared to be an inherent trait of orchids which gets evoked under certain physico-chemical stimulus under *in vitro* condition. The *in vitro* sourced leaf explants can be profitably used for micropropagation. The main advantage of this technique is that in selected single plant hybrids where keiki production is low, once they are established *in vitro*, they can be further mass multiplied using the *in vitro* leaf as explant. The young leaf segments derived from *in vitro* established *Dendrobium* hybrids were used as explant. Swelling of cut leaf edges and callus initiation studies were conducted with two

levels of CH (500 mg l^{-1} and 250 mg l^{-1}) in half strength MS medium containing BAP 5 mg l^{-1} + KN 5 mg l^{-1} + CH 500 mg l^{-1} + CW 200 ml l^{-1} + AC 1 gl^{-1}. Out of this CH at 500 mg l^{-1} was found to be the best for callus initiation and subsequent development of plantlet. The callus obtained from the inoculation medium, irrespective of the levels of CH used, were grouped together and cultured on regeneration media with two different levels of CH, *viz.*, CH at 250 mg l^{-1} and no CH. Presence of CH at 250 mg l^{-1} was found to have significant effect on shooting response. The optimum medium combination for shoot initiation was found to be half strength MS medium with BAP 5 mg l^{-1} + KN 5 mg l^{-1} + CH 250 mg l^{-1} + CW 200 ml l^{-1} + AC 1 gl^{-1}. Out of the different strengths of MS medium tried with *in vitro* leaf explant, half strength MS medium exhibited early establishment of plantlet and recorded minimum number of days for deflasking. Half strength MS with NAA 0.1 mg l^{-1} + BAP 0.5 mg l^{-1} + AC 1 gl^{-1} + CW 200 mg l^{-1} was found to be the best medium combination for better *in vitro* rooting and early establishment of plantlet. Plantlets were ready for plant out in six months.

Random amplified polymorphic DNA (RAPD) analysis was employed for the molecular characterization of the 40 selected *Dendrobium* hybrids. The eight primers, selected from the 40 initially screened primers, generated 69 scorable bands of which three were monomorphic and the remaining 66 were polymorphic (95.65 %). All primers produced polymorphic amplification products, however, the extent of polymorphism varied with each primer. Statistical analysis was carried out using NTSYS-PC software and a dendrogram was generated using Jaccard's similarity coefficients. The overall similarity coefficient ranged from 0.29 to 1.00. The forty selected *Dendrobium* hybrids that were studied formed 11 clusters in UPGMA cluster analysis. The grouping of hybrids in the cluster was largely consistent with what is known about their breeding history. The cluster based on RAPD analysis using eight primers clearly demonstrates the existence of genetic variation within the 40 selected *Dendrobium* hybrids. Polymorphism obtained in the present study can be used as fingerprints of the forty selected *Dendrobium* hybrids.