MICROPROPAGATION, BIOCHEMICAL AND MOLECULAR STUDIES OF ORCHIDS OF WESTERN GHATS OF GOA AND ITS ENVIRONS

Thesis submitted to the
Goa University for the Award of Degree of

DOCTOR OF PHILOSOPHY
IN
BOTANY

By

Govind V. Parab

Certified that all the corrections suggested by the examiners have been incorporated in this thesis.

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May 2009

STATEMENT

As required by the University Ordinance 0.19.8(ii), I state that the present thesis "**Micropropagation, Biochemical and Molecular Studies of Orchids of Western Ghats of Goa and its Environs**", is my original contribution and the same has not been submitted on any occasion for any other degree or diploma of this University or any other University/ Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Govind 12/5/09

Place: Goa
Date: 12/05/2009.

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CERTIFICATE

As required by the University Ordinance 0.19.8(IV), this is to certify that the thesis entitled "**Micropropagation, Biochemical and Molecular Studies of Orchids of Western Ghats of Goa and its Environs**", submitted by **Mr Govind V. Parab** for the award of the degree of Doctor of Philosophy in Botany, is based on his original and independent work carried out by him during the period of study, under our supervision.

The thesis or any part thereof has not been previously submitted for any other degree or diploma in any University or Institute.

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Govind V. Parab

Dedicated to the Memories of My

Beloved Father Shri Vasant G. Parab

CONTENTS

ABBREVIATIONS

%	Percent
±	Plus or minus
°C	Degree centigrade
µM	Micro mole
µm	Micrometer
µl	Microliter
BA	6-benzylamino purine
bp	Base pair
c	About
cv	Cultivar
CW	Coconut water
2, 4-D	2, 4-Dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetraacetic acid
Fl	Flower
g	Grams
h	Hour
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
ISSR	Inter simple sequence repeat
KIN	Kinetin
Km	Kilometer
kb	Kilo base pair

l	Litre
m	Meter
M	Molar
mA	Milli ampere
mg	Milligram
MI	Mitra *et al.*, medium
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
mg L^{-1}	Miligram per liter
MS	Murashige and Skoog medium
N	Normal
NAA	1-Naphthaleneacetic acid
NaOH	Sodium hydroxide
ng	Nanogram
nm	Nano meter
OD	Optical density
PGRs	Plant growth regulators
PLBs	Protocorm- like bodies
ppm	Part per million
psi	Pounds per square inch
RAPD	Random amplified polymorphism DNA

rpm	Revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sq. Km	Square kilometer
sec	Seconds
Var	Variety
UV	Ultra violet
Vern	Vernacular
v/v	Volume by volume
VW	Vacin and Went medium
w/v	Weight by volume
WAP	Weeks after pollination

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1. INTRODUCTION

Orchidaceae is among the most species rich plant families with an estimated 30,000-35,000 species. They are distributed in every ecological situation and different habitats (Kull *et al.*, 2006; Singh *et al.*, 2007). India accounts for 1141 species of orchids belonging to 166 genera. They found mostly in the North Eastern region and several are scattered in the Eastern and Western Ghats (Bhanwra *et al.*, 2006). Orchids occupy top position among all flowering plants valued for cut flowers and as potted plants. Taxonomically they are the most highly evolved family among monocotyledons (Cozzolino and Widmer, 2005). Orchids are well known for their beauty (Griesbach, 2003). The floral characteristics of orchids cover an exceptionally wide range of different shapes, forms, sizes and colouration (Dressler, 1993; Thomas and Michael, 2007). Orchid flowers have a longer shelf-life as a result they are marketed globally as cut flowers for corsages, floral arrangements and bouquets, as potted flowering plants and as bedding or aerial plants (Yadav and Basak, 1999; Lopez and Runkle, 2005; Attri *et al.*, 2008).

The world consumption of orchids was valued for more than $500 million in 2000 (Wang, 2004). Countries like China, Germany, Japan, Netherlands, Taiwan, Thailand and United States are involved in large scale orchid production (Griesbach, 2000). Recently, India has started producing orchids for export (Laws, 2004).

In vitro rapid multiplication methods of orchids are essential to cope up the commercial demand and conservation of wild orchids (Baskar and Narmatha Bai, 2006). Plant tissue culture offers opportunities to multiply large number of plants in a shorter time and to conserve threatened and overexploited plant species (Johansen and Rasmussen, 1992; Decruse *et al.*, 2003; Thompson *et al.*, 2006).

Orchid seeds are minute and have no endosperm. In nature they must be symbiotic with some kinds of fungi in order to germinate. The technique of asymbiotic seed germination by *in-vitro* culture was first introduced by Knudson (1946). Morel (1960) developed meristem culture technique which produces identical plants from single piece of apical tissue. Since then, *in-vitro* seed germination protocols have been established for many orchid species (Arditti, 1977, 1992; McKendrick, 1996; Stenberg and Kane, 1998; Kauth *et al.,* 2006; Scade *et al.,* 2006; Stewart and Kane, 2006). The orchids were the first one to be propagated through tissue culture for the commercial use (Arditti and Ernst, 1993). Regeneration from seeds via protocorm-like bodies has become the preferential method for the production of orchids (Das *et al.*, 2007). Mostly horticultural trade depends on wild orchid populations which are not propagated commercially. Hence, *in vitro* propagation techniques are needed for the continuous survival of the wild populations (Kalimuthu *et al.*, 2007).

Biodiversity is categorized into three fundamental levels such as ecological, species and genetic by the International Union for Conservation of Nature and Natural Resources (IUCN) (Mcneely *et al.*, 1990). The ability of population to adopt environmental changes decreases due to human activities like mass collection, habitat destruction and logging which ultimately decreases the biodiversity on the earth (Chung and Park, 2008). In this scenario maintenance of biodiversity is important and global issue (Izawa *et al.*, 2007).

Due to habit specificity, most of the orchids are susceptible to habitat deterioration and fragmentation (IUCN/SSC Orchid Specialist Group, 1996). At present orchids are considered to be at a high risk of extinction and hence most of the orchids are included in the conservation lists (IUCN/SSC Orchid Specialist Group, 1996). Pradhan (1985)

reported that in India, reduction in number of orchid species are taking place mainly due to uncontrolled collection and habitat destruction.

The long distance seed dispersal of orchids by wind was reported by Arditti and Ghani (2000). This particular trait is responsible for high rates of gene flow which results in higher level of genetic variation within populations and low degree of genetic differentiation among populations (Cozzolino and Widmer, 2005).

The understanding of the level of genetic variation within and among populations is needed for conservation and sustainable utilization. Population genetic studies are essential for conservation program and restoration of threatened populations. Evaluation of genetic diversity plays an important role for their conservation management (Hamrick and Godt, 1996).

Comparative population studies using molecular and biochemical markers are needed to collect information on the level and pattern of genetic diversity of wild orchids, which is the initial step towards their conservation (Moller and Spoor, 1993; Geburek, 1997). To obtain consistent information on the existing genetic diversity, a number of reliable and widely used markers have been developed in orchids.

RAPD and ISSR analysis is a popular method for estimating genetic diversity in plant populations with several advantages such as speed, low cost and the use of small amounts of plant material. RAPD and ISSR techniques are also best suited for most the orchids since no sequence information is required (Huff *et al.*, 1993; Zietkiewicz *et al.*, 1994; Ge *et al.*, 1999; Nybom and Bartish 2000; Kingston *et al.*, 2004).

Knowledge about the population genetics of orchid species are limited however, efforts have been made in recent years toward this direction (Ackerman and Ward, 1999; Wong and Sun, 1999; Alexandersson and Agren, 2000; Ehlers and Pedersen, 2000; Sun and Wong, 2001; Li *et al.,* 2002; Forrest *et al.,* 2004; Hollingsworth *et al.,* 2004; Goh *et al.*, 2005; Li and Ge, 2006; Xiaohong *et al.*, 2007; Wang *et al.*, 2008).

SDS-PAGE is useful tool for studying genetic diversity of wild and cultivated plant species (Mukhlesur *et al.*, 2004; Takehisa *et al.*, 2001; Ahmad *et al.*, 2003; Asghar *et al.*, 2004; Neto *et al.*, 2002; Farshadfar and Farshadfar, 2008). However, genetic diversity studies for orchid species using methods such as SDS-PAGE is still limited.

The conservation of biodiversity is a universal issue, which is predicated by governments and communities (Sinclair *et al.*, 1995). Biodiversity can also provide financial returns (Daily *et al.*, 2000). Biodiversity of the Western Ghats is decreasing due to human and other development activities. Hence, it is essential to document and conserve these biological resources to utilize them for the welfare activities.

OBJECTIVES OF THE PRESENT INVESTIGATION

With the above background, the present study aimed at developing simple and rapid regeneration protocol using immature seeds as explants to make use of the embryogenic potential of the callus as an effective system of micropropation. *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl., the most common epiphytic orchid species, valued for their beautiful flowers, attractive colour and long inflorescences were selected during this work.

As a part of the study, we have investigated the genetic variation of these two orchid species using fingerprinting techniques such as RAPD, ISSR and SDS-PAGE protein profiles. Such information will contribute to the better understanding of the genetic profile that can be used to develop strategies for their conservation and sustainable utilization. This will also form a starting point for future research on population and evolutionary genetics of these species. Hence, the present work was undertaken with the following specific objectives.

1. Surveying of attractive wild orchid species from Western Ghats of Goa and adjacent regions for micropropagation.
2. To develop and standardize the protocol for *in-vitro* mass multiplication, regeneration and hardening of selected orchid species.
3. Biochemical studies to characterize the relationship among the populations of above selected orchid species.
4. Molecular characterization to understand the genetic variability among the populations of above selected orchid species.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2. 1. Geographical Distribution

Orchids are distributed all over the world except in the Antarctica. They are abundantly found in tropical countries in South East Asia, South and Central America and South Africa. Also found distributed in China, Japan, Philippines, Europe and Australia (Rao, 1979). Epiphytic orchids are mainly distributed in the northern Andes of South America, Madagascar, Sumatra and Borneo while, Indo-China harbor both epiphytic and terrestrial species and South Western Australia believed to be the centre for terrestrial orchids (Cribb *et al.*, 2003).

India is very rich in orchid flora. Orchids are found distributed in different parts of the country having different altitude, rainfall and temperature. They are located at an altitude up to 5000 meters from the sea level and in areas with rainfall ranging from 60 to 1100 cm per year. Epiphytic orchids are distributed up to 1800 meters and their frequency decreases with the increase in altitude. Most of the terrestrial orchids are limited to the temperate zone. In India, five major orchid zones such as plains, tropical, sub-tropical, temperate and sub-temperate, alpine and sub-alpine zone are classified based on their occurrence in different vegetations (Bose *et al.*, 1999). Majority of orchids in India are distributed in the Eastern Himalayas, the Western and South Indian hills.

2. 2. Economic Importance and Uses

Orchids are unique group of plants that exhibit an incredible range of diversity in size, shape, structure, colour and fragrance of the flower (Thomas and Michael, 2007). Many orchids are useful for small scale industries. Fresh bulbs of *Coelogyne asperata* are used as black board erasers in Sumatra (Withner, 1959). Glue prepared from the species of

Crytopodium, Geodorum nutans and *Bletilla* are utilized for the manufacture of musical instruments. Yellow pseudostems of *Dendrobium utile* are used as ornaments in New Guinea by tribal people (Bose *et al.*, 1999). *Dendrobium* stems are used in making baskets in the Philippines, Indonesia and New Guinea (Yadav and Basak, 1998).

In several parts of the world, orchids are used as food. Roasted Bulbs of *Gastrodia sesamoides* were used as food during emergency situation (Lawler and Slaytor, 1970). Dried leaves of *Dendrobium salaccense* are used as flavoring agent during cooking of rice. In islands of Cyprus, milk custard drink is prepared from dried ground tubers of *Orchis anatolica.* Pseudobulbs of *Cymbidium madidum* and *Dendrobium speciosum* are also reported to be used as food (Bose *et al.*, 1999). Root tubers of *Disa*, *Habenaria* and *Satyrium* are used to make food dish called Chikanda or Kinaka in Zambia (Davenport and Bytebier, 2004). Powdered roots of *Vanda tessellate* are used as antidotes of poisoning, rheumatic pain and abdominal complaints (Amin *et al.*, 2004).

Different parts of orchid are used in treatment of various diseases. Juice obtained from the leaves of *Cymbidium aloifolium* are used for the treatment of otitis and other inflammatory conditions. In Malaysia, the decoction of leaves prepared from *Nervilia aragoana* is used as a protective medicine after childbirth. Roots of *Vanda tessellate* which forms constituents of medicated oils and used for rheumatic swellings and nervous disorders (Anon, 1966; Husain, 1992; Yoganarasimhan, 1996). In the Khasi hills, juice prepared from the leaves of *Cymbidium giganteum* is used to prevent blood flow in wound, while powdered flower of *Vanda spathulata* is effective against hysteria. *Dendrobium fimbriatum* is used against liver upsets and nervous debility (Bose *et al.*, 1999). Stem paste of *Vanilla walkeriae* is given orally along with feed to treat fever in cattle and also used as nutritive supplement for cattle (Balasubramanian *et al.*, 2000). *Cyrtorchis arcuata*

is employed in the treatment of diabetes (Morris, 2003). Gastrodin is active ingredient of *Gastrodia elata*, lowers blood pressure and protects the central nervous system due to its anti-delirium and anti-convulsive activity (Sun *et al.*, 2004). Paste made from pseudobulbs of *Ansiella africana* used as a contraceptive in Africa (Bulpitt, 2005). *Bletilla striata* is used as anti-haemorrhage agent in Chinese medicine (Bulpitt *et al.*, 2007).

Orchids are valued for cut flowers due their wonderful nature of flowers and extended keeping qualities (Amin *et al.*, 2004). Orchids are mostly grown in parks, gardens and urban areas (Borys *et al.*, 1999; Lopez and Runkle, 2005). Some orchids, flowers are long lasting for one to three months when remain attached to the plant while, cut flowers remains fresh for one to four weeks. It was reported that flowers of *Dendrobium crumenatum* last for a day, while, the flowers of *Grammatophyllum multiflorum* last for about 270 days (Avadhani *et al.*, 1994). Attri *et al.* (2008) reported that un-pollinated flowers of *Aerides multiflora* and *Rhynchostylis retusa* can remain fresh for 17 and 24 days, respectively.

Commercialization of orchids has taken place in many countries such as Thailand, Singapore, Malaysia, Japan, Korea and Taiwan for both plant sale and cut flowers (Gavinlertvatana and Prutpongse, 1991). Sales of potted orchid plants in the United States have increased from $100 million in 2001 to $144 million in 2005 (Jerardo, 2006).

In India, commercial orchid growing is limited, as it is in the hands of dealers who collect orchids from the wild populations (Rao 1977). Many of the Indian orchids such as *Cymbidium, Dendrobium, Paphiopedilum, Renanthera* and *Vanda* are currently in the International market. It was reported that about 70% of orchid plants are illegally imported by USA from India and Thailand (Chadha, 1992).

2.3. Orchid Flora

Orchidaceae are characterized by a diverse range of life histories, reproductive biology and distributions which reflects varieties of patterns of orchid populations (Scacchi *et al.*, 1990; Peakall and Beattie, 1996; Sun, 1996; Gustafsson, 2000; Tremblay and Ackerman, 2001; Wallace, 2002; Forrest *et al.*, 2004). It consists of five sub-families (Dressler, 1981; Chase *et al.*, 2003) and about 35,000 species belong to 800 genera distributed throughout the world (Singh *et al.*, 2007). Two third of orchids are epiphytes and lithophytes, while, one third contributed by terrestrial orchids (IUCN, 1999).

India accounts for wide range of variations among orchids due to its unique geographical position and geological history. Orchids in India, are represented by 1141 species belonging to 166 genera. They are mainly distributed in the Himalayan, northeastern and peninsular regions of the country (Bhanwra *et al.*, 2006).

The Western Ghats of Goa covers about 600 sq. km. with a length of 125 km from North to South. The major forest types that predominantly found in Goa are dense and open type forests, inhabited by a number of terrestrial and epiphytic orchids (FSI, 1997). Rao (1986) reported 29 species of orchids belonging to 18 genera covering entire Goa, Diu, Daman, Dadra and Nagarhaveli regions.

2. 4. Growth Habits

Orchids have varying habit to adjust different environmental conditions in which they grow. They include terrestrial, epiphytic and saprophytic forms (Jonathan and Raju, 2005).

2. 4. 1. Terrestrial orchids

These orchids grow on land and do not require a host plant for their survival. The orchids which grow in cooler regions are remains dormant during the dry season and produces new shoots during wet season (Freudenstein, 1994; Bose *et al.*, 1999). Orchids in the tropical and sub tropical regions are generally evergreen and possess pseudo bulbs (Jonathan and Raju, 2005).

2. 4. 2. Epiphytic orchids

Epiphytic orchids are entirely depending on trees for structural and nutritional support. They are not parasitic and do not feed on the host plants (Dematte and Dematte, 1996; Jonathan and Raju, 2005). Orchids obtain nutrients from rains and debris which accumulated on the barks of host plant. They have adapted to have aerial roots and do not have the advantage of absorption of water from the soil. Usually in orchids, water is absorbed by a spongy structure called "velamen tissue" which formed by dead cells layers in the roots. Orchids withstand severe drought, however, they die if there is inadequate drainage (Bomba, 1975; Batchelor, 1981; Dematte and Dematte, 1996).

2. 4. 3. Saprophytes

A very small number of orchids have peculiar characteristic possessing mycorrhizal fungus in their root for the supply nutrients. These plants do not have chlorophyll and reduced in size with bunch of coralloid roots. They are of underground and emerged out of the ground only during flowering. Fischer (1928), reported three saprophytic species such as *Didymoplexis pallens*, *Epipogum nutans* and *Aphyllorchis montana* from South India. Varjravelu and Radhakrishnan (1968) have reported fourth saprophytic species namely, *Alphyllorchis prainii* from the forest of Attapadi. Taxonomic review of saprophytic orchid species were reported from Arunachal Pradesh (Rao, 1988;

Hegde, 2001). Total of 42 saprophytic species belong to 18 genera were reported from Indian regions (Pradhan, 2004). A rare and giant saprophytic orchid *Galeola falconeri* Hook. f. was reported from lower Subansiri district of Arunachal Pradesh (Das and Khumbongmayum, 2006).

2. 5. Characteristics of Orchids

2. 5. 1. Leaves

Orchids have typical monocotyledonous pattern of leaves which are generally green in colour. Sometimes they have golden or silvery veins that provide attractive ornamentation as in *Anoectochilus* species. Leaves are either long, plicate or short, elliptic oblong or linear ovate and always with sheathing bases in terrestrial orchids. In epiphytic species, leaves are thick and coriaceous. In species such as *Vanda*, the leaves act as a storage organ. Leaves are laterally compressed and succulent in *Oberonia* and *Podochilus* (Bose and Bhattacharjee, 1980; Bose *et al.*, 1999).

2. 5. 2. Inflorescences

The origin of inflorescence is specific to each genera and species. They are either terminal or lateral in terrestrial orchids while, in epiphytic orchids inflorescences are lateral. Exceptionally, in few epiphytic orchids like *Porpax and Polystachya* inflorescence is terminal. Usually, inflorescences are developed from a leafy shoot, but in some cases leaves are shed before flowering like in *Bulbophyllum fimbriatum.* Only leafless flowering shoot appears in saprophytes, (Bose and Bhattacharjee, 1980; Bose *et al.*, 1999).

2. 5. 3. Flower

Orchid flowers have tremendous diversity in morphology (Mondrago'n-Palomino and Theiben, 2008). The orchid *Sorbralia macrantha,* known for largest flower sized 15 to

30 cm across while, *Bulbophyllum minutissimum* possess tiniest flower of pin head size. The principal shades are white, yellow, green and purple of in every possible combination of colours. The important characteristic of orchid flowers is zygomorphic nature of flowers (Bose *et al.*, 1999).

Orchids flowers usually composed of three outer tepals (sepals) in the first floral whorl, two lateral inner tepals (petals) and a median inner tepal termed as the labellum or lip in the second whorl (Rudall and Bateman, 2002; Mondrago'n-Palomino and Theiben, 2008). The primary characteristics of orchid flowers are the fusion of male and female organs within a single structure located at the centre of the flower, pollinia and *zygomorphic* nature of flowers (Van der Pijl and Dodson, 1966; Montalvo and Ackerman, 1987; Proctor and Harder, 1994; Nazarov and Gerlach, 1997; Freudenstein and Rasmussen, 1997; Pacini and Hesse, 2002). The labellum is morphologically complex structure in orchids which primarily attracts the pollinators such as insects (Nilsson, 1992; Rudall and Bateman, 2002). Labellar structure consists of fused style and stamens of the gynostemium which is diagnostic character for orchids (Bateman and Rudall, 2006).

2. 6. Harvesting of fruits

The orchid fruit is a capsule and it takes a long time to mature. Each ripened capsule contains numerous dust-like seeds (about 4 million) (Arditti and Ghani, 2000). The orchid seeds are the smallest among the flowering plants (Arditti *et al.*, 1980). They vary in size, morphology, structures and colour. Orchid seeds vary in size from 150 to 6000 μm, whereas in most of the taxa it ranges from 300-800 μm (Molvray and Kores, 1995). Shape of the seed varies from filiform to fusiform, clavate to ellipsoidal and rarely winged. In general seed coat is papery and loosely surrounds the embryo and rarely seeds are covered with hard coat (Molvray and Kores, 1995). Usually harvesting of the capsules is done

before the dehiscence and normally it takes about 4-10 months to mature and ripen. Time taken for ripening of capsules varies among the species (Hegde, 1984). Abraham and Vatsala (1981) recommended the early harvesting of pods to prevent fungal or bacterial infection of seeds.

2. 7. Cultivation of Orchids

2. 7. 1. Orchid House

Generally two different types of greenhouses (un-cooled and controlled) are used for orchid cultivation. Tropical warm orchids are mostly grown in un-cooled greenhouse which protects from direct sun light which reduces the inside temperature and increases humidity and favoring luxuriant growth and flowering of orchids (Bose *et al.*, 1999). In controlled greenhouse, temperature, light and humidity are controlled which provides favorable environment for growing high value orchids.

2. 7. 2. Selection of Orchid Species

A systematic and careful selection of orchid species is required for the successful cultivation. Many orchids are of commercial importance as a cut flower and potted plants, however cannot be grown in a particular climatic condition. The choice of the orchid species depends on the climatic conditions in which they are to be grown. The availability of the area and the choice of the grower are other important criteria for selecting the orchid species. Always selected plants are to be healthy and free from pest and pathogens (Black, 2003).

2. 7. 3. Containers Used for Growing Orchids

Different types of containers are used for growing orchids. Most commonly used are clay pot, wooden and galvanized wire baskets, tree fern blocks and logs of woods. The

containers such as clay pots have several cuts and holes to facilitate adequate aeration and proper drainage are routinely used. In the recent times, plastic containers are gaining importance due to their less weight, easy to transport, free from deposition of algae or salts on their sides and longer moisture retaining capacity than clay pots (Black, 2003).

2. 8. 4. Potting Media

A suitable growing media for orchids is necessary for the plant growth and development and flowering. Ideal media should support the plant, supply water and nutrients to the roots and provide excellent drainage and aeration. For several years the *Osmunda* fiber was used as potting medium, either alone or in combination with moss which increases water holding capacity. Recent years, orchids have been successfully grown in various potting media (Tan and Lee, 2001; Lauzer *et al.*, 2007).

Use of broken tiles along with charcoal chips (1:2) was reported in blue *Vanda* (Seeni and Latha, 2000). Temjensangba and Deb (2005) used mixture of charcoal pieces, brick pieces, coconut husk and decayed powder (1:1:1:1) with a layer of moss to achieve 60% survival of hardened plants. Requirement of pre hardening medium was reported for *Zygopetalum intermedium* (Nagaraju and Mani, 2005). The rooted plantlets were cultured on pre-hardening media containing paclobutrazol (0.25 mg l^{-1}) and activated charcoal (1.5 mg l^{-1}) for a period of two months. Subsequently, after pre-hardening, the plants were transferred to community pots containing mixture of sterilized cocco peat and tree fern (1:1). Chen *et al.* (2005) successfully utilized mixture of perlite, sand, charcoal and soil (3:2:2:3) for hardening *Cymbidium faberi* plants. Regenerated plants of *Oncidium* sp. (Dancing doll) were grown in the greenhouse after transferring to perforated plastic pots containing mixture of charcoal and brick pieces (1:1) (Kalimuthu *et al.*, 2007). Roy *et al.*

(2007) reported in *Dendrobium chrysotoxum,* 80% survival rate using dried coconut husk, brick pieces and charcoal in the ratio of (1:1:1).

2. 7. 5. Fertilization

The supply of nutrients in liquid forms improves the growth and flowering of orchids. In nature, orchids get their nutrients from the decayed organic matter and soil on which they are grown and also from the atmosphere by the rains (Bose *et al.*, 1999). During cultivation all the nutrients are required to be supplied to the orchids regularly for their growth and development and flowering (Black, 2003). Several fertilizer solutions have been recommended by various workers.

Kumaria and Tandon (1994) reported in *Dendrobium fimbriatum* var. *oculatum* that feeding the plantlets with MS liquid medium (10 times diluted) was found beneficial to the developing hardened plantlets. Similar recommendation of use of MS medium (10 times diluted) was made in *Arachnis labrosa* (Temjensangba and Deb, 2005), *Cymbidium devonianum* (Das *et al.*, 2007) and *Vanilla planifolia*, (Janarthanam and Seshadri, 2008). Seeni and Latha (2000) used foliar spray of Vijay complex, NPK 17:17:17 (Madras Fertilizer Co, Madras) at weekly intervals in case of blue *Vanda*. Park *et al.* (2002) used hyponex solution (6.5N:4.5P:19K) at 15 day intervals in case of *Phalaenopsis*. A mixture of two commercial fertilizer i.e DAP (Di Ammonium phosphate) and NPK (20:10:10) was used at weekly intervals for hardening of *Vanda coerulea* (Malabadi *et al.*, 2004). Thomas and Michael (2007) used full strength macro elements of MS medium for *in vitro* regenerated plants of *Rhynchostylis retusa* during hardening. 150 ppm of N:P:K balanced liquid fertilizer (Peter's 20:20:20, The Scott's Company, Marysville, OH) was used weekly as a source of nutrient for greenhouse acclimation of *Bletia purpurea* and *Cyrtopodium punctatum* (Dutra *et al.*, 2008; Dutra *et al.*, 2009).

2. 7. 6. Watering

In orchid cultivation, time and quantity of water is very important for the survival of plant. It is difficult to prescribe a schedule of watering for orchids, since the number of watering necessary to keep the plants alive depends on the climatic conditions, type of media used and type and size of the pots used during the cultivation (Black, 2003). For most of the orchids watering should be done only when the medium completely dry, however, in case of *Paphiopedium* and *Cymbidium*, require water around their roots at all times. Over watering should be avoided for wild orchid species such as *Vanda* which requires minimal supply of water usually absorbed through the velamen roots (Bose *et al.*, 1999). Over watering blocks the air passages in the roots and asphyxiated (Abraham and Vatsala, 1981). Wrinkling of pseudobulbs and yellowing of leaves are the initial sign of over watering, at this point of time watering should be stopped immediately and confine to aerial spraying. In general, watering twice in a day would be more suitable (Seeni and Latha, 2000; Chen *et al.*, 2005), however, there are reports of watering once in day (Jaime *et al.*, 2006; Dutra *et al.*, 2009).

2. 8. Conservation of Orchids

Orchidaceae consist of a high number of threatened species (Swarts and Dixon, 2009). Orchid species are under major threat worldwide than any other plant family due to over exploitation by collectors and enthusiasts (IUCN, 1999). They are highly vulnerable to changes in ecosystem equilibriums such as availability of nutrients, light, water and competition that can affect the survival of both seedling and mature plants (Swarts and Dixon, 2009). The major threat to orchid populations are human activities, land clearing for agriculture purposes, mining and urban development, weed invasion, grazing and collection of plants for medicinal, horticulture and ethnobotanical reasons (Lokesha and Vasudeva, 1992; Sosa and Platas, 1998; Coates and Dixon, 2007). The habitat

fragmentation, removal of key species from the particular ecosystem, susceptibility to fire threats, pollinator decline and introduction of undomesticated animals are resulted in drastic losses in orchid diversity and populations (Sosa and Platas, 1998; Coates and Dixon, 2007). Smaller size of the population and isolation due to habitat destruction and degradation leads in to significant losses of unique evolutionary lineages (Coates, 2000; Hopper, 2000).

During the formulation policies for conservation programmes, several aspects such as existing and future environmental threats, taxonomic distinctiveness, geographic distribution, habitat specialization, reproductive biology, evolutionary processes influencing population structure and *ex situ* conservation methods should be taken in to consideration (Swarts and Dixon, 2009). *Ex situ* conservation is often taken as the key conservation aspects, but preservation of germplasm off-site should be considered as an 'emergency ward' targeting at extinction of those species under the immediate threat. Despite of off-site conservation such as seed and germplasm banks and *in vitro* propagation, the *in situ* conservation and conservation via assisted migration are the premier approaches for global biodiversity conservation (Swarts and Dixon, 2009). The effective conservation strategies must be developed to avoid further loss of essential species and ecosystem (Swarts and Dixon, 2009). The adaptive management approach was one of the useful methods for linking research to operational practicality in management and restoration of species and ecosystems (Bormann *et al.*, 2007). Integrated conservation approach mainly depends on the understanding of ecological and genetic studies, *in situ* research and *ex situ* propagation (Falk, 1990; Ramsay and Dixon, 2003).

2. 9. Tissue Culture of Orchids

Plant tissue culture plays an important role of mass multiplication and conservation of endangered plant species (Grell *et al.*, 1988; Rubluo *et al.*, 1989; Gangaprasad *et al.*, 1999; Seeni and Latha, 2000; Decruse *et al.*, 2003). Morel (1960) successfully cultured *Cymbidium* shoot apices on Knudson C agar medium. Later, this method of clonal propagation technique created interest among orchid grower and nurseries.

2. 9. 1. Choice of explants

The kind of explants, play a major role in mass multiplication and regeneration of plants *in vitro*. Various explants like shoot tip, leaf and immature seeds are variously used for micropropagation.

2. 9. 1. 1. Leaf

The first finding of the formation of protocorm-like bodies (PLBs) from orchid leaf tissue was made by Wimber (1965). Tip of young leaves from mature plant of *Cattelya* was able to proliferate into protocorm-like bodies. This method was developed to limit the need to sacrifice entire plant or shoots and to provide a simple procedure for clonal propagation of *Cattleya* (Churchill *et al.*, 1971). Leaf segments of infants and young seedlings proved relatively easy to form the PLB in both *Phalenopsis* and *Vanda*, but leaf segment derived from adult plants did not produce the PLBs (Tanaka *et al.*, 1975). Tanaka and Sakanishi (1977) induced the formation of PLBs on leaf segments excised from the shoots originated from a flower stalk by aseptic culture. Churchill *et al.* (1973) used different explants (leaf tip, petioles, various section of leaf blade) from *Epidendrum* and *Laeliocattleya.* However, he was successful to get PLBs only from leaf tip, which produced callus initially. According to him, the production of plantlets from leaf tips is interesting because whole plant originates from a region which is destined to mature into

foliar mesophyll and epidermis of the plant, there-by losing all meristematic capacity. Direct organogenesis from leaf segment was reported by Vij *et al.* (1984) in *Rhynchostylis retusa*. Regeneration of large number of plantlets from leaf explants of *Acampe praemorsa* was reported by Nayak *et al.* (1997), which could be used for both large-scale propagation and *ex situ* conservation of this important orchid species. Murthy and Pyati (2001) developed an *in vitro* propagation system for the rare/endemic orchid species *Aerides maculosa* using juvenile leaf explants.

Recently, a thin cell layer method has been shown to be an efficient means of plant regeneration in orchids. A thin section culture system for rapid regeneration of the monopodial orchid hybrid *Aranda* 'Deborah' has been developed by Lakshmanan *et al.* (1995). Similarly, Van *et al.* (1999) obtained high frequency shoot regeneration of *Rhynchostylis gigantea* using thin cell layer of leaves. Thin leaf section compared to thick one proved to be ideal explants for the production of PLBs in *Doritaenopsis* hybrid and can be exploited as a method of rapid plant propagation (Park *et al.*, 2002). Kuo *et al.* (2005) developed a protocol, which provides a simple way to regenerate transgenic plant through direct somatic embryogenesis in *Phalaenopsis* var. Little Steve using leaf explants. For the first time plant regeneration through direct and secondary somatic embryogenesis in *Phalaenopsis amabilis* using leaf tips was carried out (Chen and Chang, 2006). Similar morphogenetic response of direct and secondary embryogenesis was also reported in *Dendrobium* cv. Chiengmai Pink using leaf tips (Chung, *et al.*, 2007).

2. 9. 1. 2. Shoot tips

Cymbidium was the first orchid genus which was propagated by shoot tip culture (Morel, 1960). Sagawa *et al.* (1966) also developed technique in *Cymbidium* using shoot tip, which can also produce plantlets quickly. Mitra (1971) reported tissue culture method

in *Arundina bambusifolia* using shoot tips from mature plant. Kunisaki *et al.* (1972) propagated *Vanda* Miss Joaquim by shoot tip culture. Strap leafed *Vanda* was successfully propagated by shoot tip culture by Teo *et al.* (1973). In hybrid *Aranda* 'Deborah' noticeable swelling was observed from all the shoot tip explants after 3-4 weeks in culture (Goh, 1973). Intuwong and Sagawa (1974) successfully developed method for the culture of *Phalaenopsis* shoot tips. Devi and Laishram (1998) cultured shoot tip and axillary bud from young shoot of six *Dedrobium* hybrids. *In vitro* clonal multiplication of *Dendrobium* cv. Sonia was investigated using offset segments (Pathania and Sehgal, 1999). Gangaprasad *et al.* (2000) successfully miropropagated *Anoectochilus regalis* using shoot tip. Development of plantlets from shoot tip derived suspension cultures of *Phalaenopsis* was reported by Tokuhara and Mii (2003). A rapid and efficient procedure for *in vitro* clonal propagation of an elite cultivar of jewel orchid *Anoectochilus formosanus* was reported (Ket *et al.*, 2004). Shoot regeneration of *Vanda coerulea*, an endangered epiphytic orchid was reported by using thin shoot tip sections (Malabadi *et al.*, 2004). Successful initiation of PLBs and *in vitro* regeneration of *Cymbidium elegans* was achieved using shoot tip in presence of an endogenous plant growth regulator epi-brassinolide (Malabadi and Nataraja, 2007).

2. 9. 1. 3. Inflorescence

Flower stalks of *Dendrobium* having well developed buds were used for the multiplication (Singh and Sagawa, 1972). Two stage sterilization procedure using chlorax was developed by Intuwong *et al.* (1972) for *Phalaenopsis* using flower stalk cuttings. Intuwong and Sagawa (1973) developed effective clonal propagation method without sacrificing a mother plant in *Ascofinetia* using inflorescence. Chen and Chang (2000) were successful in producing somatic embryos from the segments of flower stalk internodes of *Oncidium* cultivar Sweet Sugar. Tokuhara and Masahiro (2001) reported micropropagation

method of *Phalaenopsis* using shoot tip explants excised from flower stalk buds. High proliferation rate of both the callus and cell suspension culture without loss of embryogenic potential for at least 5 years makes this method highly efficient. An efficient and rapid *in vitro* method for regeneration of *Phalaenopsis* using flower stalk nodes was developed by Park *et al.* (2002). Role of thidiazuron (TDZ) in direct PLB formation from flower stalk internodes of *Epidendrum radicanson* was reported by Chen *et al.* (2002). Thin sections of inflorescence axis were used for the micropropagation of *Phalaenopsis amabilis* cv. Cool Breeze (Sinha *et al.*, 2007).

2. 9. 1. 4. Immature seed

The horticultural trade currently depends on wild orchid populations as a source of stock plant (Kalimuthu *et al.*, 2007). Although many commercially exploited orchid species are common, no sufficient artificial propagation methods are developed to reduce collection pressure. Orchid capsule contains millions of seeds however, it lacks metabolic machinery and do not have any endosperm. Despite the production of large number of seeds in orchids, only 0.2-0.3% of seeds germinates in nature (Singh, 1992; Murthy and Pyati, 2001). Knudson (1951) successfully germinated *Cattelya* seeds without any symbiotic association of fungus on nutrient medium. Since then vast extensive work using mature seeds have been reported (Buyun *et al.*, 2004; Sinha and Roy, 2004; Kauth *et al.*, 2006; Manrique and Gutie, 2006; Johnson and Kane, 2007; Johnson *et al.*, 2007; Dutra *et al.*, 2008; Dutra *et al.*, 2009). Sharma *et al.* (2004) reported that slow growth and extended time taken for flowering are the limitations for the use of mature seeds as a explants for micropropagation. The technique of culturing of orchid seeds prior to maturity is referred as ovule/embryo/green pod/green fruit culture (Sagawa, 1963). Sauleda (1976) reported the optimal time for harvesting of capsules of a number of orchid species and hybrids. In

green pod culture, the immature seeds are obtained from early stages of development starting from fertilization to maturity (De *et al.*, 2006).

Micropropagation methods for several orchids using immature seed have reported earlier. Mitra *et al.* (1976) was successful in achieving the differentiation of protocorms in seed callus of *Dendrobium fimbriatum* in absence of any kind of auxin or cytokinin. Seedling from immature seed of *Vanda coerulea* (four month after self pollination) was used by Seeni and Latha (2000) and achieved rapid multiplication for horticultural use. *Arachnis labrosa* a rare and threatened monopodial epiphytic orchid was successfully regenerated by culturing immature seeds 16-18 week after pollination (Temjensangaba and Deb, 2005). In China many elite varieties of *Cymbidium faberi* has been cultivated with a very limited number of plants for more than 100 years, but all the cultivars were developed from it natural variants and its cross breeding has not been recorded (Wu, 1992). Chen *et al.* (2005) were of the opinion that lack of cross breeding was mainly due to absence of efficient protocol of plant regeneration from the seeds. They further established *in vitro* plant regeneration protocol for immature seeds of *Cymbidium faberi.* Sharma *et al.* (2005) developed *in vitro* protocol for rapid micropropagation and conservation of *Dendrobium fimbriatum* using immature seeds (70 days old). *In vitro* regeneration, multiplication and rooting of plantlets were achieved from immature seed of *Oncidium* sp. cultured on MS medium (Kalimuthu *et al.*, 2007). Thompson *et al.* (2007) developed dual phase protocol for ten *Disa* species. Dual phase cultures were initiated using immature seed from six week old capsule, two weeks prior to dehiscence. This novel system was adapted to test the germination response.

2. 10. Molecular Studies in Orchids

Orchid seeds are very small and easily get transported to long distances by wind (Arditti and Ghani, 2000). This particular characteristic is responsible for high rate of gene flow which results in higher level of genetic variation within the populations (Cozzolino and Widmer, 2005). An understanding of the level genetic variation within and among populations is essential for developing appropriate conservation and sustainable utilization methods (Xiaohong *et al.*, 2007). Knowledge on genetic diversity forms a base for conservation (Geburek, 1997). Population genetic studies have been carried out to understand the loss of genetic diversity and also to restore the threatened populations (Hamrick and Godt, 1996).

Despite the tremendous diversity within the family Orchidaceae, very little is known about the genetic diversity within the natural populations (Xiaohong *et al.*, 2007; Wang *et al.*, 2008). Loss of genetic variation is a major problem in conservation of particular species, which can prevent a species from natural selection and limit its evolutionary potential (Qamaruz-Zaman *et al.*, 1998). Depletion of orchid populations are mainly due to habitat destruction, macroclimatic changes and shifting cultivation (Subba Rao, 2005). Various developmental and human activities are responsible for habitat destruction and fragmentation which leads to isolation of population (Sosa and Platas, 1998; Coates and Dixon, 2007). Significance of habitat destruction and over exploitation of wild orchids from different parts of world has been well reported (Soucy, 1979; Stolzenburg, 1993; Tsi *et al.*, 1999).

In orchids, number of markers have been developed as reliable tool to obtain more consistent information on the existing genetic diversity (Cambell *et al.*, 2002; Alexandersson and Aegren, 2000; Flanagan *et al.*, 2007; Dueck and Cameron, 2008;

Rajkumar *et al.*, 2008; Jacquemyn *et al.*, 2009). Molecular markers have been proved to be valuable techniques in the characterization and evaluation of genetic diversity. It has been shown that different markers might reveal different classes of variation (Powell *et al.*, 1996; Russell *et al.*, 1997). The techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) are used frequently to study orchid diversity (Chang *et al.*, 2000; Schluter *et al.*, 2007; Pillon *et al.*, 2007; Boonsrangsom *et al.*, 2008; Pinheiro *et al.*, 2008; Pellegrino *et al.*, 2008; Rajkumar *et al.*, 2008; Jacquemyn *et al.*, 2009; Wang *et al.*, 2008; Verma *et al.*, 2009).

2. 10. 1. Use of RAPD marker

The RAPD technique has many advantages such as simplicity and rapidity of analysis, low cost, availability of a large number of primers and the requirement of small amount of DNA for analysis (Williams *et al.*, 1990; Huff *et al.*, 1993; Ge *et al.*, 1999; Nybom and Bartish, 2000; Kingston *et al.*, 2004). RAPD technique is more suited to orchids since very little is known about the genetic diversity within the natural populations (Xiaohong *et al.*, 2007).

The identification and mapping of DNA polymorphisms using RAPD technique reveals species-specific and genus specific traits, speciation, morphological evolution and molecular change in plants (Benner *et al.*, 1995). Lim *et al.* (1999) confirmed strap and terete leaved *Vanda* as phylogenetically distinct based on RAPD analysis. He also recommended RAPD analysis for the determination of genetic background of the plant used in hybridization programme. Wong and Sun (1999) studied *Goodyera procera*, a terrestrial orchid using RAPD and found out that the diversity varied greatly both at

species and at population level. Wallace (2002) used RAPD loci to assess the potential effects of fragmentation and reduced population size on the future viability of *Platanthera leucophaea*, a threatened species. Li *et al.* (2002) conducted a preliminary analysis of the level and apportionment of genetic diversity in *Paphiopedilum micranthum* using RAPD analysis. Besse *et al.* (2004) detected low levels of genetic diversity in *Vanilla planifolia* in cultivation areas such as Reunion Island and Polynesia (Pacific Ocean). Goh *et al.* (2005) demonstrated that RAPD markers are useful tool for identifying *Phalaenopsis* orchids up to the specific and/or sub generic levels. Li and Ge (2006) investigated the level and apportionment of genetic diversity of this species using RAPD technique in *Changnienia amoena* endemic to China. Based on the results they proposed conservation managements for this endangered species, including habitat protection along with the protection of their pollinators, artificial pollination as well as *ex situ* conservation. Low level of diversity at species and population level between glaciated and un-glaciated sites of *Cypripedium reginae* was reported based on RAPD (Kennedy and Walker, 2007). No difference of RAPD genetic diversity in wild and cultivated group of *Vanilla planifolia* was observed by Schluter *et al.* (2007). Minoo *et al.* (2008) discriminated different species of *Vanilla planifolia* and its related species using RAPD analysis that were usually differentiated by presence/absence of leaves and flower colour.

2. 10. 2. Use of ISSR marker

ISSR markers are used for the detection of polymorphism in inter-microsatellite loci using specific primers (Wu *et al.*, 1994; Zietkiewicz *et al.*, 1994). ISSR markers show dominant inheritance, which are being used as an alternate tool in genetic diversity studies (Tsumura *et al.*, 1996). Since, no sequence information of a target DNA of particular species is required, ISSR technique is especially suited to plant groups such as orchids (Xiaohong *et al.*, 2007).

Smith *et al.* (2002) reported very low genetic diversity for terrestrial woodland orchid *Tipularia discolor* in eastern United States based on ISSR markers, with polymorphism ranging from 0.00 to 18.2% and gene diversity ranging from 0.00 to 0.069. Wallace (2003) successfully proved the recurrent origin of polyploidy in orchid *Platanthera huronensis* from the allopolyploid species of *Platanthera dilatata* and *Platanthera aquilonis* using ISSR and RAPD technique. Comparison of genetic variation between allopolyploid and diploid species of *Platanthera* species was successfully done by Wallace (2004). Li and Yang (2006) studied genetic diversity of wild *Cymbidium goeringii*, an endangered orchid from Hubei Province of China using ISSR primers and based on the result obtained, they proposed conservation strategies for survival. Wu *et al.* (2006) reported higher genetic variation in natural than the cultivated populations of *Gastrodia elata* using ISSR. The genetic uniformity due to loss of genetic diversity in cultivated populations was caused by a genetic bottleneck during human domestication and clonal reproduction over generations (Wu *et al.*, 2006). *Ex situ* conservation measures such as seed banking and vegetative propagation of key genotypes along with *in situ* conservation was suggested by Xiaohong *et al.* (2007) in *Cymbidium goeringii* based on the ISSR analysis. Shen *et al.* (2006) used ISSR for authentication of eight wild populations of *Dendrobium officinale*, since the selection of genuine population is key issue in the traditional medicine. Use of ISSR technique for genotyping system in *Cymbidium goeringii* was reported by Wang *et al.* (2008) and this genotyping system was used efficiently for tagging genes for commercially important traits such as flower colours and structural alterations. Verma *et al.* (2009) for the first time reported inter relationship amongst the cultivated, wild and hybrid of *Vanilla* using ISSR along with RAPD analysis.

2. 11. Use of Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) Protein marker

The study of SDS-PAGE protein profiles in orchids to understand the genetic variation is poorly represented. However, SDS-PAGE protein profiles have been used in other group of plants to understand genetic variations. SDS-PAGE of seed storage proteins was used to investigate genetic variation and to classify plant varieties of common beans (*Phaseolus vulgaris*) (Brown *et al.*, 1981; Gepts *et al.*, 1986; Gepts and Bliss, 1988) and mung bean (*Vigna radiata*) (Tomooka *et al.*, 1992). Similary, *Phaseolus coccineus* cultivars were classified using SDS-PAGE on the basis of vacilin and lectin variability (Brown *et al.*, 1981). Durante *et al.* (1989) classified *P. coccineus* cultivars on the basis of legmin, vicilin and phytohaemagglutein variability. Yamaguchi and Kosuge (1991) and Mori *et al.* (1992) have reported variation in seed protein among azuki cultivars.

SDS-PAGE of seed storage proteins has proven to be a simple and effective method for distinguishing the cultivars of the largely cross-fertilized pasture grasses and legumes despite their high innate genetic variability (McCausland and Wrigley, 1977; Shewry *et al.*, 1978; Heisel *et al.*, 1986; Ferguson and Grab, 1986; Gardiner and Forde, 1988; Sammour, 1988; Radovic and Vapa, 1996). Variation in number of bands, width and intensity of bands were observed in different peas (*Lathyrus sativus*) of same geographical origin (Przybylska *et al.*, 1999; Dellagatta *et al.*, 2002).

SDS-PAGE analysis was used to evaluate the intra-specific variation and geographical distribution in Asia in case of *Vigna angularis* (Takehisa *et al.*, 2001). Existence of variation between accessions of *Brachiaria* using SDS-PAGE was shown by Neto *et al.* (2002). Ahmad *et al.* (2003) revealed variations among the local genotypes with immense potential for future improvement in Seabuckthorn on the basis of SDS-PAGE.

Electrophoretic pattern of the protein fractions are directly related to the genetic background and can be used to certify the genetic makeup. The accessions of rice germplasm were analyzed for total seed protein through SDS-PAGE and observed considerable variation in protein banding pattern (Asghar *et al.*, 2004). Dendrogram generated by Mukhlesur *et al.* (2004) using SDS-PAGE data clearly separated genotype of *Brassica rapa* from Bangladesh, Japan and China.

Rout and Chrungoo (2007) determined intra-specific variability in *Fagopyrum* spp. using SDS-PAGE marker. Genetic variation among *Vicia faba* beans cultivars from Egypt was measured using SDS protein electrophoresis (Mustafa, 2007). High genetic variability among the accessions of different geographical regions and a low variability among the accessions of the same region in *Lathyrus sativus* were reported by Sammour *et al.* (2007). Mirza *et al.* (2007) observed variation between irrigation-associated and rain associated plants of *Avena fatua* based on SDS-PAGE protein polymorphism. Similarity of genetic structure between the landraces and wild species of *Phaseolus vulgaris* was confirmed by Igrejas *et al.* (2009) based on the SDS-PAGE profile.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study was carried out as a part of Ph.D. programme of the Department of Botany, Goa University during the period from July 2004 to June 2008. Details of the methodology used for the study is presented in this chapter.

3. 1. Study area

Goa is a small state with an area of 3702 sq. km. and having length of 105 km. and width of 60 km. It is located between 73° 40'-74° 20' E longitudes and 14° 53'-15° 47' N latitudes. The Western Ghats of Goa covering about 600 sq. km. which is extended in the form of an arc for a length of 125 km from north to south. The two major rivers, Mandovi (16.6 km. long) and Zuari (62.4 km. long) are flowing in these regions (Rao, 1985).

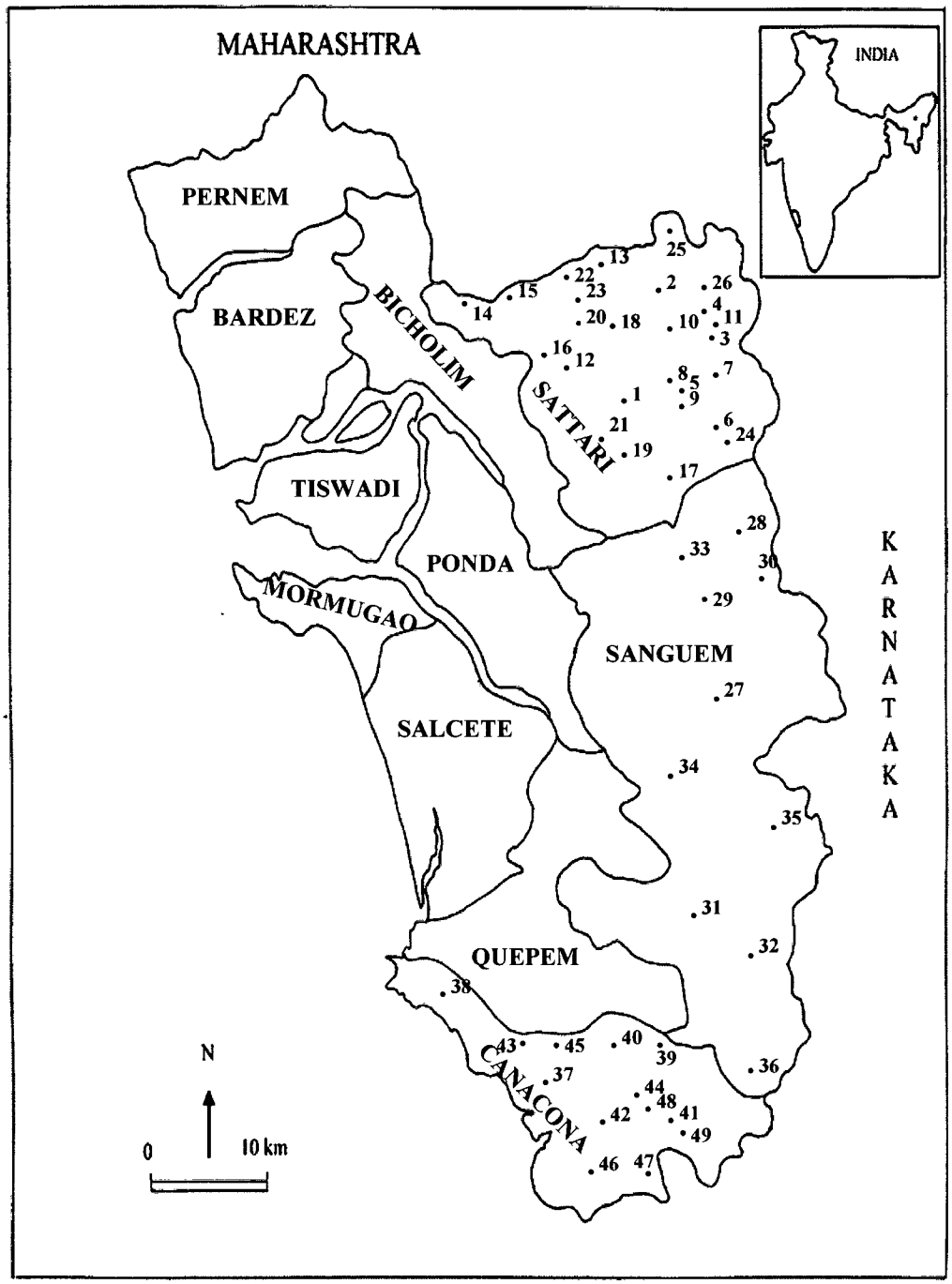
The summer season in Goa is from March to May, the South West monsoon from June to September, the post monsoon from October to November and the winter season from December to February. Rainfall is the major climatic factor which controls the distribution of flora. The humidity in these regions is usually from 70-95%. The maximum temperature ranging from 30-37°C and minimum temperature ranges from 15-20°C (Rao, 1985).

The Western Ghat areas of Goa mainly cover the talukas namely, Sattari, Sanguem and Canacona. These regions were thoroughly surveyed and sampled for the collection of orchid species during the study period. The details of the location visited are given in the (Table 1; Fig. 1).

Table 1. Survey and collection sites of Western Ghats of Goa Region.

Sl. No.	Location	Taluka	Sl. No.	Location	Taluka
1	Valpoi	Sattari	26	Codal	Sattari
2	Thane	"	27	Pikulewadi	Sanguem
3	Ustem	"	28	Collem Railway Track	"
4	Dhave	"	29	Mollem	"
5	Tar-Savordem	"	30	Anmod Ghat	"
6	Kumtol	"	31	Netorlim	"
7	Bondir	"	32	Verlem Ghat	"
8	Karmali	"	33	Bolcornem	"
9	Shir	"	34	Ugem	"
10	Brahma-karmali	"	35	Potrem	"
11	Nanoda	"	36	Salginim	"
12	Bhuipal	"	37	Canacona	Canacona
13	Chorla Ghat	"	38	Khola	"
14	Parye	"	39	Ambe Ghat	"
15	Keri	"	40	Gavdongri	"
16	Harvalem	"	41	Kotigao	"
17	Tambdi Surla	"	42	Poinguinium	"
18	Vagueri	"	43	Karmal Ghat	"
19	Gulelim	"	44	Partagal	"
20	Saleli	"	45	Gulem	"
21	Bhironda	"	46	Balli	"
22	Anjuna	"	47	Sadolxem	"
23	Ajobachi rai	"	48	Satpali	"
24	Caranzol	"	49	Bhatpal	"
25	Surla	"			

Fig. 1. Map of Goa showing collection sites of orchid species.



1. Valpoi; 2. Thane; 3. Ustem; 4. Dhave; 5. Tar-Savordem; 6. Kumtol; 7. Bondir; 8. Karmali; 9. Shir; 10. Brahma-karmali; 11. Nanoda; 12. Bhuipal; 13. Chorla Ghat; 14. Parye; 15. Keri; 16. Harvalem; 17. Tambdi Surla; 18. Vagueri; 19. Gulelim; 20. Saleli; 21. Bhironda; 22. Anjuna; 23. Ajobachi rai; 24. Caranzol; 25. Surla; 26. Codal; 27. Pikulewadi; 28. Collem Railway rack; 29. Mollem; 30. Anmod Ghat; 31. Netorlim; 32. Verlem Ghat; 33. Bolcornem; 34. Ugem; 35. Potrem; 36. Salginim; 37. Canacona; 38. Khola; 39. Ambe Ghat; 40. Gavdongri; 41. Kotigao; 42. Poinguinium; 43. Karmal Ghat; 44. Partagal; 45. Gulem; 46. Balli; 47. Sadolxem; 48. Satpali; 49. Bhatpal.

3. 2. Collection and Identification

During field survey different species of orchids were uprooted or detached carefully and brought to the laboratory, photographed and maintained in the shade net condition. Collected orchid species were identified by using standard literature including local/regional floras and revision/monograph (Cooke, 1967; Bose and Bhattacharjee, 1980; Abraham and Vatsala, 1981; Rao, 1986; Nageswara Rao, 1986; Bose *et al.*, 1999; Manilal and Sathish Kumar, 2004).

3. 3. Micropropagation studies

3. 3. 1. Sterilization procedures

3. 3. 1. 1. Glassware and culture vials

The glassware and culture vials used for culturing were first washed in tap water, and soaked in dilute hydrochloric acid solution for 2 h followed by a detergent wash and later rinsed with distilled water and drained. Glassware was sterilized by exposure to hot dry air at 130-170°C for 2-4 h in a hot air oven.

3. 3. 1. 2. Instruments

Instruments used during inoculation were sterilized by wet sterilization method. Forceps, blade holder, cutting board and cotton were wrapped in aluminum foil and subjected to wet sterilization in an autoclave at 121°C and 15 psi pressure for 20 min. The sterilized equipments were transferred to a laminar air-flow hood.

3. 3. 1. 3. Culture room

The culture room was maintained sterile by fumigating the area once in a month. Fumigation was carried out by vaporizing 1:2 ratio of ethanol and formaldehyde solution and the vapors allowed to remain in the culture room for 24 h. The formalin vapors were absorbed by placing ammonia solution in a Petri dish.

3. 3. 1. 4. Laminar air-flow system

The inoculation procedures were carried out in a laminar air-flow system. Prior to this the inner surface of the system was swabbed with dettol solution followed by alcohol using clean cotton swab or tissue paper. Then the laminar air-flow hood was exposed to germicidal UV (253.7nm) light for about 20 min. All material that was needed for inoculation like instruments, culture medium, explants etc were then brought to the laminar air-flow hood.

3. 3. 2 Media preparation

Different medium such as Murashige and Skoog (1962) (MS), Vacin and Went (1949) (VW) and Mitra *et al.* (1976) (MI) were used during this study. The culture medium consisted of micronutrients, macronutrients, vitamins, amino acids, carbon source, plant growth regulators and a gelling agent. All the required chemicals for the preparation of medium were weighed with the help of electronic balance.

The chemical composition of media used in the study is provided in Table 2-4. Iron stock solution of MS medium was prepared by weighing out the desired amount of sodium EDTA and iron sulfate separately and dissolved each in 50 ml of double distilled water. After complete dissolution, the two solutions were combined with continuous stirring and stored in amber colored bottle at 4°C.

Table 2. Composition of MS Medium (1962) (Stock solution).

Stock solutions	Constituents	Amount (g/l)
A. 10 X (Macronutrients)	Potassium nitrate Ammonium nitrate Calcium chloride Magnesium sulfate	19.0 16.5 4.4 3.7
B. 10 X (Phosphate)	Potassium phosphate Sodium phosphate	1.7 1.7
C. 100 X (Micronutrients)	Boric acid Manganese sulfate Zinc sulfate	0.62 1.69 0.86
D. 1000 X (Micronutrients)	Potassium iodide Sodium molybate	0.83 0.25
E. 10000 X (Micronutrients)	Copper sulfate Cobalt chloride	0.25 0.25
F. 100 X (Iron/ EDTA)	Sodium EDTA Iron sulfate	0.372 0.278
G. 1000 X (Vitamins)	Glycine Nicotinic acid Pyridoxine Hcl Myo- inositol	200.0 50.0 50.0 10,000 } mg/100ml

Table 3. Composition of Vacin and Went medium (1949) (Stock solution).

Stock solutions	Constituents	Amount (g/l)
A	Ammonium sulfate	50.0
	Magnesium sulfate	25.0
	Potassium nitrate	52.5
	Potassium phosphate	25.0
	Manganese sulfate	0.75
B	Calcium phosphate	20.0
C	FeNaEDTA	3.7

Table 4. Composition of Mitra *et al.*, medium (1976) (Stock solution).

Stock solutions	Constituents	Amount (g/l)
A. 100 X	Calcium nitrate	20.0
B. 100 X	Potassium nitrate	18.0
	Ammonium sulfate	10.0
	Sodium dihydrogen phosphate	15.0
	Magnesium sulfate	25.0
C. 100 X	$Na_2FeEDTA$	0.3
D. 1000 X	Potassium iodide	0.03
	Manganese chloride	0.4
	Zinc sulfate	0.05
	Boric acid	0.6
	Copper sulfate	0.05
	Sodium molybdate	0.05
	Cobalt nitrate	0.05
E. 10000 X	Thamine Hcl	0.3
	Pyridoxine Hcl	0.3
	Nicotinic acid	1.25
	Riboflavin	0.05
	Biotin	0.05
	Folic acid	0.3

3. 3. 2. 1. Preparation of MS medium

Poured 400 ml of double distilled water into 2 liter flask and added 100 ml of stock solution A and B, 10 ml of stock solution C and F, 1 ml of stock solution D and G and 0.1 ml of stock solution E with constant stirring. Later, added 30 g of sucrose with stirring and brought volume to 900 ml with distilled water. The pH of the medium was adjusted to 5.7 ± 0.2 using 0.1 N NaOH or 0.1 N HCl after adding required amount of auxins and cytokinins. After addition of agar (8 g), the volume was made to one liter with double distilled water. The medium was boiled, allowed to cool at 60°C and later was dispensed into culture tubes and autoclaved.

3. 3. 2. 2. Preparation of VW medium

Poured 400 ml of double distilled water into a 2 liter flask and added 10 ml of stock solutions A, B and C. To this was added 30 g of sucrose with constant stirring until it got dissolved and the volume was brought to 900 ml with double distilled water. Required amounts of auxins and cytokinins were added and the pH was adjusted to 5.7 ± 0.2 using 0.1N NaOH or 0.1 N HCl. After addition of 8 g of agar, the volume was made to one liter with double distilled water. The medium was made to boil, allowed to cool at 60°C and later was dispensed into culture tubes and autoclaved.

3. 3. 2. 3 Preparation of Mitra *et al.* medium

Poured 400 ml of double distilled water into 2 liter flask and added 10 ml of stock solution A, B and C and 1 ml of stock solutions D and E. To this medium was added 30 g of sucrose with constant stirring until it got dissolved and the volume was brought to 900 ml with double distilled water. Required amounts of auxins and cytokinins were added and the pH was adjusted to 5.7 ± 0.2 using 0.1 N NaOH or 0.1 N HCl. After addition of 8 g of

agar, the volume was made to one liter with double distilled water. The medium was boiled, allowed to cool at 60°C and later was dispensed into culture tubes and autoclaved.

As per the need, the medium was supplemented with different plant growth regulators (Table 5) and coconut water (CW) either alone or in combinations. After dispensing the prepared medium into culture tubes, the medium was autoclaved at 121°C and 15 psi for 20 min.

3. 3. 3. Culture condition

The cultures were maintained at 25 ± 2°C under fluorescent light of about 40 μ mole m^2 S^{-1} for 16 h photoperiod. The results were recorded on the basis of visual and microscopic observations.

3. 3. 4. Plant Materials

During this study 26 species of orchids were collected from Western Ghats of Goa. Among the above species, *Rhynchostylis retusa* (L.) Bl. and *Aerides maculosa* Lindl. were chosen for mass multiplication due to their attractive flower and long inflorescences. Healthy plants of both these species were maintained in the orchidarium of Zonal Agricultural Office, Tiswadi, Goa.

3. 3. 5 Explants

Seeds of immature capsules collected from healthy plants, 10-20 weeks after pollination (WAP) (*R. retusa*) and 5-10 WAP (*A. maculosa*) were used for *in vitro* propagation.

Table 5. Plant growth regulators (PGRs) used for micropropagation study.

Sl. No.	PGRs	Molecular weight	Solubility	Stock solution prepared (M)
1	2,4-D	221.04	Ethanol	10^{-3}
2	IBA	203.24	Dilute NaOH	10^{-3}
3	IAA	175.19	Ethanol	10^{-3}
4	NAA	186.21	Ethanol	10^{-3}
5	BAP	225.25	Ethanol	10^{-3}
6	KIN	215.22	Dilute NaoH	10^{-3}

3. 3. 5. 1. Isolation of seeds and surface sterilization

Collected capsules were soaked in aqueous solution of commercial detergent (Teepol) for 15 min. Dirt particles adhering to the surface of capsules were removed using tissue paper. The capsules were surface sterilized in absolute alcohol for 30 sec., followed by quick flaming. After surface sterilization, the capsules were taken in a sterile Petri dish containing filter paper, cut longitudinally and yellowish seeds were inoculated on to the medium.

3. 3. 6. Inoculation

Prior to inoculation all the required instruments and culture tubes were kept inside the laminar air-flow chamber in the presence of UV light for about 30 min. Green capsules were given two longitudinal cut with a sterile surgical blade to expose the immature seeds. The seeds were scooped out with the help of spatula and were cultured on different culture media (Table 2-4). After inoculation of seeds, the culture tubes were sealed with sterile cotton plugs and labeled. The inoculated culture tubes were maintained in the culture room.

3. 3. 7. *Ex vitro* establishment

The rooted plants of 2-3 cm height were removed from culture tubes and thoroughly washed with sterile distilled water to remove adhering agar completely without causing damage to the roots. The plantlets were transferred to conical flask containing sterilized charcoal pieces with 10 ml of $1/10^{th}$ diluted Vacin and Went medium in culture room with a cotton plug on the conical flask for three weeks. Later, cotton plugs were removed and plantlets maintained in culture room for a week. They were then treated with fungicide solution (bavistin) at 0.1% concentration for 5 min. and transferred to perforated

plastic pots. Plastic pots were filled with a mixture of different potting media combinations (Table 6). Plastic pots along with media were treated with fungicide (Bavistin) at 0.1% concentration. After transplanting, the plants were sprayed with coconut water 3 times a day for 2 weeks. After two weeks, it was sprayed with $1/10^{th}$ diluted Vacin and Went medium once a day up to one month. Later, plants were watered regularly twice daily. Care was taken so that roots of the seedling passed through the space in between charcoal and brick pieces. To prevent fungal and bacterial infections, 0.1% bavistin and 2 ppm of Ceftriaxone as antibiotic were sprayed once in 15 days. The potted plants were kept under a greenhouse (25% light) and mist irrigated. Reading was taken after 60 days.

3. 4. Biochemical Study

3. 4. 1. Plant Materials

Two orchid species were analyzed in the present study namely, *Rhynchostylis retusa* and *Aerides maculosa.* Seven and eight different natural populations of *R. retusa* and *A. maculosa* respectively were studied for genetic variation. The physical characteristics of these study areas are given in Table 7 and the map showing the study sites is provided in Fig. 2. Leaf samples from the respective populations were brought to the laboratory using zip-lock plastic bags and maintained at 4°C. Samples were cleaned with the help of moist tissue paper to remove any dirt adhering to the leaf tissue.

3. 4. 1. 1. Extraction of proteins

Leaf tissue (0.5 g) was homogenized in the presence of Tris buffer at pH 7.5. In order to avoid polyphenol interference, mercaptoethanol (100 µl g^{-1} tissue) was incorporated while grinding. The homogenate was centrifuged at 10,000 rpm for 20 min. at 4°C. The protein was estimated using spectrophotometer at 660 nm following Lowry *et al.*

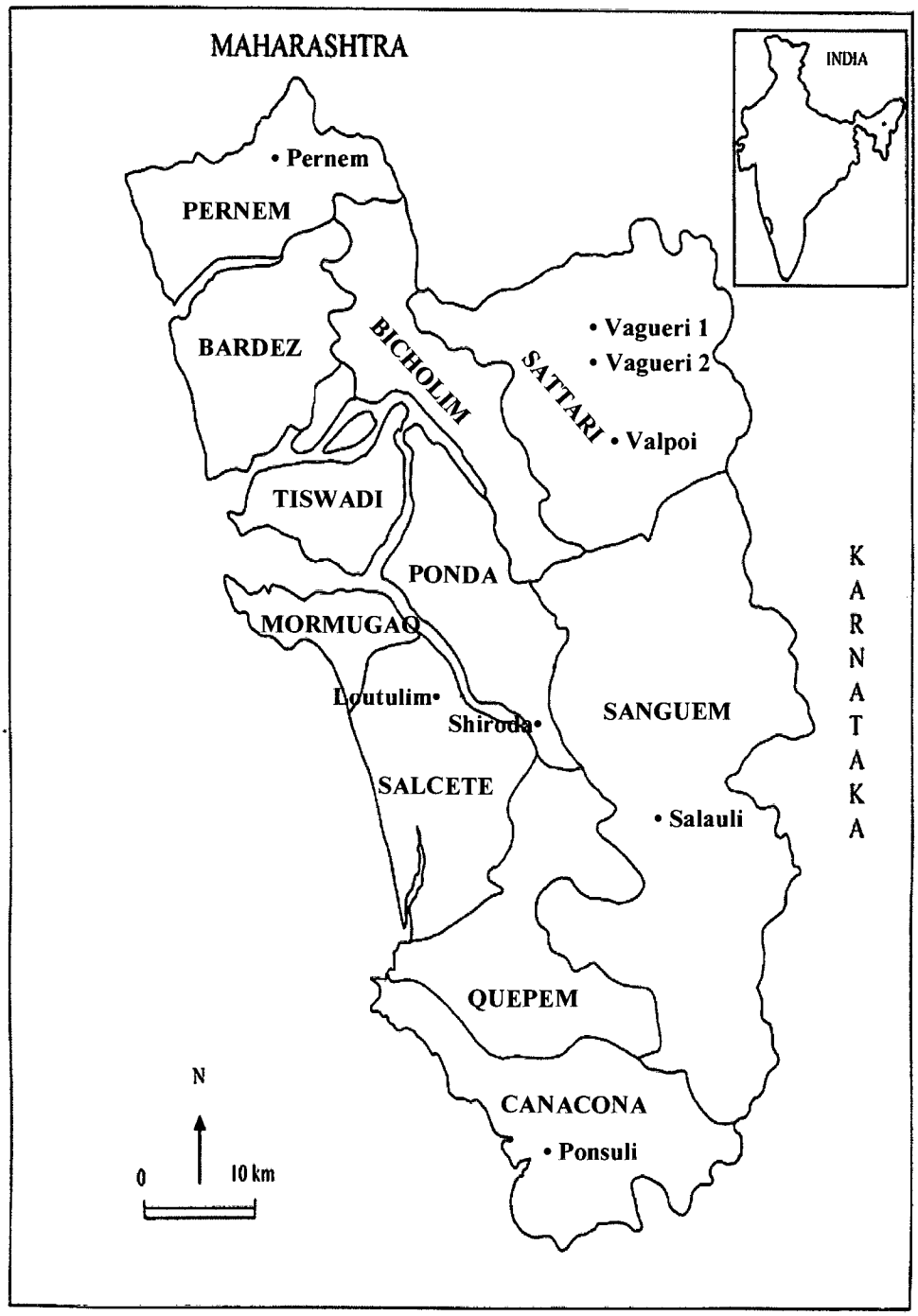
Table 6. Different potting mixture used for the *ex vitro* acclimatization study.

Sl. No.	Treatments
1	Small charcoal pieces (approx 2 cm in size)
2	Small brick pieces (approx 2 cm in size)
3	Small charcoal pieces + coconut husk
4	Small brick pieces + coconut husk
5	Small charcoal pieces + brick pieces(1:1)
6	Small charcoal pieces + brick pieces (1:1) + coconut husk

Table 7. Collection sites of populations used for molecular and biochemical studies.

Population	Abbreviation	Latitude	Longitude	Altitude (m)
Ponsuli	PO	14° 59' 56.9"N	74° 02' 34.6" E	10
Pernem	PE	15° 43' 31.6"N	73° 47' 47.2" E	15
Salauli	SA	15° 09' 28.1"N	74° 13' 47.7" E	76
Valpoi	VA	15° 33' 26.1"N	74° 04' 36.1" E	71
Shiroda	SH	15° 27' 31.1"N	73° 59' 55.6" E	102
Loutulim	LO	15° 23' 06.6"N	73° 56' 22.5" E	103
Vagueri 1	VG 1	15° 34' 59.8"N	74° 05' 47.6" E	295
Vagueri 2	VG 2	15° 35' 34.8"N	74° 06' 04.9" E	720

Fig. 2. Map of Goa showing collection sites of orchid populations for molecular and biochemical studies.



method (Lowry *et al.*, 1951). Fraction of the protein sample was treated with sodium dodecyl sulfate (SDS) buffer and stored at 4°C for later use in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Quality chemicals were procured from Sisco Research Laboratory Pvt. Ltd., E. Merck (India) Ltd., Mumbai; Hi media Laboratory Ltd, Mumbai, and used during this study.

3. 4. 1. 1. 1. Composition of Tris buffer (0.1M)

Tris base : 1.21 g

Tris base was dissolved in 50 ml of distilled water and the pH was adjusted to 7.5 using 1N HCl. The volume of the solution was adjusted to 100 ml.

3. 4. 2. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS gel electrophoresis allows the separation of proteins in denatured condition. Migration of polypeptides is based on the molecular weight. Sodium dodecyl sulfate is an anionic detergent that denatures protein by wrapping around the polypeptide backbone. In doing so, SDS confers a negative charge to the polypeptide in proportion to its length. As a result, polypeptides become rod of negative charge with equal densities or charge per unit length. The samples were electrophorised in a vertical gel electrophoretic unit (Hoefer Mighty Small, USA) according to the procedure described by Laemmli (1970).

3. 4. 2. 1. Preparation of reagents

The reagents used for SDS-PAGE include monomer solution, resolving gel buffer, stacking gel buffer, electrode buffer, treatment buffer, initiator and SDS solution. The detailed procedures followed for preparation of various reagents are as follows:

3. 4. 2. 1. 1. Monomer solution (30% Acrylamide, 2.7% Bisacrylamide)

Composition

Acrylamide	:	30 g
Bis acrylamide	:	0.8 g

The volume of the solution was adjusted to 100 ml. The solution was stored at 4°C in amber coloured bottle. (Extreme care should be taken while handling the monomer solution to avoid spill over).

3. 4. 2. 1. 2. 4X Resolving gel buffer (1.5 M Tris Cl, pH 8.8)

Composition

Tris base	:	18.5 g

Tris base was dissolved in about 50-60 ml of distilled water, pH adjusted to 8.8 with 1 N HCl and stored at 4°C. The volume of the solution was adjusted to 100 ml.

3. 4. 2. 1. 3. 4X Stacking gel buffer (0.5 M Tris-Cl, pH 6.8)

Composition

Tris base	:	6 g

Tris base was dissolved in about 50-60 ml of distilled water, pH adjusted to 6.8 with 1 N HCl and stored at 4°C. The volume of the solution was adjusted to 100 ml.

3. 4. 2. 1. 4. Electrode buffer (0.025 M Tris, pH 8.3, 0.192 M Glycine)

Composition

Tris base	:	1.525 g
Glycine	:	7.2 g
SDS	:	0.5 g

The volume of the solution was adjusted to 500 ml. The solution was stored at room temperature

3. 4. 2. 1. 5. 2X Treatment buffer (0.125M Tris-Cl)
Composition

4X Tris-Cl, with pH 6.8	:	2.5 ml
Glycerol	:	2.0 ml
2-Mercaptoethanol	:	0.2 ml
Bromophenol blue	:	0.2 g
SDS (10%)	:	4.0 ml

The volume of the solution was adjusted to 10 ml.

3. 4. 2. 1. 6. Initiator (10% APS)
Composition

Ammonium persulphate	:	0.1 g
Distilled water	:	1 ml

This solution was prepared fresh, before use.

3. 4. 2. 1. 7. SDS (10%)
Composition

Sodium dodecyl sulphate	:	10 g

The volume of the solution was adjusted to 100 ml.

3. 4. 2. 2. Casting of the gel

The Hoefer Mighty Small™ gel system of Hoefer Pharmcia Biotech., California, USA, was used. The glass plates were cleaned with tissue paper soaked in 95% ethanol. They were kept apart by 0.75 mm thick spacer coated with sealing wax to avoid any leakage. The spacer and the two glass plates were then assembled using the clamp. The clamp was tightened by aligning the glass plates with spacer on the casting stand.

3. 4. 2. 3. Preparation of sample for loading

The extracted protein sample was mixed with the treatment buffer in the ratio of 1:1 and was heated at 100°C for two min. for denaturation and used for electrophoresis.

3. 4. 2. 4. Preparation of gel

3. 4. 2. 4. 1. Resolving gel

Different combinations of the resolving gel were tried out to select the optimum combination. The composition of resolving gel is given in Table 8.

3. 4. 2. 4. 2. Stacking gel

Composition

Monomer	:	0.67 ml
4X Stacking gel buffer	:	1.25 ml
SDS 10%	:	0.05 ml
Distilled water	:	3 ml
APS 10%	:	25 µl
TEMED	:	3 µl

Respective stock solutions of resolving gel were mixed serially in a beaker. The mixture was then poured between the glass plates with the help of a micropipette up to a desired height (leaving 2 cm at the top). Small quantity of distilled water was over laid to allow polymerization of resolving gel to take place for 30 min. After complete polymerization of the resolving gel, distilled water was drained off. The comb was placed properly and then stacking gel was carefully poured without leaving any air bubble. It was allowed to polymerize for 15 min.

After complete polymerization, the comb was removed and the gel slab was carefully taken out from the casting unit, washed with the electrode buffer and transferred

Table 8. Different composition of resolving gel used for SDS-PAGE.

Components	Resolving gel %		
	7.5	**10**	**15**
Monomer (ml)	2.49	3.33	4.99
4 X Resolving buffer (ml)	2.5	2.5	2.5
SDS 10% (ml)	0.1	0.1	0.1
Distilled water (ml)	4.85	4	2.36
APS 10% (µl)	50	50	50
TEMED (µl)	5	5	5
Total (ml)	10	10	10

to electrophoresis unit. The upper and lower tanks were filled with electrode buffer. The sample was loaded in the well. Protein molecular weight marker (Genei, Bangalore, India) was loaded as standard. The upper tank buffer was connected to cathode and lower to the anode of the power pack. A constant power supply of 50 mA was maintained throughout the run till the tracking dye reaches the anode.

3. 4. 2. 5. Staining of protein

Silver staining procedure was followed for staining the gel (Hames, 1998). Silver stain method depends on the reduction of silver ions to provide metallic silver images. Selective reduction of silver ions to metallic silver at gel sites occupied by protein depends on difference in the oxidation reduction potentials in the sites occupied by the protein in comparison with adjacent sites in the gel which do not contain proteins.

3. 4. 2. 5. 1. Fixing the gel

After the run, the gel was separated carefully and transferred to fixing solution (300 ml) and shaken gently at 40 rpm. The composition of the Fixing solution is as follows:

3. 4. 2. 5. 2. Fixing solution (30% ethanol, 10% acetic acid)

Composition

Ethanol	:	270 ml
Glacial acetic acid	:	90 ml

The volume of the solution was adjusted to 900 ml.

The gel was retained in the fixing solution initially for 10 min. after which it was replaced with fresh fixing solution. Three such changes were allowed for fixing the polypeptides. The gel was taken out and washed free of fixative by gently shaking it in

distilled water allowing three changes at intervals of 10 min. each. The gel was then transferred to staining solution.

3. 4. 2. 5. 3 Staining solution

1.5 ml of silver nitrate (25%) was diluted to 150 ml with distilled water. This was mixed well and used for staining.

3. 4. 2. 5. 4. Developer solution

Fifteen ml of developer I (Na_2CO_3 25%) was diluted to 150 ml with distilled water. To this solution 85 µl of developer II (formaldehyde) was added and mixed gently. The developer solution was prepared fresh at the time of use just after the staining procedure was done.

3. 4. 2. 5. 5. Stop solution (1%)

Three ml of glacial acetic acid (99%) diluted to 300 ml using distilled water and stored at room temperature.

3. 4. 2. 5. 6. Reducer solution

One ml of reducer solution A (saturated potassium dichromate), 2 ml of reducer solution B (sodium thiosulphate 25%) and 350 µl of reducer C (formaldehyde 40% w/v) were mixed together and mixture was diluted to 300 ml with distilled water.

3. 4. 2. 5. 7. Staining the gel

The fixed gel was transferred to staining solution in a plastic acrylic tray, so that it is completely immersed in the solution. Gentle shaking was done for 30 min. at room temperature. This was followed by rinsing the gel in distilled water for 10-20 sec., and

immediate placing of the gel in the developer solution. The gel was shaken gently for 5-8 min. or till the protein bands were properly developed. When they were clear enough, it was transferred to stop solution for 5 min. The excess stain was removed by placing in the reducer solution for 10-30 sec. Later the gel was washed with distilled water. The protein profile was viewed in trans-illuminator and photographed using Gel Documentation System (Alpha Digi Doc^{TM}, USA).

3. 4. 3. Data Analysis

The relative migrations of polypeptides were considered for data analysis. Bands were scored as present (1) or absent (0) for each plant sample. Dendrogram and genetic distance were generated by clustering according to the Unweighted Paired Group Method with Arithmetic Mean (UPGMA) using the NTSYS-pc Version-2 computer software (Rohlf, 1992).

3.5 Molecular study

3. 5. 1. Plant Material (Refer section 3.4.1)

3. 5. 2. Genomic DNA Extraction

High levels of secondary metabolites including mucilage and polyphenolic compounds are present in the leaf tissue of orchid species which makes difficult for the extraction of DNA. To overcome these problems, a modified protocol of CTAB method (Cetyl Trimethyl Ammonium Bromide) was used (Edwards *et al.*, 1991). The extracted DNA was further purified from RNA and proteins by standard procedures (Sambrook *et al.*, 1989).

For each accession, about 5 g of bulked leaf tissue from 50 plants was ground to a fine powder using liquid nitrogen. This was then suspended in 20 ml of extraction buffer (20 mM EDTA at pH 8.0, 100 mM Tris-HCl at pH 8.0, 1.5 M NaCl, 2% CTAB and 1% β-mercaptoethanol). The suspension was mixed by inverting the tubes three to four times and incubated at 60°C for 45 min. with gentle mixing in water bath. After 45 min. equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12000 rpm for 20 min. at 4°C. Supernatant was carefully transferred to the new tube and 0.6 volume of ice cold isopropanol was added. The tubes were incubated at -20°C for 1 h. The DNA was pelleted down by centrifugation at 12,000 rpm for 10 min. at 4°C. After centrifugation, the supernatant was discarded and the pellets containing DNA was dissolved in 700 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) using new Eppendorf tube. After complete dissolution, the purification of DNA was done by adding equal volume of phenol:chloroform mixture (1:1) and inverting the tubes three to four times followed by centrifugation at 8000 rpm for 10 min. at room temperature (RT). Later, supernatant was transferred to a new Eppendorf tube adding equal volume of chloroform. The content was mixed three to four times and centrifuged at 8000 rpm for 10 min. at RT. The supernatant was transferred to a new tube adding equal volume of 3 M sodium acetate (pH 5.2) and three volumes of ethanol (100%) and then incubated at -20°C for 2 h followed by centrifugation at 8000 rpm for 15 min. at RT. Supernatant was discarded. 1 ml of ethanol (70%) was added to the pellet, gently vortexed and centrifuged at 8000 rpm for 10 min. at RT. The pellets were air dried followed by incubation at 37°C for 15 min. DNA pellet was dissolved in 100 µl of sterile distilled water and kept at 37°C for overnight. The concentration of DNA was estimated by agarose gel electrophoresis and staining with ethidium bromide and also spectroscopically. To check the purity of the DNA, the optical density (OD) ratio of 260:280 nm was taken.

3. 5. 3. Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis of genomic DNA was carried out using 10 base pair universal random oligonulceotide primers (Operon Technologies, USA). The details of the primers used are given in Table 9.

3. 5. 3. 1. Amplification reaction

Each Polymerase Chain Reaction (PCR) amplification was carried out in final volume of 25 µl, containing 50 ng of genomic DNA, 2.5 µl of 10X Taq assay buffer (100 mM Tris-HCl, pH 8.3; 20 mM $MgCl_2$; 500 mM KCl; 0.1% gelatin), 2.5 µl of 2.5 mM dNTPs, 15 ng of primers and 0.5 units of Taq polymerase (Genei, Bangalore, India).

3. 5. 3. 2. Amplification condition

PCR amplification was carried out in a DNA thermal cycler (Perkin-Elmer 480, USA). First cycle of the amplification consisted of an initial denaturation step of 3 min. at 94°C, followed by 40 cycles of 94°C for 1 min. (denaturation), 37°C for 1 min. (annealing) and 72°C for 2 min. (extension) and 10 min. for final extension step at 72°C. The amplified products were analysed on 1.4% agarose gels in 1X TAE buffer (50X stock 40 mM Tris HCl; 1mM EDTA, pH 8.0) with molecular weight marker (1Kb Marker, GeneRuler™, MBI Fermentas, USA). The gel was stained with ethidium bromide, visualized under ultraviolet light and photographed using Gel Documentation System (Amersham Pharmcia Biotech VDS Image Master, USA).

3. 5. 4. Inter simple sequence repeat (ISSR) analysis

ISSR analysis was carried out using 11 to 19 base pair universal random oligonulceotide as primers (Operon Technologies, USA). The details of the primers used are given in Table 10.

Table 9. RAPD random primers (Operon Technologies, USA) **used for analysis.**

Sl. No.	Primers	Sequence 5' to 3'
1	OPA 03	AGTCAGCCAC
2	OPA 04	AATCGGGCTG
3	OPA 05	AGGGGTCTTG
4	OPA 06	GGTCCCTGAC
5	OPA 07	GAAACGGGTG
6	OPA 08	GTGACGTAGG
7	OPA 09	GGGTAACGCC
8	OPA 12	TCGGCGATAG
9	OPA 13	CAGCACCCAC
10	OPA 14	TCTGTGCTGG
11	OPD 02	GGACCCAACC
12	OPD 03	GTCGCCGTCA
13	OPD 04	TCTGGTGAGG
14	OPD 05	TGAGCGGACA
15	OPD 06	ACCTGAACGG
16	OPD 07	TTGGCACGGG
17	OPD 08	GTGTGCCCCA
18	OPD 10	GGTCTACACC
19	OPD 11	AGCGCCATTG
20	OPD 20	ACCCGGTCAC

Table 10. ISSR primers (Operon Technologies, USA) **used for analysis.**

Sl. No.	Primers	Sequence 5' to 3'
1	HB 8	GAG AGA GAG AGA GG
2	HB 9	GTG TGT GTG TGT GG
3	HB 10	GAG AGA GAG AGA CC
4	HB12	CAC CAC CAC GC
5	HB13	GAG GAG GAG GC
6	HB 14	CTC CTC CTC GC
7	HB15	GTG GTG GTG GC
8	814	CTC TCT CTC TCT CTC TTG
9	814 A	CTC TCT CTC TCT CTC TAC
10	844 B	CTC TCT CTC TCT CTC TGC
11	P2	CTG AGA GAG AGA GAG AGA G
12	17898A	CAC ACA CAC ACA AC
13	17898B	CAC ACA CAC ACA GT
14	17899A	CAC ACA CAC ACA AG
15	17899B	CAC ACA CAC ACA GG

Table 11. List of ISSR primers with their annealing temperatures.

Sl. No.	ISSR primers	Annealing temperature
1	HB 10, HB12, HB13, HB 14, HB15	39°C for 30 Sec.
2	17898A, 17898B, 17899A, 17899B	44° C for 30 Sec.
3	HB 8, HB 9	46° C for 30 Sec.
4	814, 814 A	53° C for 1 min.
5	844 B, P2	57° C for 30 Sec.

3. 5. 4. 1. Genomic DNA Extraction (Refer section 3. 5. 2.)

3. 5. 4. 2. Amplification reaction

The amplification was performed in a 25 µl reaction volume containing 5 ng of genomic DNA, 2.5 µl of 10X Taq assay buffer (100 mM Tris-HCl, pH 8.3; 20 mM $MgCl_2$; 500 mM KCl; 0.1% gelatin), 2.5 µl of 2.5 mM dNTP's, 25 pmoles/µl of primer and 0.6 units of Taq polymerase.

3. 5. 4. 3. Amplification condition

Initial denaturation step was carried out for 5 min. at 94°C, followed by 35 cycles of 1 min. at 94°C for denaturation, annealing temperatures varies for different primers (Table 11) and 2 min. at 72°C for primer extension. An additional cycle of 10 min. at 72°C was used for primer final extension. The PCR products were analyzed on 1.2% agarose gels and stained with ethidium bromide, photographed under ultraviolet light by using gel documentation system. Molecular weight was estimated by using 1Kb DNA ladder (GeneRuler™, MBI Fermentas, USA).

3. 5. 5. Data Analysis

ISSR and RAPD amplified fragments named by primer code and molecular weights (bp) were scored. Each informative RAPD and ISSR band was scored independently as 1 for presence and 0 for absence. Percentage of polymorphism was calculated as the proportion of polymorphic bands over the total number of bands. Dendrogram and genetic distance were generated by clustering according to the Unweighted Paired Group Method with Arithmetic Mean (UPGMA) method using the NTSYS-pc Version-2 computer software (Rohlf, 1992).

4. RESULTS

The results reported here are based on the investigation of micropropagation and biochemical and molecular studies of two orchid species namely *Rhynchostylis retusa* and *Aerides maculosa.* During this study, (i) Western Ghats of Goa and adjacent regions were surveyed for collection of orchids, collected orchid species were photographed and systematically identified; (ii) developed and standardized the protocol for *in-vitro* mass multiplication and plant regeneration of *Rhynchostylis retusa* and *Aerides maculosa*; (iii) studied genetic variation among the populations of these two orchid species using RAPD and ISSR molecular markers, and (iv) analyzed the genetic variation among the populations of above species at protein level using SDS-PAGE electrophoresis.

4. 1. Study area

Western Ghats talukas of Goa region namely Sattari, Sanguem and Canacona were sampled. Field trips were made to 49 locations in these three talukas (Table 1; Fig.1). The collected specimens belonged to 20 genera and 26 species (Table 12).

4. 2. Taxonomic treatment

Key to the genera:

1. Plants terrestrial .....(2)
1. Plants Epiphytic herbs.....(7)

2. Leaves thin; petals more or less equal to sepals .....(3)
2. Leaves stout; petals narrower than dorsal sepals.....*Pecteilis*

3. Inflorescences de-curved; lip attached to the foot of the column.....(4)
3. Inflorescences erect; lip adnate to the foot of the column.....*Eulophia*

4. Lip spurred, spur projecting beyond the lateral sepals .....(5)
4. Lip not spurred, often saccate at base.....(6)

5. Sepals subequal, more or less connvient; stigmatic surface
in form of small swellings on the edge of lips.....*Peristylus*
5. Sepals unequal, lateral spreading or reflexed; stigmatic lobes
standing out as stalked appendages.....*Habenaria*

6. Lip with basal auricles; column very short, entire.....*Malaxis*
6. Lip without basal auricles; column long, margined or
winged toward the apex.....*Liparis*

7. Leaf persistent, sub-orbicular, not long slender.....*Porpax*
7. Leaves leathery, linear, long slender .....(8)

8. Pseudobulbs present.....(9)
8. Pseudobulbs absent.....(13)

9. Pseudobulbs discoid, depressed .....*Eria*
9. Pseudobulb conical or elongated.....(10)

10. Pseudobulbs of single nodes.....(11)
10. Pseudobulbs with 2 or more nodes.....(12)

11. Petals ovate, triangular, three nerved.....*Bulbophyllum*
11. Petals not ovate, triangular, one nerved.....*Pholidota*

12. Column-foot present .....*Dendrobium*
12. Column-foot absent .....*Cymbidium*

13. Leaves sessile, fleshy, laterally compressed.....*Oberonia*
13. Leaves petiolate, not fleshy, not laterally compressed.....(14)

14. Lip not with spur nor saccate at base.....*Cottonia*
14. Lip with spur or saccate at base.....(15)

15. Stem cylindrical, erect often woody.....(16)
15. Stem not cylindrical, not erect, not often woody.....(17)

16. Leaves apex bilobed.....*Cleisostoma*
16. Leaves apex not bilobed..... *Luisia*

17. Leaves lobed at apex.....(18)
17. Leaves toothed at apex..... *Rhynchostylis*

18. Inflorescences always drooping..... *Aerides*
18. Inflorescences erect, not drooping.....(19)

19. Sepals and petals with purple horizontal striations.....*Acampe*
19. Sepals and petals without any horizontal striations..... *Smithsonia*

Table 12. List of Orchid species collected from Western Ghats of Goa region.

Sl. No	Botanical Name	Locality
1	*Acampe praemorsa* (Roxb.) Blatt. & McC.	Bhuipal
2	*Aerides crispum* Lindl.	Ambeghat, Canacona
3	*Aerides maculosa* Lindl.	Tambdi Surla, Sanguem, Mollem
4	*Bulbophyllum neilgherrense* Wight	Ustem, Canacona
5	*Cottonia peduncularis* (Lindl.) Rchb. f.	Mollem
6	*Cymbidium aloifolium* (L.) Sw.	Canacona, Sanguem
7	*Dendrobium lawianum* Lindl.	Mollem
8	*Dendrobium macrostachyum* Lindl.	Bondir
9	*Dendrobium ovatum* (L.) Kranzl.	Anmod Ghat
10	*Eria dalzellii* Lindl.	Valpoi, Verlem
11	*Eria reticosa* Wight	Surla, Verlem
12	*Eulophia spectabilis* (Dennst.) Suresh	Salginim
13	*Habenaria crinifera* Lindl.	Surla, Sattari
14	*Habenaria heyneana* Lindl.	Canacona, Sanguem, Verlem
15	*Habenaria marginata* Coleb.	Quepem, Sattari
16	*Liparis odorata* (Willd.) Lindl.	Surla, Verlem
17	*Luisia zeylanica* Lindl.	Valpoi
18	*Malaxis rheedii* Swartz.	Verlem, Surla, Ambeghat
19	*Oberonia brunoniana* Wight	Chorla Ghat, Netravali, Vaddem
20	*Pecteilis gigantea* (L.) Rafin.	Canacona, Sattari, Sanguem
21	*Peristylus goodyeroides* (Don.) Lindl.	Terrestrial
22	*Pholidota imbricata* W. J. Hook.	Dhave, Sanguem
23	*Porpax jerdoniana* (Wight) Rolfe	Bondla
24	*Rhynchostylis retusa* (L.) Bl.	Valpoi
25	*Cleisostoma tenuifolium* (L.) Garay	Nanoda
26	*Smithsonia viridiflora* (Dalz.) Saldanha	Tambdi Surla

ACAMPE Lindley

Acampe praemorsa (Roxb.) Blatt. & McC.

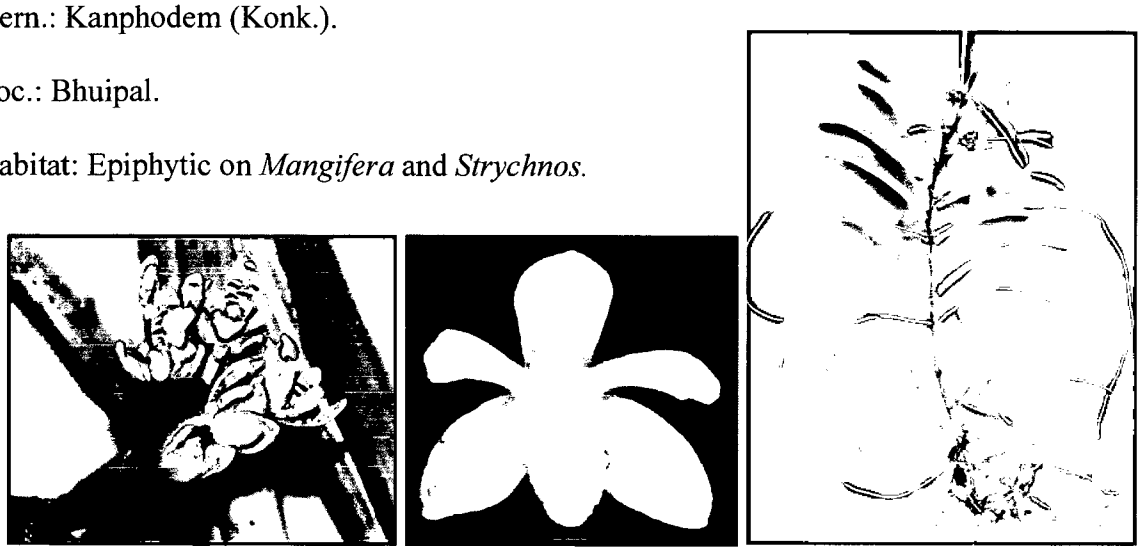
Stem woody, sheathed, longitudinally striated, up to 50 cm tall, leafy. Leaves thick, fleshy, about 30 cm long. Inflorescence approximately 6 cm tall, rarely branched, compact. Flowers about 1 cm across, dark yellow with dark brownish stripes. Lip creamy yellow with minute red streaks.

Fl.: April-August.

Vern.: Kanphodem (Konk.).

Loc.: Bhuipal.

Habitat: Epiphytic on *Mangifera* and *Strychnos.*



AERIDES Lour.

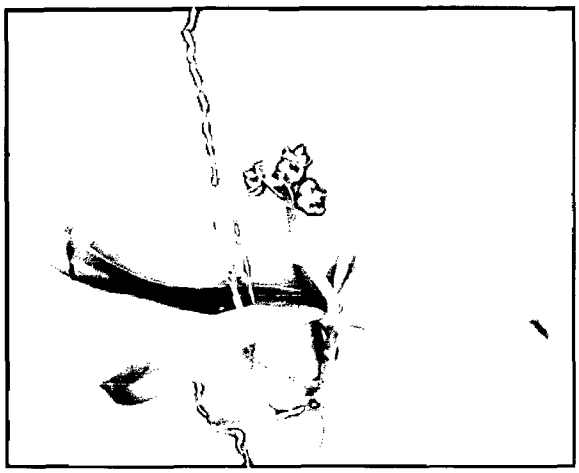
Key to the species:

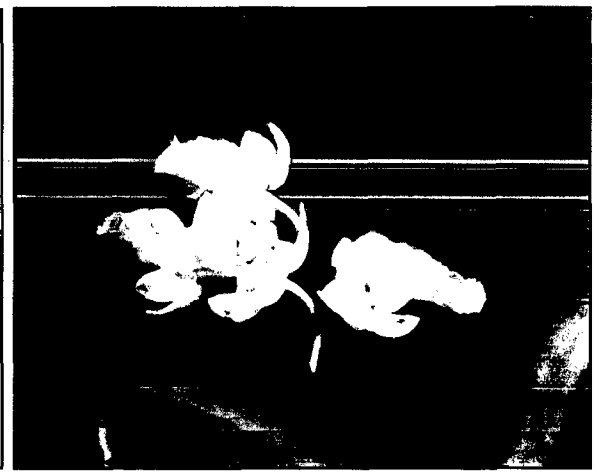
1. Flowers sweetly fragrant; lateral sepals obliquely oblong; petals pinkish-white, not speckled; midlobe of lip ovate ..... *A. crispa*
1. Flowers almost inodorous; lateral sepals obovate; peatal rose colored, speckled with dots of a deeper colour; middle of lip quadrate-oblong..... *A. maculosa*

Aerides crispa Lindl.

Stem tall, robust and erect, brownish violet. Leaves deep green, spreading, 15-20 cm long and 4-8 cm broad, flat, bilobed, cut at the apices. Inflorescence generally racemose but

often branched. Flowers up to 5 cm across, scented, white flushed with purplish rose behind. Lip trilobed, margin crenulate; spur strongly incurved.





Fl.: May-June.

Loc.: Ambeghat, Canacona.

Habitat: Epiphyte on *Atrocarpus heterophyllus*.

Aerides maculosa Lindl.

Slow growing plant of rather stiff and dwarf habit. Leaves leathery, up to 22 cm long and

5 cm broad, thick, fleshy and rounded at the apex. Inflorescence longer than the leaves, pendulous and branched, many-flowered. Flowers large, about 4 cm long, pale rose coloured, fragrant.

Fl.: June-July.

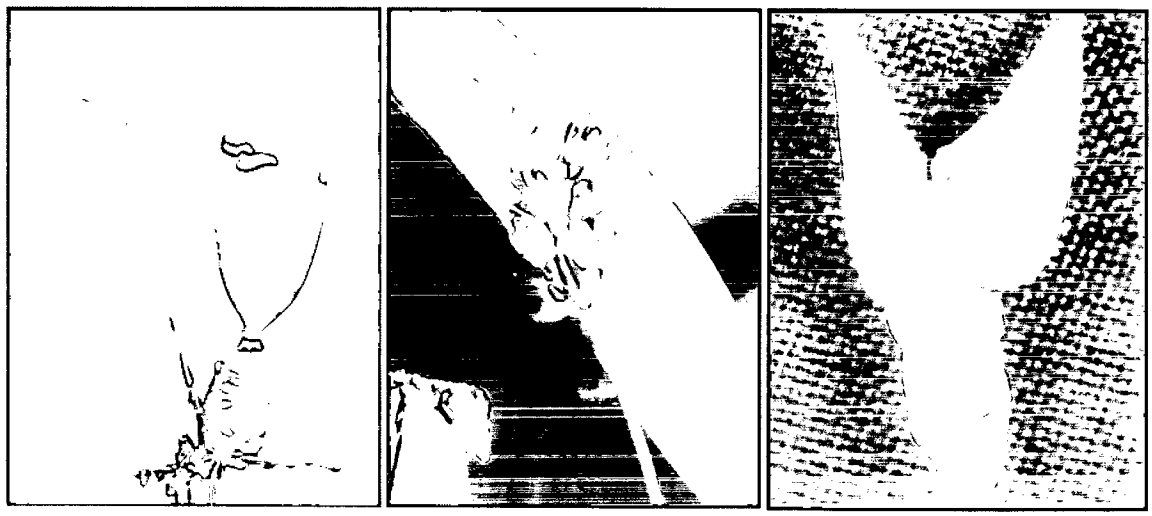
Loc.: Tambdi Surla, Sanguem, Mollem.

Habitat: Epiphytic on *Ficus* sp. and *Terminalia paniculata;* generally found in open moist and semi-evergreen forests.

BULBOPHYLLUM Thou.

Bulbophyllum neilgherrense Wight

Rhizome stout, creeping. Pseudobulbs smooth, ovoid, up to 4 cm long. Leaves thick, fleshy, oblong-obtuse, up to 17 cm long. Scape stout, elongate; sheaths few, distant; racemes lax-flowered; bracts acute, much shorter than the flowers. Flowers *c.* 1 cm across, brownish yellow or greenish purple; sepals oblong, dorsal sepal broad ovate; petals triangular-ovate. Lip with entire or toothed auricles.



Fl.: December-February.

Loc.: Ustem, Canacona.

Habitat: Epiphytic on *Careya arborea* and *Syzygium* sp.

COTTONIA Wight

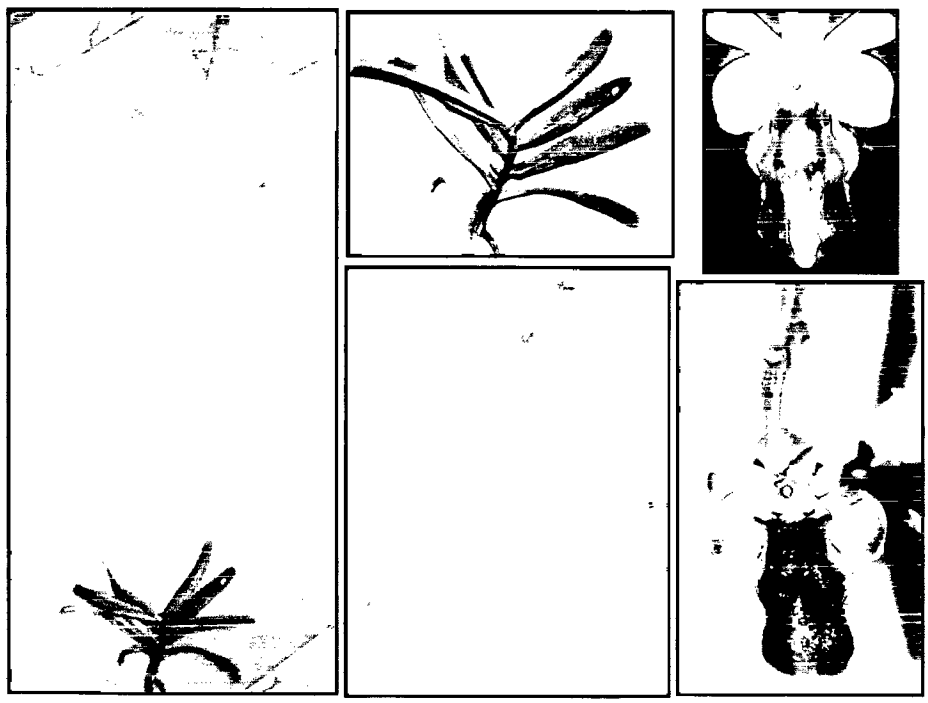
Cottonia peduncularis (Lindl.) Rchb. f.

Stem elongated, leafy. Leaves linear, obtuse, about 25 cm long and 1.5 cm wide. Scape up to 30 cm long, erect, tipped by short raceme, pedicels long, slender, bracts ovate. Flowers *c.* 2.5 cm in diameter; sepals oblong, subspathulate; petals dirty orange with red streaks. Lip dark purple; margin broad, villous, golden; side lobes with 3 calli between them, disc of midlobe with medium callus.

Fl.: March-June.

Loc.: Mollem.

Habitat: Epiphyte in semi-evergreen and moist deciduous forests.



CYMBIDIUM Sw.

Cymbidium aloifolium (L.) Sw.

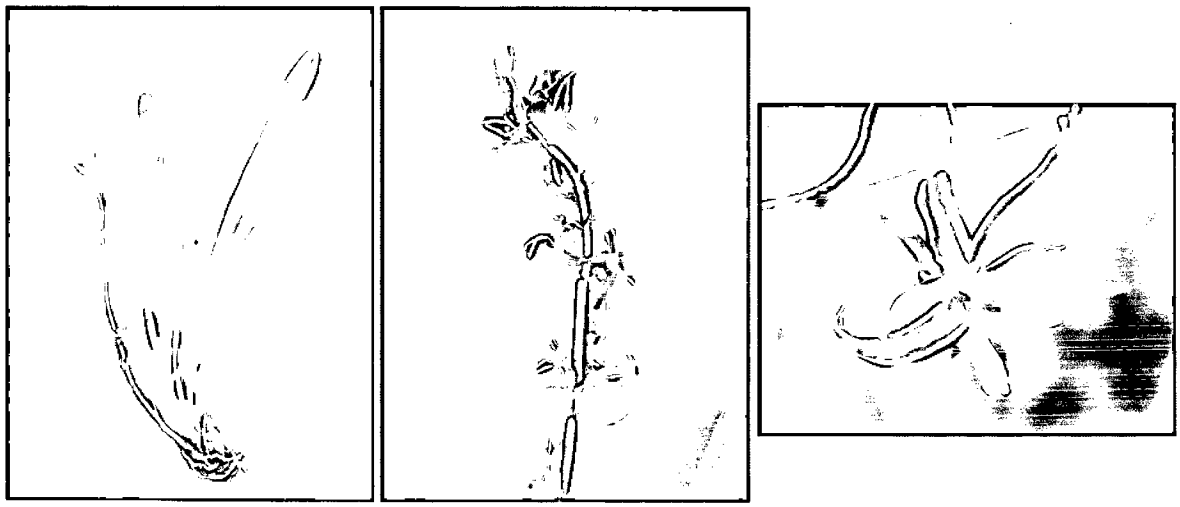
An epiphytic herb. Stem short and stout. Leaves linear-oblong, obtuse, up to 50 cm long and 3 cm broad, not attenuated towards the notched tip. Scape and raceme up to 50 cm long, erect below, decurved above. Flowers *c.* 4 cm across, yellowish red or brownish red; sepals and petals linear-oblong, subacute. Lip oblong with two lamellae notched at the middle; end lobe ovate-oblong with a white patch at its base, otherwise purple with darker lines.

Fl.: April-May.

Vern.: Sonu (Konk.).

Loc.: Canacona, Sanguem.

Habitat: Epiphyte on *Tectona grandis.*



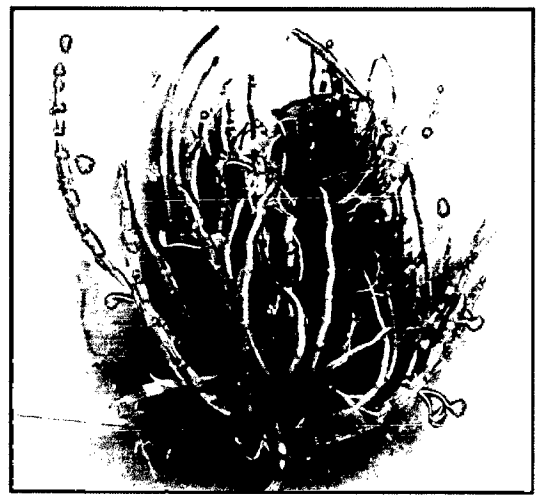
DENDROBIUM Sw.

Key to the species:

1. Flowers in racemes.....*D. ovatum*
1. Flowers in fascicles.....(2)

2. Sepals and petals waxy white flushed with rose purple..... *D. lawianum*
2. Sepals and petals, pale green of yellow often tinged with pink.....*D. macrostachyum*

Dendrobium lawianum Lindl.



Pseudobulbs 5-22 cm long, curved. Leaves 5-10 cm long, lanceolate or oblong-lanceolate, acute. Flowers solitary or in pairs; sepals and petals waxy, white, flushed with rose-purple towards the apex, 5-nerved; column stout.

Fl.: May-July.

Loc.: Mollem.

Habitat: Epiphyte on *Artocarpus heterophyllus*

Dendrobium macrostachyum Lindl.

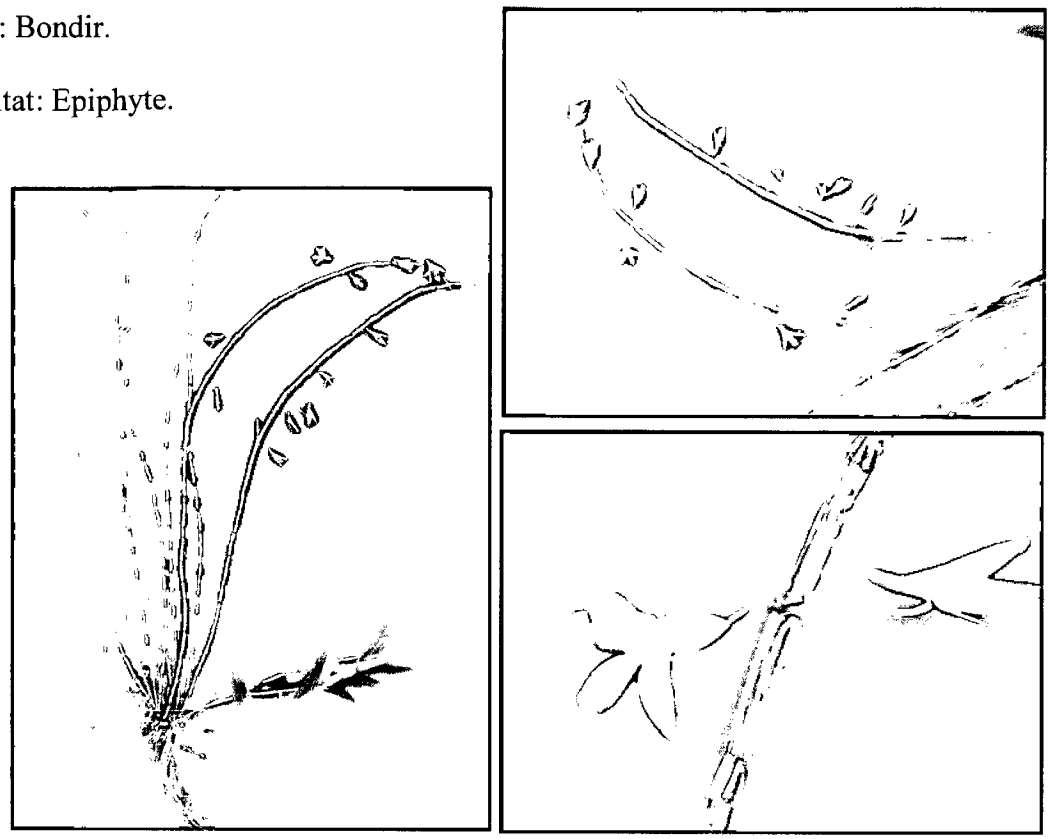
Stem slender, up to 60 cm long, pendulous, terete. Leaves up to 10 cm long and 2 cm broad, membranous, ovate-oblong, acute. Inflorescence 2 to 4 flowered lateral racemes. Flowers *c.* 2.5 cm long, fragrant, pale green or yellow, often tinged with pink; sepals and petals equal in length, acute; sepals oblong-lanceolate; petals linear-lanceolate. Lip longer, ovate-oblong, long-clawed, finely pubescent.

Fl.: June-August.

Vern.: Joivansi (Mar.).

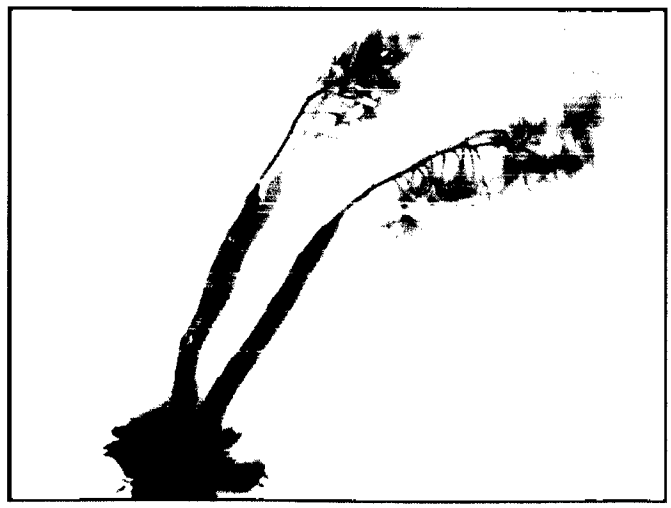
Loc.: Bondir.

Habitat: Epiphyte.



Dendrobium ovatum (L.) Kranzl.

Pseudobulbs up to 50 cm long, mauve-brown, leafless at the time of flowering. Leaves up to 10 cm long and 1.5 cm broad, lanceolate or oblong-lanceolate, acute. Inflorescence in racemes from the apical part of the pseudobulbs, raceme up to 15 cm long, several flowered. Flowers 1.5 cm long, cream-coloured, lip greenish; internal sepals oblong, lanceolate, subacute, dorsal oblanceolate, obtuse; mentum acute. Lip flat; side lobes small, rounded, midlobe large, subquadrate.



Fl.: November-January.

Loc.: Anmod Ghat.

Habitat: Epiphyte.

ERIA Lindl.

Key to the species:

1. Inflorescence 1-flowered ..... *E. reticosa*
1. Inflorescence many flowered .....*E. dalzellii*

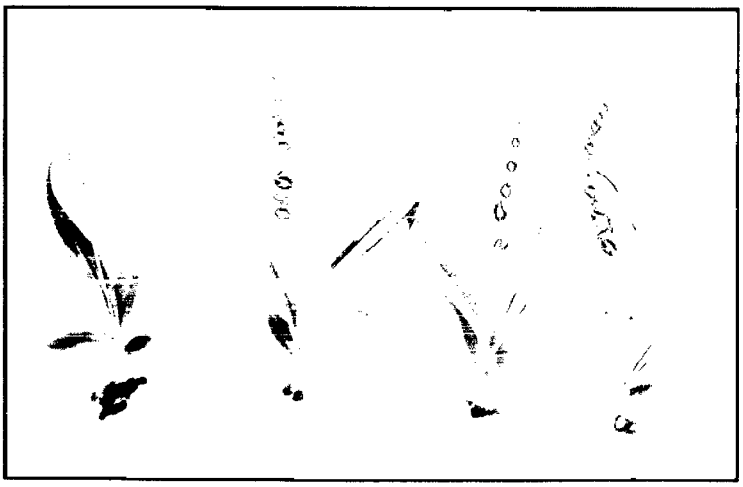
Eria dalzellii Lindl.

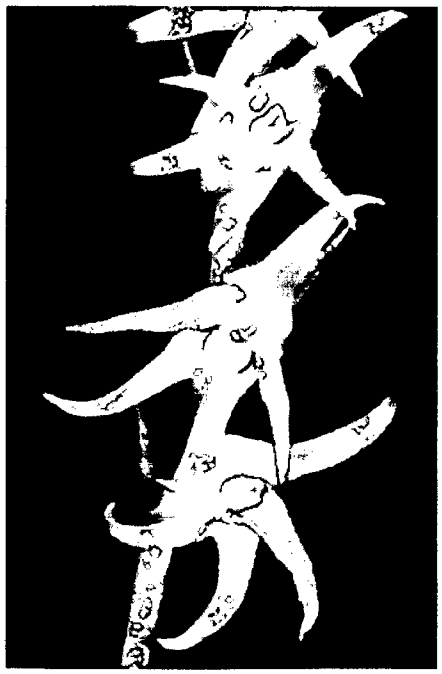
Pseudobulbs clustered, depressed, up to 1.5 cm in diameter. Leaves linear to oblanceolate, tip rounded, apiculate, up to 7 cm long, Inflorescence few-flowered, up to 12 cm long, bracts lanceolate. Flowers *c.* 7 mm long, white or yellowish; sepals 1 to 3 nerved, dorsal ovate-lanceolate, lateral falcately ovate or lanceolate; petals lanceolate, 1 to 3 nerved, subsimilar. Lip very small, ovate-lanceolate.

Fl.: July-August.

Loc.: Valpoi, Verlem.

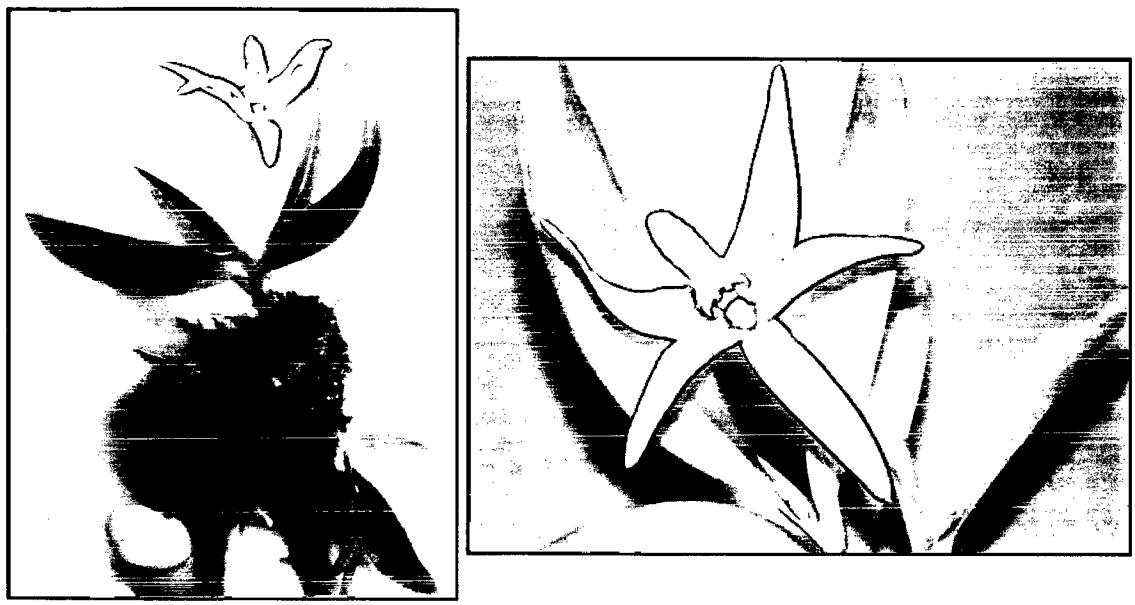
Habitat: Epiphyte on *Mangifera indica.*





Eria reticosa Wight

Pseudobulbs disc-like, 1-1.5 cm across, covered with reticulate sheath. Leaves 3.5-4.5 cm long and 1.5-2 cm broad, ovate to oblong-lanceolate, acute. Inflorescence solitary. Flowers 2.5-3.5 cm long, simultaneous with leaf pair, white scented; pedicels 3-4 cm long with the ovary; sepals 2-3.5 cm long, oblong-lanceolate; petals nearly equal to sepals, oblong-lanceolate. Lip trilobed, lateral narrowly oblong, with red margin, keels yellow, midlobe oblong, crenulate.



Fl.: July-August.

Loc.: Surla, Verlem.

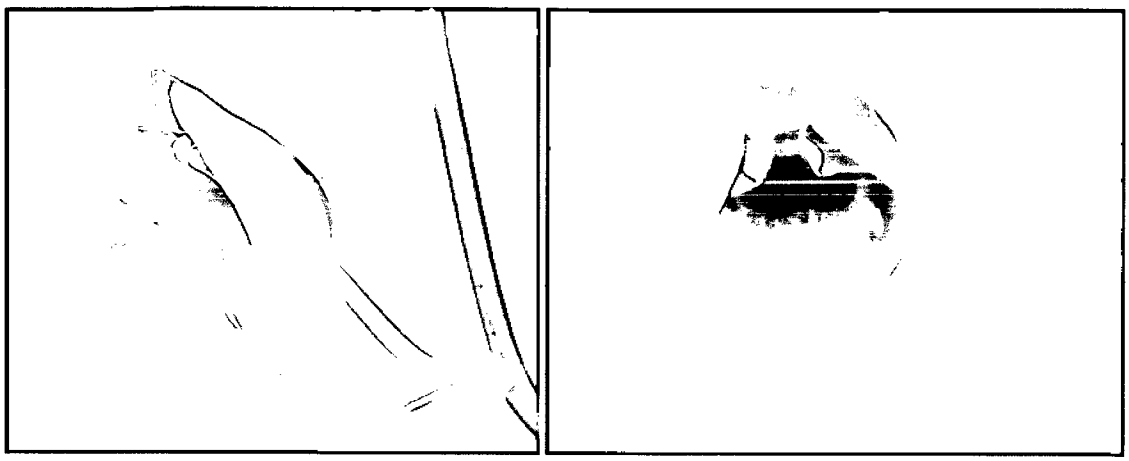
Habitat: Epiphyte on *Mangifera indica.*

EULOPHIA R. Br.

Eulophia spectabilis (Dennst.) Suresh

Pseudobulbs round, enveloped by lanceolate sheaths. Inflorescences raceme up to 70 cm long, up to 12 flowered; bracts, linear, 1-1.5 cm long, persisting even after flowers have

fallen off. Flowers deep purple, *c.* 2 cm long and 1 cm width, sepals 2.5 cm long, 0.5 cm wide. Petals compressed, ridged.



Fl.: April-June.

Loc.: Salginim.

Habitat: Terrestrial.

HABENARIA Willd.

Key to the species:

1. Leaves cauline; inflorescence 1-sided ..... *H. heyneana*
1. Leaves radical, flat on the ground or slightly above the ground; inflorescence Not 1-sided.....(2)

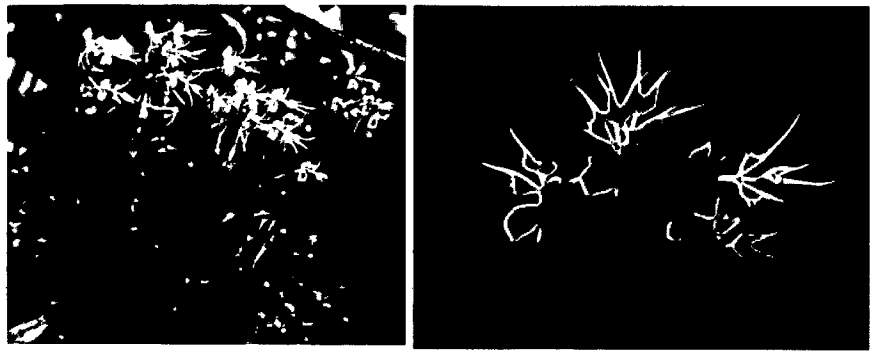
2. Inflorescence 1-4 flowered terminal lobe of lip divided.....*H. crinifera*
2. Inflorescence many flowered lateral lobes of lip narrow than midlobe.....*H. marginata*

Habenaria crinifera Lindl.

Stem up to 50 cm tall, leafy near the base. Leaves oblong or lanceolate, acute, up to 15 cm long and 4 cm broad. Inflorescence up to 10 cm long, dense-flowered; Bracts ovate-lanceolate, acuminate. Flowers *c.* 3.5 cm long, white; sepals broad, obtuse, petals linear, acuminate. Lip clawed; side lobe cuneiform; midlobe flabelliform; spur slender, incurved.

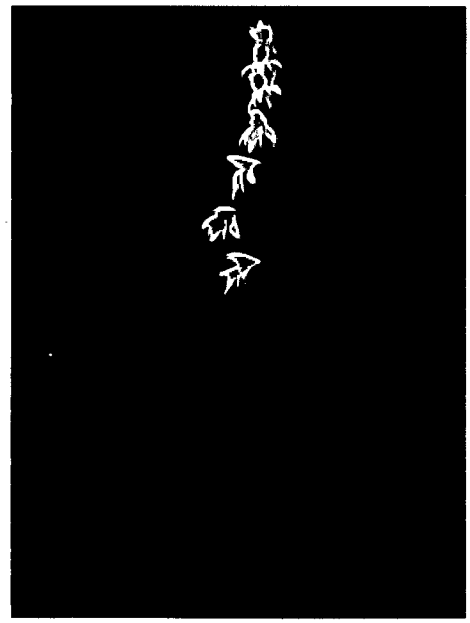
Fl.: August-October.

Loc.: Surla, Sattari.



Habitat: Epiphytes in forest openings of semi-evergreen forests and moist deciduous forests.

Habenaria heyneana Lindl.



Stem up to 35 cm tall; tubers small, ovoid or oblong. Leaf solitary, basal, oblong-lanceolate, pointed, short-stalked, up to 7 cm long. Inflorescence up to 10 cm long, many-flowered. Flowers 1.2 cm across, greenish yellow, sepals subequal, obtuse; petals as long as sepals, narrow, linear-oblong. Lip divided into three linear segments almost to the base; side lobes linear, obtuse; midlobe broader; spur subclavate.

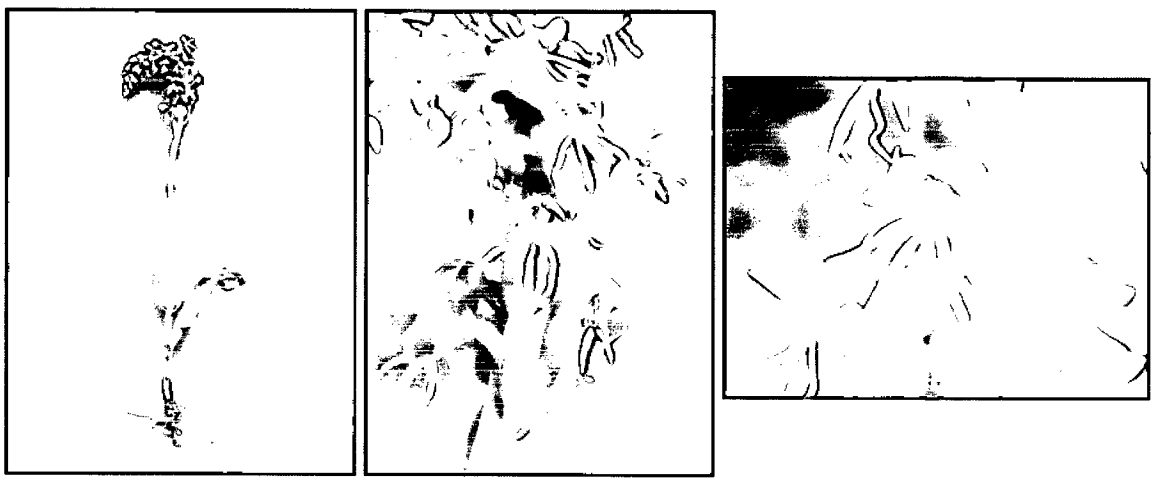
Fl.: August-September.

Loc.: Canacona, Sanguem, Verlem.

Habitat: Terrestrial.

Habenaria marginata Coleb.

Stem stout up to 30 cm tall. Leaves oblong or linear-oblong, obtuse or acute, up to 12 cm long and 2.7 cm broad, margins yellow. Inflorescence many-flowered; scape about 25 cm long; bracts lanceolate, ciliolate. Flowers *c.* 1.2 cm across, yellow; sepals subequal, laterals oblong-lanceolate; dorsal sepals broadly ovate; petals ovate, falcate. Lip longer than the sepals; side lobes linear, slender; midlobe linear, obtuse; spur stout, as long as ovary.



Fl.: September-October.

Loc.: Quepem, Sattari.

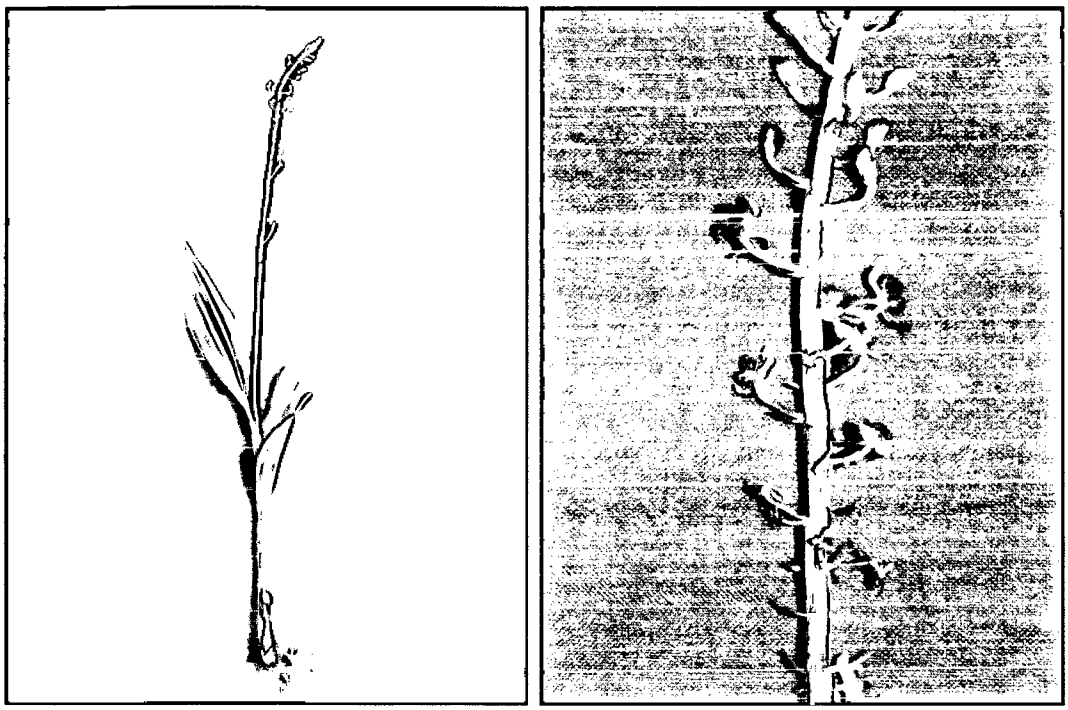
Habitat: Terrestrial.

LIPARIS L. C. Rich.

Liparis odorata (Willd.) Lindl.

Stem short, up to 7 cm long, with pseudobulbous base, sheaths few. Leaves membranous, opposite, tapering at the base of the apex, ovate- oblong, 7-nervered, up to 12 cm long. Inflorescence up to 20 cm long, slender and many- flowered; floral bracts linear, acute to

apiculate. Flowers brownish, *c.* 8 mm across; petals like sepals, narrowly oblong, obtuse.



Fl.: July-August.

Loc.: Surla, Verlem.

Habitat: Terrestrial.

LUISIA Gaudich.

Luisia zeylanica Lindl.

Stem slender, up to 30 cm long. Leaves very variable in thickness, sometimes very long and slender, green up to 17 cm long. Flowers a few *c.* 1.2 cm long, yellowish with a purple tinge; petals much longer than the sepals, narrow. Lip dark purple with patch on either side, as long as the

petals, narrowly pandurate, convex, base broad, flat.

Fl.: July-August.

Vern.: Amb-keli (Konk.).

Loc.: Valpoi.

Habitat: Epiphytic on *Mangifera indica.*

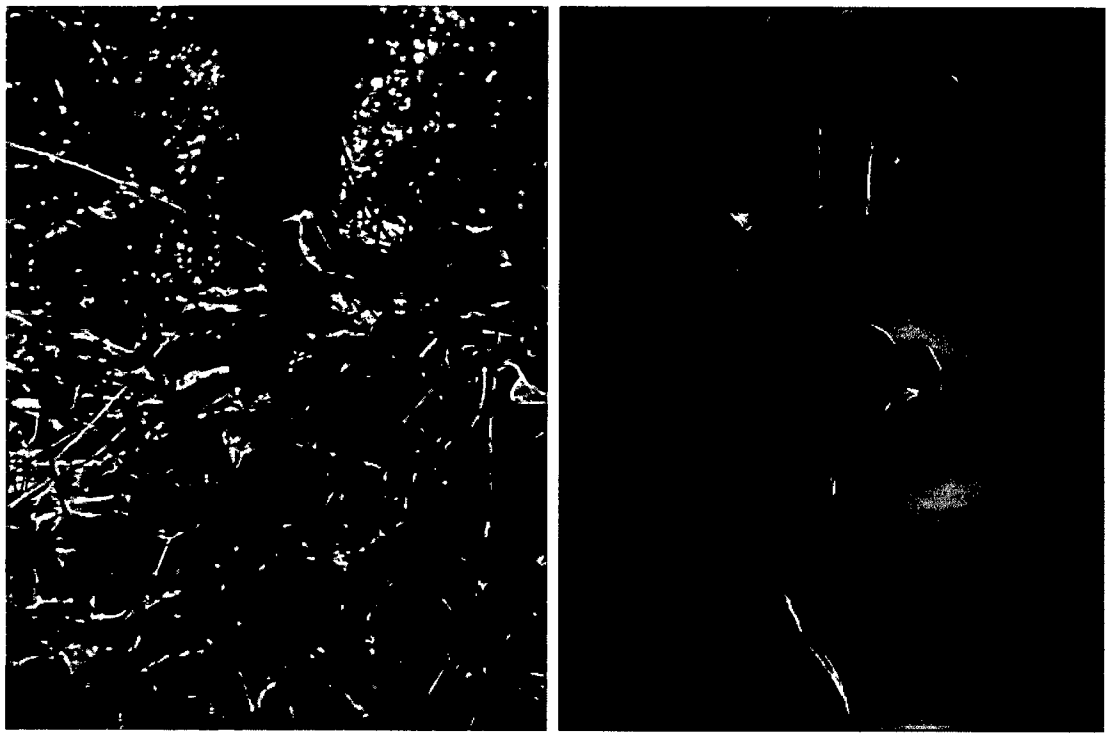
MALAXIS Soland. ex Sw.

Malaxis rheedii Swartz.

Stem stout, up to 15 cm long, stoloniferous. Leaves 3-5, shortly petiolated, broadly ovate or elliptic, acute or acuminate, up to 17 cm long and 7.5 cm broad. Inflorescence up to 30 cm long, stout or slender, elongate, lax-flowered racemes. Flowers very variable in size and colour, greenish yellow or purplish, sweet-scented; sepals obtuse. Lip reniform or fan-shaped pectinate.

Fl.: July-September.

Vern.: Dande (Konk.).



Loc.: Verlem, Surla, Ambeghat.

Habitat: Terrestrial.

OBERONIA Lindl.

Oberonia brunoniana Wight

Stem elongate. Leaves linear-oblong, ensiform, very coriaceous, succulent, up to 35 cm long and 3 cm broad. Inflorescence up to 20 cm long. Flowers sessile, densely imbricated, *c.* 4 mm across; sepals and lip dark brownish; petals pale yellowish, narrow. Lip orbicular with a short terminal auricle.

Fl.: February-March.

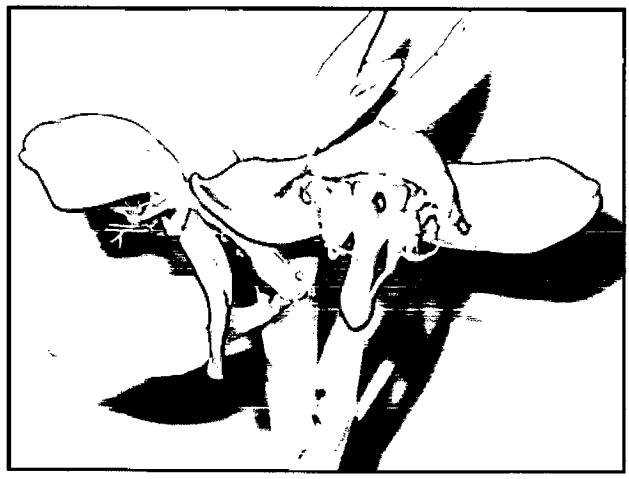
Loc.: Chorla Ghat, Netravali, Vaddem.

Habitat: Rare in deciduous forest and on trees in forest openings.

PECTEILIS Rafin.

Pecteilis gigantea (L.) Rafin.

Tubers large, about 10 cm long. Stem leafy almost throughout, tall, robust. Leaves ovate-oblong, upper sheathing, the largest leaf up to 12 cm long and 5 cm broad, sessile. Inflorescence up to 20 cm long, 4-10 flowered,



bracts large. Flowers very large, *c.* 10 cm in diameter, subsessile, white, fragrant; sepals spreading, lateral sepals obtuse; petals small. Lip trilobed to the base; side lobs fringed with long narrow teeth; spur twice as long as the ovary.

Fl.: September-October.

Loc.: Canacona, Sattari, Sanguem.

Habitat: Terrestrial ground orchid; occasional on rocky slopes along forest edge.

PERISTYLUS Blume

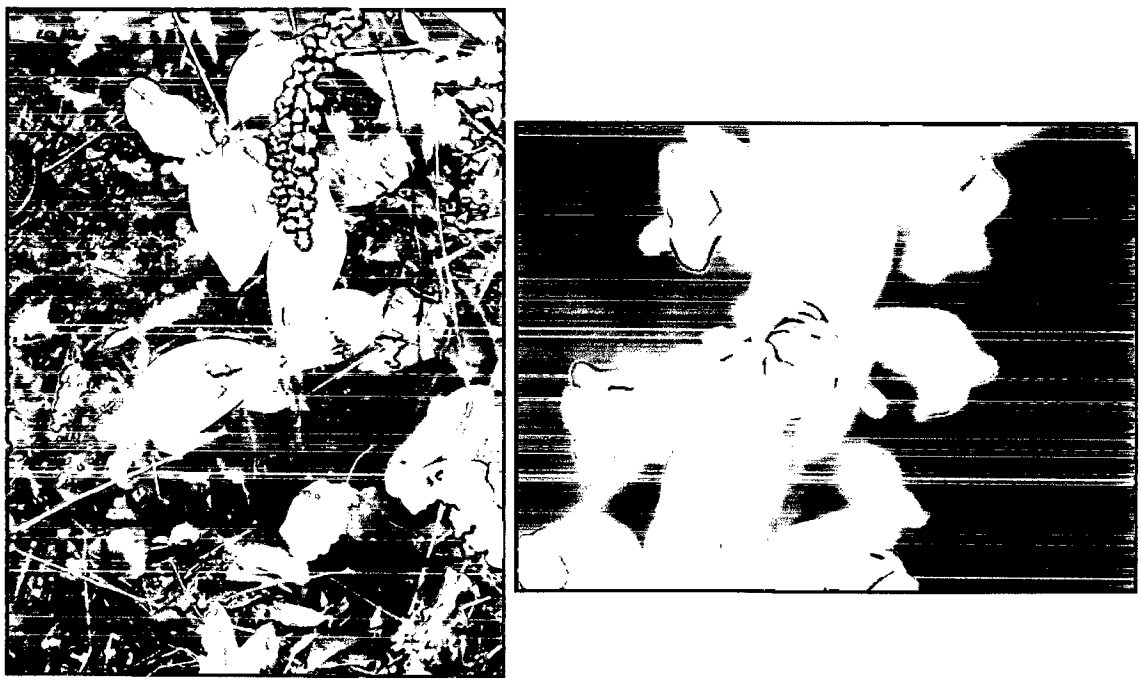
Peristylus goodyeroides (Don.) Lindl.

Robust plants, with inflorescence up to 30-40 cm long, leaves 2-4, very coriaceous, often with brownish tinge, 5-8 cm long and 3-4 cm wide. Flowers greenish-white, *c.* 0.5 cm across. Sepals greenish or some time brownish, petals and lip white.

Fl.: July-September.

Loc.: Surla.

Habitat: Terrestrial.



PHOLIDOTA Lindl.

Pholidota imbricata W. J. Hook.

Pseudobulbs tufted, up to 6 cm long, broadly conical, not angled, bearing a single leaf, slender, conical with an ovoid base. Leaves up to 30 cm long and 6 cm broad, acute, broadly elliptic-lanceolate. Inflorescence drooping, densely flowered, up to 25 cm long. Flowers small, insignificant, *c.* 7 mm long, musk-scented or odorless, pale pink; sepals broad, concave; petals narrower, pointed. Lip with two hatchet-shaped side lobes, a rectangular middle part; midlobe spreading downwards, deeply bilobed, often with yellow spots.

Fl.: June-July.

Vern.: Tolasi (Konk.).

Loc.: Dhave, Sanguem.

Habitat: Epiphytic.

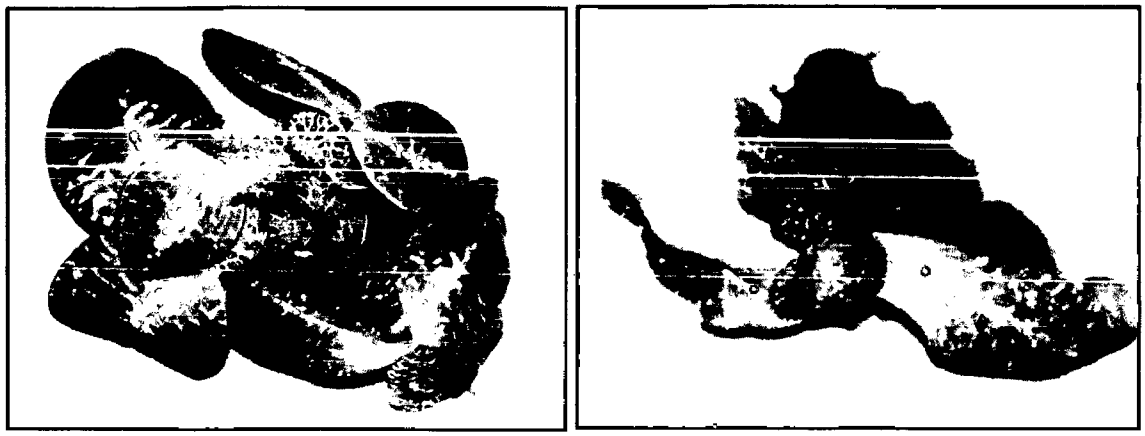
PORPAX (Wight) Rolfe

Porpax jerdoniana (Wight) Rolfe

Pseudobulbs *c.* 1.5 cm in diameter, aggregated. Leaves persistent, suborbicular, up to 3 cm long. Flowers red; lateral sepals connate to above the middle, concave, pubescent, dorsal sepals nearly free, broadly oblong; petals linear-spathulate, very small. Lip minute, shortly clawed, ovate-cordate, toothed.

Fl.: July-October.

Loc.: Bondla.



Habitat: Epiphytes on *Tectona grandis* and also in semi-evergreen forests.

RHYNCHOSTYLIS Bl.

Rhynchostylis retusa (L.) Bl.

Stem stout, woody, clothed with sheaths of fallen leaves, up to 60 cm long. Leaves leathery, strap-shaped, linear, deeply channelled, gracefully arching, up to 30 cm long and 2.5 cm broad, apex subequally bilobed. Inflorescence pendulous, compact, many-flowered, cylindrical, up to 60 cm long. Flower *c.* 1 cm in diameter, fragrant, long lasting, white, spotted with bluish purple, lip purple; sepals oval, oblong, acute; petals narrowly oblong, acute. The basal part of the lip forming a wide and deep sac, the apical part obovate.

Fl.: May-June.

Vern.: Panas koli (Konk.), Foxtail orchid (Eng.).

Loc.: Valpoi.

Habitat: Epiphytic on *Mangifera indica* and *Atrocarpus heterophyllus.*

CLEISOSTOMA Bl.

Cleisostoma tenuifolium (L.) Garay

Stem terete, slender, leafy, pendulous, up to 30 cm long. Leaves linear, straight or falcately curved, obtusely acuminate, up to 50 cm long, and 1 cm broad, sheaths ribbed. Inflorescence up to 12 cm long, a slender raceme. Flowers *c.* 6 mm across, yellow with red margins, lip white or yellowish; sepals elliptic, obtuse; petals spathulate. Side lobes of lip small acute; mid lobe small, triangular.

Fl.: July-August.

Loc.: Nanoda.

Habitat: Epiphyte on *Mangifera indica.*

SMITHSONIA Saldanha

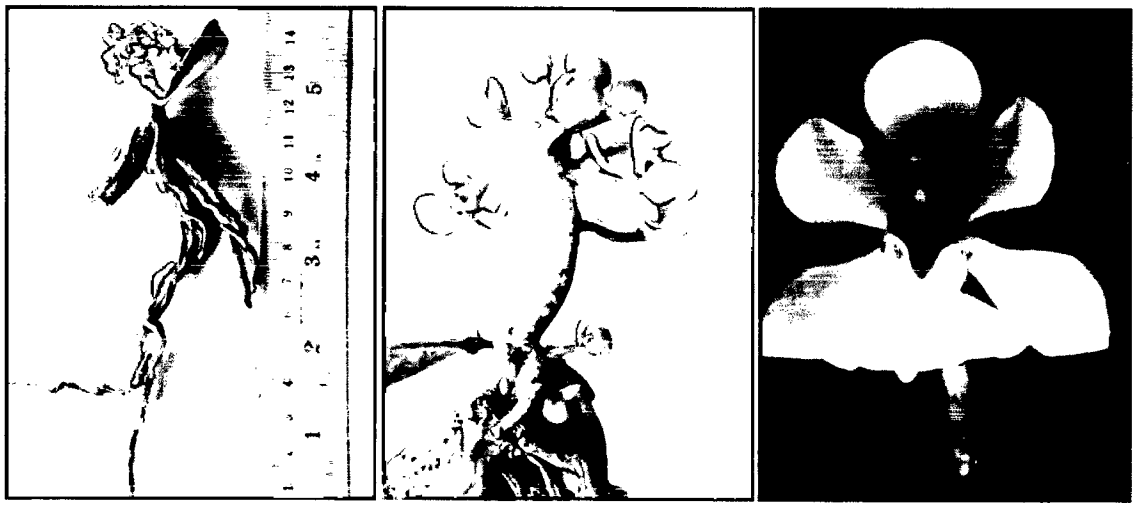
Smithsonia viridiflora (Dalz.) Saldanha

Leaves 2.5-6.5 cm long and 1 cm broad, lanceolate. Inflorescence 3 to 5 flowered raceme; bracts short, obtuse. Flower 1-1.5 cm in diameter, greenish white. Lip clouded with rose-coloured blade placed at the mouth of the spur, trilobed, side lobes free, rounded. Spur very short, conical acute, glabrous, parallel to the blade of the lip.

Fl.: May-June.

Loc.: Tambdi Surla.

Habitat: Epiphyte on *Artocarpus heterophyllus*.



4.3 Micropropagation

Aerides maculosa Lindl. and *Rhynchostylis retusa* (L.) Bl. are the most important epiphytic orchid species that grows in the broad leaved forests, valued for its beautiful inflorescence/flowers. These species ranks high among the important Indian ornamental orchids because of its beautiful flowers. Due to their attractive colour and long inflorescences, both the species were selected for the micropropagation (Table 12).

During this study, totipotent callus were established and plantlets were regenerated via protocorm-like bodies (PLBs) for both the orchid species *Rhynchostylis retusa* and *Aerides maculosa.*

4. 3. 1. *Aerides maculosa*

4. 3. 1. 1. Explants

Different parts from the mature plant such as shoot tip, leaf and root was initially used as explants for the micropropagation study. However, explants from the mature plant did not show any sign of growth up to 10 to 12 weeks after culture. Further sub culturing of the explants to fresh medium resulted in necrosis.

Yellowish green seeds from immature capsules of 5-10 weeks after pollination (WAP) (Plate 1a) were collected from the nursery grown plants. These seeds were placed on different media for callus induction (Table 13).

4. 3. 1. 2. Induction of callus

Immature seeds were used for callus induction. Seed were inoculated on the medium containing different growth regulators and growth adjunct. Detail responses of immature seeds to different growth regulators and growth adjunct are given in Table 13. In each treatment 10 replicates were used. Percentage of callus induction from each

Table 13. Effect of PGRs and growth adjunct on callus induction of immature seeds of *Aerides maculosa*.

Medium	Media supplemented with/without growth regulators and growth adjunct	Responses of immature seeds in culture		
		Morphogenesis	Callus induction %	No. of days taken
I	VW	No response	0	-
II	VW + 2,4-D 0.5 mg l^{-1}	root formation	0	-
III	VW + NAA 0.5 mg l^{-1}	root formation	0	-
IV	VW + IAA 0.5 mg l^{-1}	No response	0	-
V	VW + CW 10%	Moderate quantity of calli (++)	77 ± 0.55	30
VI	VW + CW 15%	Large quantity of calli (+++)	81.8 ± 0.82	27
VII	VW + CW 20%	Small quantity of calli (+)	78.6 ± 0.54	32
VIII	MS	No response	0	-
IX	MS + 2,4-D 0.5 mg l^{-1}	root formation	0	-
X	MS + NAA 0.5 mg l^{-1}	root formation	0	-
XI	MS + IAA 0.5 mg l^{-1}	root formation	0	-
XII	MS + CW 10%	Moderate quantity of calli (++)	57.8 ± 0.92	33
XIII	MS + CW 15%	Small quantity of calli (+)	60.6 ± 0.61	35
XIV	MS + CW 20%	Moderate quantity of calli (++)	58.4 ± 0.62	37
XV	MI	No response	0	-
XVI	MI + 2,4-D 0.5 mg l^{-1}	root formation	0	-
XVII	MI + NAA 0.5 mg l^{-1}	root formation	0	-
XVIII	MI + IAA 0.5 mg l^{-1}	No response	0	-
XIX	MI + CW 10%	Small quantity of calli (+)	49.4 ± 0.54	40
XX	MI + CW 15%	Small quantity of calli (+)	53.6 ± 0.25	38
XXI	MI + CW 20%	Small quantity of calli (+)	50.8 ± 0.64	41

Data showing the mean of 10 replicates ± standard error (SE).

PGRs: Plant growth regulators, VW: Vacin and Went medium (1949), 2, 4-D: 2, 4-dichlorophenoxyacetic acid, NAA: 1-naphthaleneacetic acid, IAA: indole-3-acetic acid, CW: coconut water, MS: Murashige and Skoog medium (1962), MI: Mitra *et al.*, medium (1976).

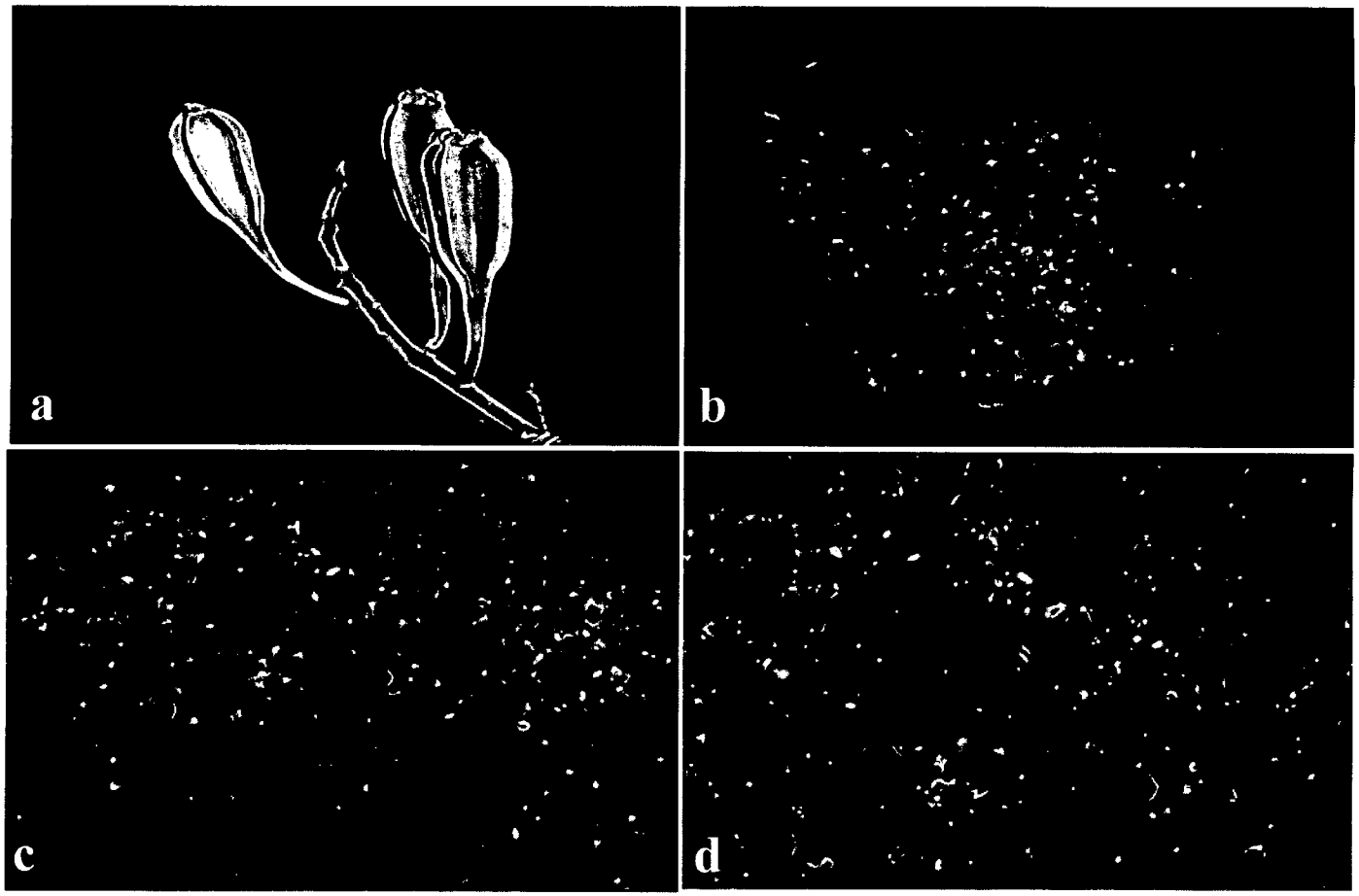


Plate 1. Callus formation from immature seeds of *Aerides maculosa.* a. Immature capsules, after 5 weeks of pollination. b. Callus developed on Vacin and Went medium supplemented with 15% coconut water. c and d. Closer view of callus.

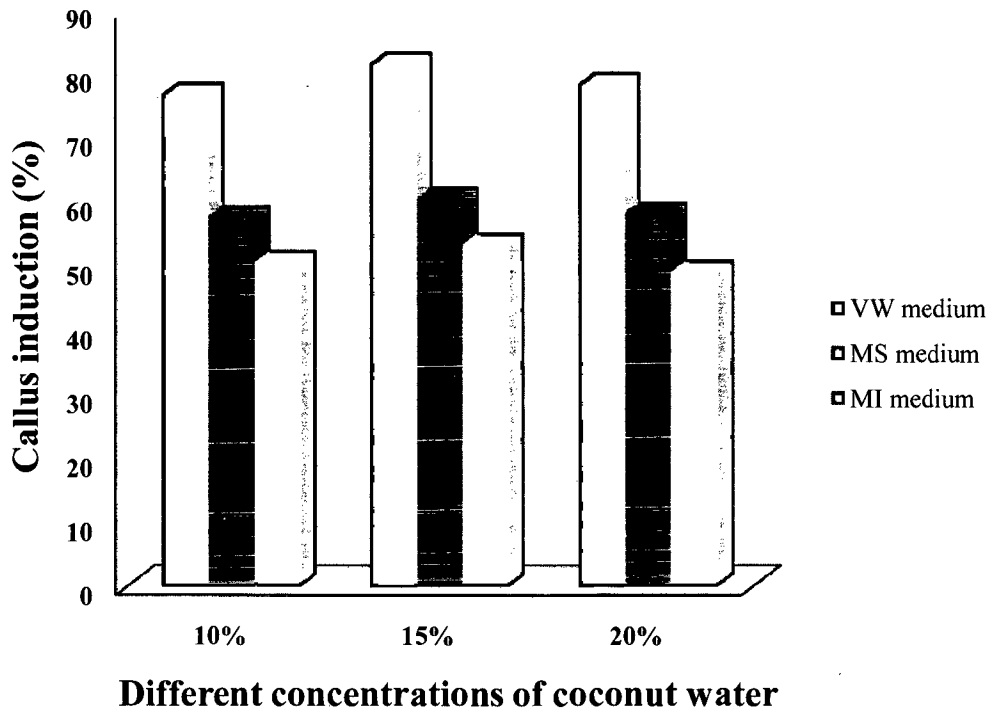
experiment was scored after one month of culture. During the study, three different media namely Murashige and Skoog (1962) (MS), Vacin and Went (1949) (VW) and Mitra *et al.* (1976) (MI) were tried. Each medium was supplemented with three different auxins (IAA, NAA, 2,4,-D) at 0.5 mg l^{-1} and different concentrations of coconut water (10, 15 and 20%). Altogether twenty one different combinations were tried for callus induction.

Among the different combinations tried no callus induction was observed in case of the basal medium (Table 13, Medium I, VIII and XV). Application of auxins i.e 2,4-D, NAA and IAA to all three media had no effect on the induction of callus. However, root formation was noticed in these treatments (Table 13, Medium II, III, IX, X, XI, XVI and XVII). Among the auxins tested, application of IAA had no effect on the induction of callus (Table 13, Medium IV and XVIII). On contrary to auxin, callus induction was observed in all three media supplemented with coconut water (Plate 1b-d). The callus was friable, opaque and creamish to light yellow in colour. The calli varied in size and were classified into small, medium and large (Table 13).

The callus formation was noted only in the media containing coconut water. When compared to MS and MI media higher response of callus induction (77-81.8%) was observed in case of VW media. It varied from 57.8-60.6% in case of MS medium and was still lower (49.4-53.6%) in MI medium (Fig. 3).

Vacin and Went medium supplemented with 10% coconut water (CW), the percentage of callus induction was 77%. When the coconut water concentration was increased to 15% the percentage of callus induction increased to 81.8%. Further increase in the concentration of coconut water to 20%, further enhancement of callus was not observed. However, increase in the concentration exhibited an inhibitory effect on callus

Fig. 3. Response of coconut water on callus induction from immature seeds of *Aerides maculosa.*



induction (78.6%). Similar trend was also observed in case of MS and MI media when supplemented with coconut water. In the present study, VW medium supplemented with 15% CW (Table 13, Medium VI) was found to be the most effective (Plate 1b-d).

The time taken for callus initiation varied amongst the three media tested. In case of VW medium, callus initiation was observed after 27 to 32 days of inoculation, while in MS medium appeared after 33 to 37 days. In MI medium callus formation was initiated after 38 to 41 days. Earliest callus initiation was noticed in VW medium supplemented with 15% coconut water after 27 days of inoculation. However, in MS and MI media the number of days taken for callus initiation was more in presence of same concentration of coconut water (Table 13).

4. 3. 1. 3. Regeneration

To evaluate the plant regeneration ability a piece of approx. 0.01 g (fresh weight) callus was cultured on VW medium supplemented with different growth regulators (Table 14). Fifteen replicates of 0.01 g callus per tube were used in each treatment. After two months of culture, the number of protocorm-like bodies (PLBs) was counted. Initially the totipotent calli turn green and continued increasing size. The callus further turned embryogenic and differentiated into PLBs that were swollen at the base and pointed at the tip. The callus exhibited high regenerative potential in the form of PLBs formation (Plate 2a-d).

Induced callus was sub-cultured on VW basal medium supplemented with various concentrations and combinations of cytokinins and auxins. Sub cultured callus displayed varied differentiation responses on VW medium (Table 14).

Table 14. Influence of PGRs on development of protocorm-like bodies from immature seed callus of *Aerides maculosa* on Vacin and Went medium supplemented with 15% coconut water.

Medium	Treatments	Development of PLBs		Development of shoot buds and plantlets from PLBs
		No. of PLBs from each callus *	Type of response	
I	VW	No response		No response
II	BA 5 + NAA 5 mg l^{-1}	No response	-	No response
III	BA 5 + NAA 2 mg l^{-1}	No response	-	No response
IV	BA 2 + NAA 2 mg l^{-1}	4.7 ± 0.36	Greenish PLBs formed	No further growth
V	BA 2 + NAA 1 mg l^{-1}	5.33 ± 0.96	Green PLBs formed	Development of shoot buds from PLBs & plantlets formation (approximately 2-3 cm)
VI	BA 1 + NAA 1 mg l^{-1}	13.93 ± 0.64	Healthy and green PLBs formed	Development of shoot buds from PLBs & plantlets formation (approx. 2-3 cm)
VII	BA 5 + KIN 5 mg l^{-1}	No response	-	-
VIII	BA 5 + KIN 2 mg l^{-1}	5.17 ± 0.31	Green PLBs formed	No further growth
IX	BA 2 + KIN 2 mg l^{-1}	9.3 ± 2.72	Green PLBs formed	No further growth
X	BA 2 + KIN 1 mg l^{-1}	10.77 ± 0.92	Green PLBs formed	No further growth
XI	BA 1 + KIN 1 mg l^{-1}	11.17 ± 1.32	Few green PLBs formed	No further growth

* Mean number of PLBs per 0.01 g fresh weight of callus.
Data showing the mean of 15 replicates ± standard error (SE).

PGRs: Plant growth regulators, VW: Vacin and Went medium (1949), BA: 6-benzylamino purine: NAA: 1- naphthaleneacetic acid, KIN: kinetin.

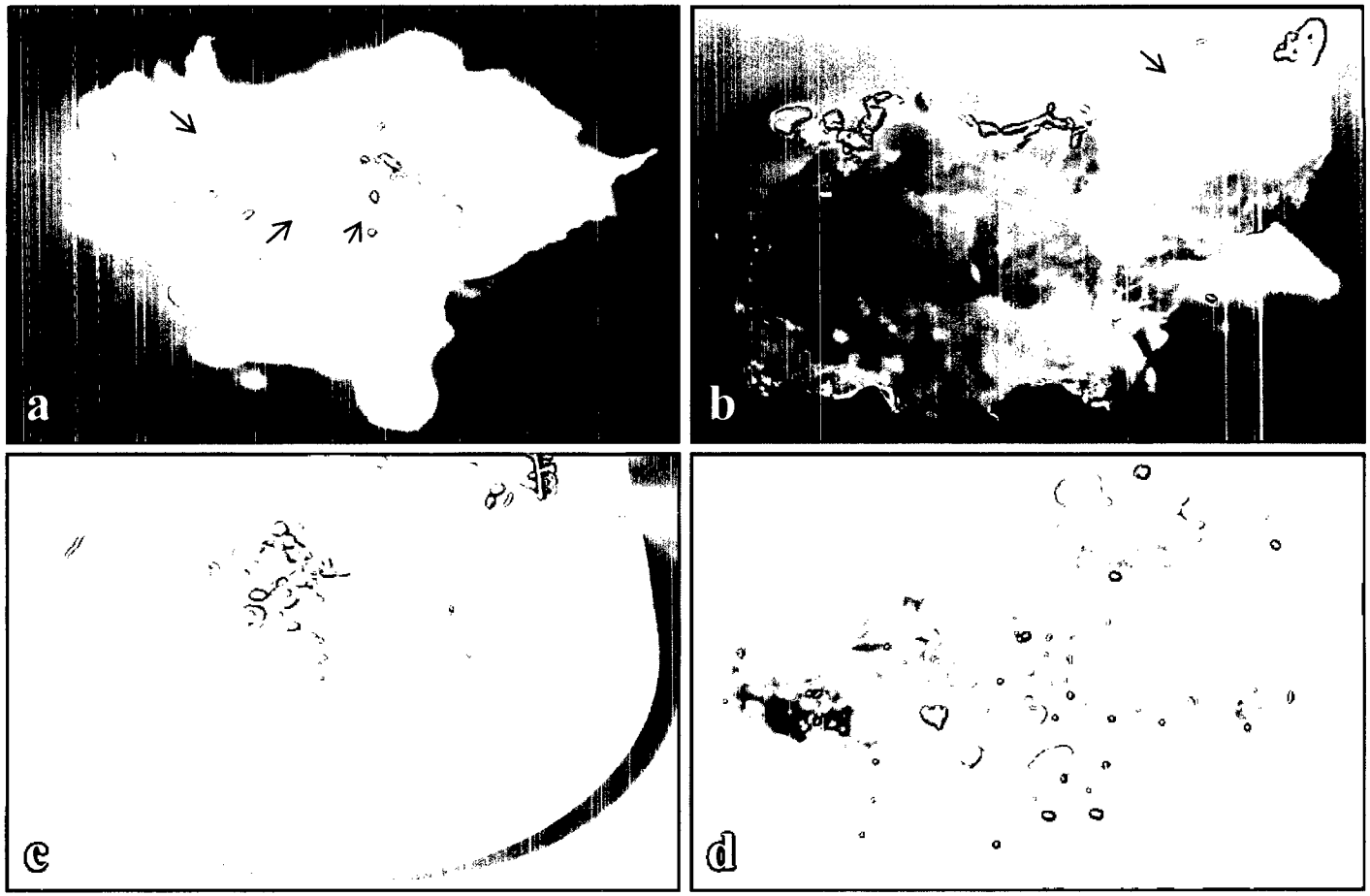


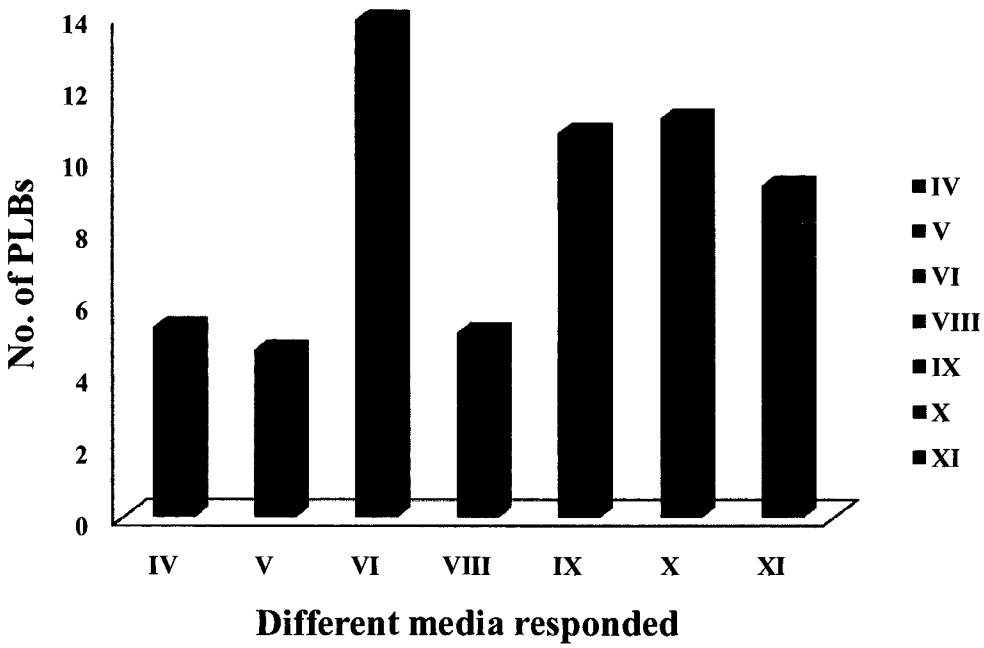
Plate 2. Development of PLBs from callus in *Aerides maculosa* on VW medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) and 15% coconut water. a. Callus mass showing PLBs in early stage of development. b. Elongation of PLBs. Note the swollen base and pointed tip of PLB. c. Clusters of PLBs formed from the callus. d. Closer view of PLB formation from callus.

No response of PLB formation was noted in presence of higher concentration of BA (5 mg l^{-1}) along with NAA (2-5 mg l^{-1}). With the combination of higher concentration of cyokinins (BA and KIN) 5 mg l^{-1} each, have also not showed any response. Among the different media tried, PLB formation was observed only in the case of medium IV, V, VI, VIII, IX, X and XI (Table 14).

The medium containing combinations of cytokinins and combinations of auxins and cytokinins showed varied responses in PLB formation (Fig. 4). Cytokinin and auxin combination was better in term of formation of PLBs and formation of plantlet. The formation of average number of PLBs per callus in BA and NAA combination ranged from (4.7 to 13.93). Maximum number of PLBs (average 13.93) per callus was noted on VW medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW (Plate 3c, d). PLB formation was low (average 5.33/callus) in presence of BA (2 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW (Table 14, Medium V). While further reduction in number of PLB formation (average 4.7/callus) was noted in presence of BA (2 mg l^{-1}) + NAA (2 mg l^{-1}) + 15% CW.

The callus was sub-cultured on the medium containing combination of BA and KIN produced PLBs ranging in number from (5.17 to11.17) per callus. Trend of reduction in PLB formation was also observed similar to that of BA and NAA combination. In this case maximum no. of PLBs per callus (11.17) was observed on medium supplemented with BA (1 mg l^{-1}) + KIN (1 mg l^{-1}) + 15% CW (Plate 3a-f). No significant reduction in PLB formation was noticed in presence of BA (2 mg l^{-1}) + KIN (1 mg l^{-1}) + 15% CW. However, in case of higher concentration of BA (5 mg l^{-1}) + KIN (2 mg l^{-1}) + 15% CW reduction in the formation of PLBs (average 5.17/callus) was noted.

Fig. 4. Response of cytokinin combinations and auxin and cytokinin combinations on PLB formation in *Aerides maculosa* on Vacin and Went medium supplemented with 15% coconut water.



IV. BA 2 (mg l^{-1}) + NAA 2 (mg l^{-1}); V. BA 2 (mg l^{-1}) + NAA 1 (mg l^{-1}); VI. BA 1 (mg l^{-1}) + NAA 1 (mg l^{-1}); VIII. BA 5 (mg l^{-1}) + KIN 2 (mg l^{-1}); IX. BA 2 (mg l^{-1}) + KIN 2 (mg l^{-1}); X. BA 2 (mg l^{-1}) + KIN 1 (mg l^{-1}); XI. BA 1 (mg l^{-1}) + KIN 1 (mg l^{-1}).

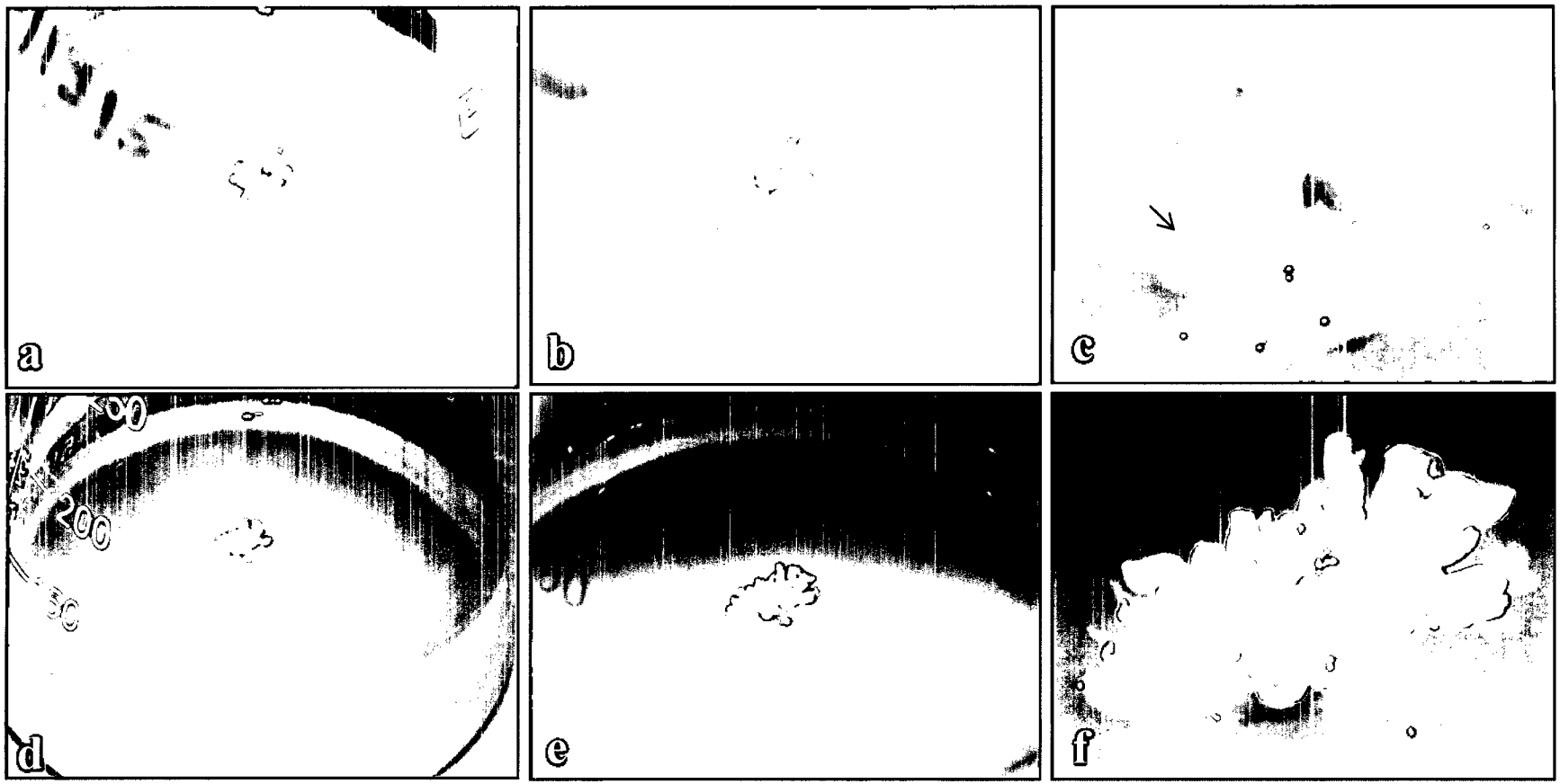


Plate 3. Different stages of development of PLB formation in *Aerides maculosa* on VW medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) and 15% coconut water. a, b, d, e. Broader view of PLBs. c and f. Closer view of PLBs. Note the globular PLBs (c) and development of leaf from PLBs (f).

Among the different media tried, VW basal medium with BA (1 mg l^{-1}) + NAA (1mg l^{-1}) + 15% CW and VW basal medium with BA (1 mg l^{-1}) + KIN (1 mg l^{-1}) + 15% CW was more suitable for PLB formation from the callus. The PLB formation was maximum on these media.

Produced PLBs were sub-cultured on the same medium for further growth. It was observed that in the case of VW medium supplemented with BA and NAA combinations, conversion of PLBs into well developed plantlets readily occurred on the same medium after 8 to 10 weeks. PLBs produced on VW basal medium with BA (1 mg l^{-1}) + NAA (1mg l^{-1}) + 15% CW produced healthy plantlet on the same medium after 8 to 10 weeks (Plate 4a-f). This combination (Table 14, Medium VI) was most suitable for plant regeneration from the callus. However, PLBs formed on VW basal medium with the different combinations of BA and KIN became brown and necrotic upon sub-culturing (Table 14, Medium VIII to XI).

4. 3. 1. 4. Acclimatization

The actively growing plants, with 3-4 leaves and 1 or 2 roots, were removed from the flask and transplanted in the greenhouse. In each treatment 50 plants were transplanted. The plants were gradually acclimatized to greenhouse conditions. The established plants produced new roots and leaves within 3 months period. The plants were exposed to 25% sunlight. The rate of survival of plants were calculated and provided in the Table 15 and Fig. 5. Survival rate of the plants was classified based on the presence of actively growing leaves. Percentage of survival among different treatments is depicted in Fig. 5. Survival rate of transferred plant in the greenhouse ranged from 8 to 60%. Maximum survival rate was observed in the combinations of charcoal pieces, brick pieces (1:1) and coconut husk (60%) (Table 15; Plate 5a-d). The charcoal pieces and coconut husk mixture showed 26%

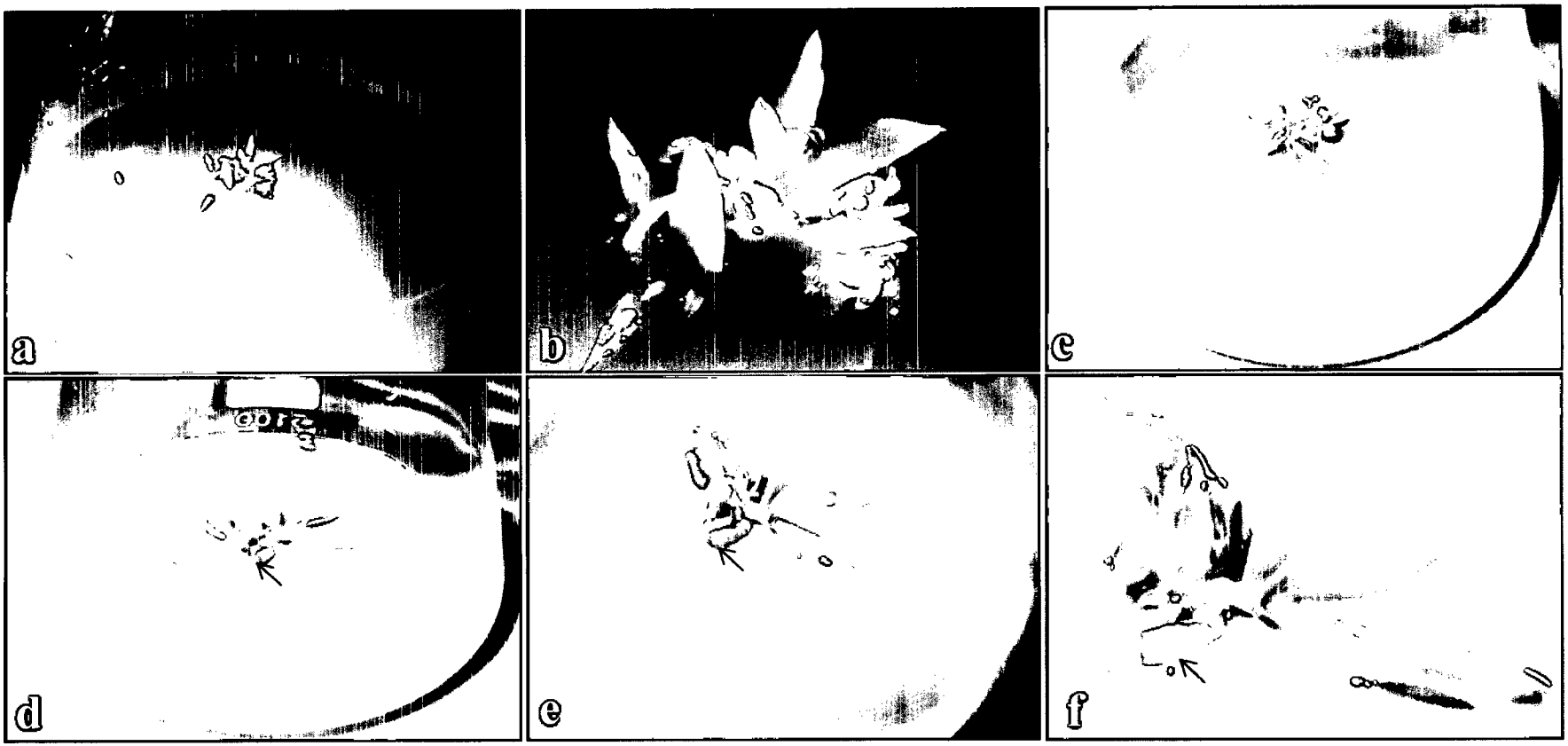
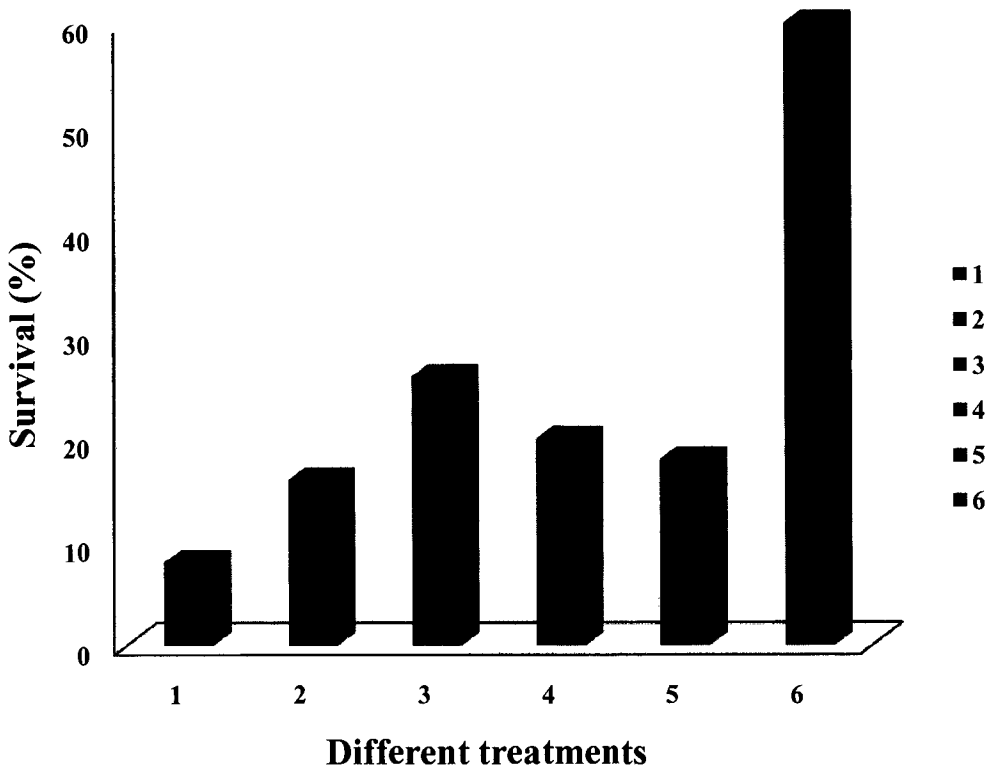


Plate 4. Development of plantlets from PLBs of *Aerides maculosa* on VW medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) and 15% coconut water. a, c, d and e. Broader view showing conversion of PLBs into plantlets. b. Closer view showing conversion of PLBs into plantlets. f. Closer view of plantlet showing prominent root development.

Table 15. Rate of survival of micropropagated plantlets of *Aerides maculosa* and *Rhynchostylis retusa* in green house after 3 months.

Treatments	Composition of potting mixture used for acclimatization	No. of plantlets Transfer-red	*Aerides maculosa*		*Rhynchostylis retusa*	
			No. of plants survived	Survival %	No. of plants survived	Survival %
1	Charcoal pieces (approx 2 cm)	50	4	8	5	10
2	Brick pieces (approx 2 cm)	50	8	16	9	18
3	Charcoal pieces + coconut husk	50	13	26	15	30
4	Brick pieces + coconut husk	50	10	20	11	22
5	Charcoal pieces + Brick pieces (1:1)	50	9	18	12	24
6	Charcoal pieces + Brick pieces (1:1) + coconut husk	50	30	60	30	60

Fig. 5. Percentage survival of plantlets of *Aerides maculosa*.



1. Charcoal pieces (approx 2 cm); 2. Brick pieces (approx. 2 cm); 3. Charcoal pieces + coconut husk; 4. Brick pieces + coconut husk; 5. Charcoal pieces + Brick pieces (1:1); 6. Charcoal pieces + Brick pieces (1:1) + coconut husk.

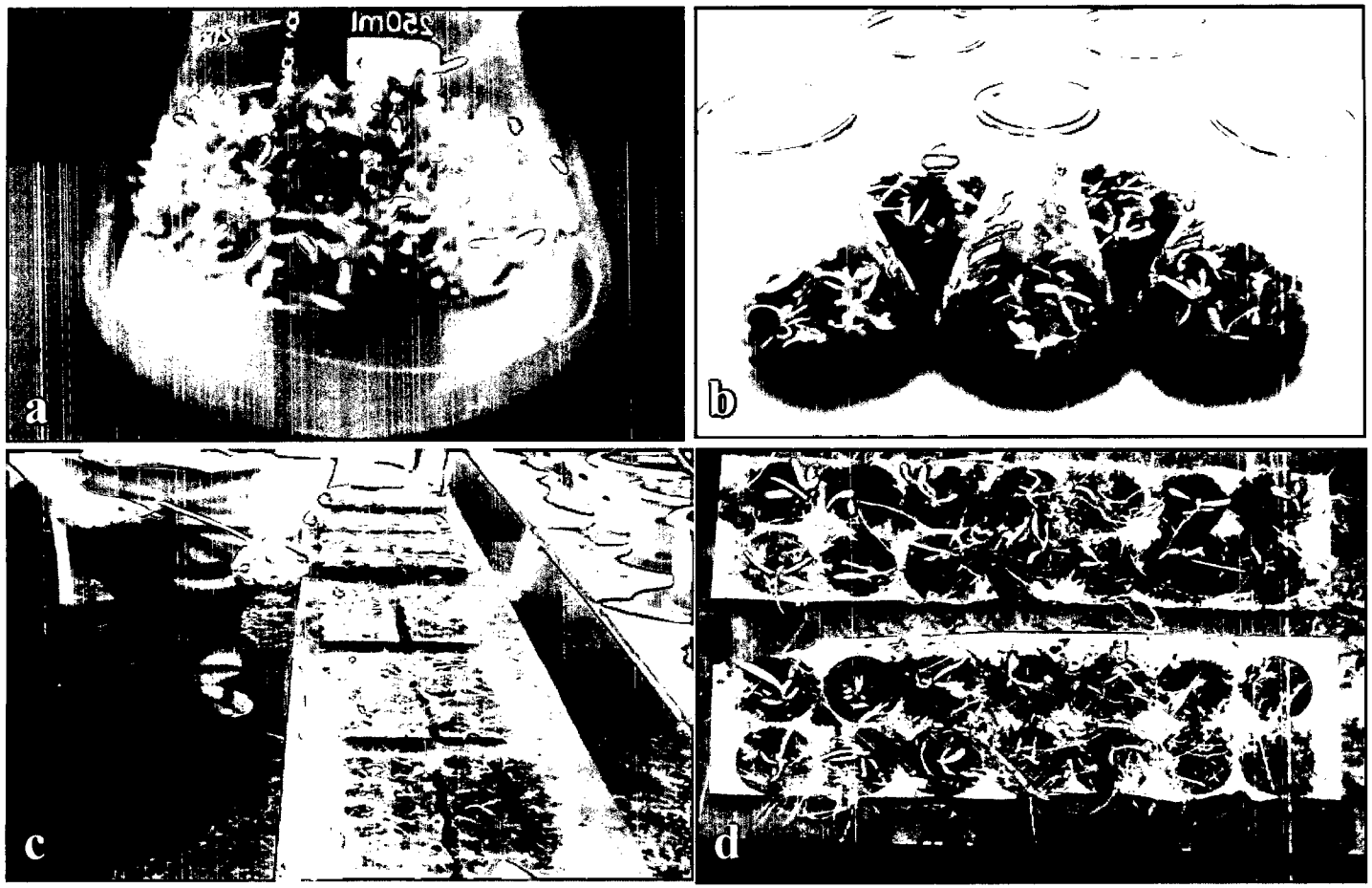


Plate 5. Acclimatization process of micropropagated plantlets of *Aerides maculosa.* a. Well rooted plantlets. b. Well rooted plantlets planted on charcoal pieces for acclimatization. c. Well rooted plantlets transferred into green house in portray. d. Closer view of transplanted plantlets in portray.

of survival, while lowest rate of survival was noted in case of charcoal pieces alone (8%). During summer season (January-May) comparatively lesser growth was observed. However, plant resumed copious growth during the onset of the monsoon.

4. 3. 2. *Rhynchostylis retusa*

4. 3. 2. 1. Explants

The immature capsules were collected from the nursery grown plants 10-20 weeks after pollination (WAP) (Plate 6a). Yellowish green seed were separated from the capsule and placed on different media for callus induction (Table 16).

4. 3. 2. 2. Induction of callus

For callus induction, immature seeds were used. Basal medium supplemented with different concentrations and combinations of growth regulators and growth adjunct were tried. Responses of immature seeds to various growth regulators and growth adjunct used are given in the Table 16. There were 10 replications per treatment. Percentage of callus induction was analysed after one month of culture. To study the effect of growth regulators and growth adjunct on callus induction, immature seeds were cultured on three different media namely, Murashige and Skoog (1962) (MS), Vacin and Went (1949) (VW), Mitra *et al.* (1976) (MI). Three different auxins such as IAA, NAA, 2,4-D at 0.5 mg l^{-1} and three concentration of coconut water (10, 15 and 20%) were supplemented to the above three basal media. Total twenty one different combinations were tried for callus induction.

In *Rhynchostylis retusa,* auxins did not respond for callus induction. In all three basal media (2, 4-D and NAA) resulted in the root formation (Table 16, Medium II, III, IX, X, XVI and XVII) while, in case of IAA, no response was observed (Table 16, IV, XI and XVIII). However, callus induction was noticed in all the concentrations of coconut water

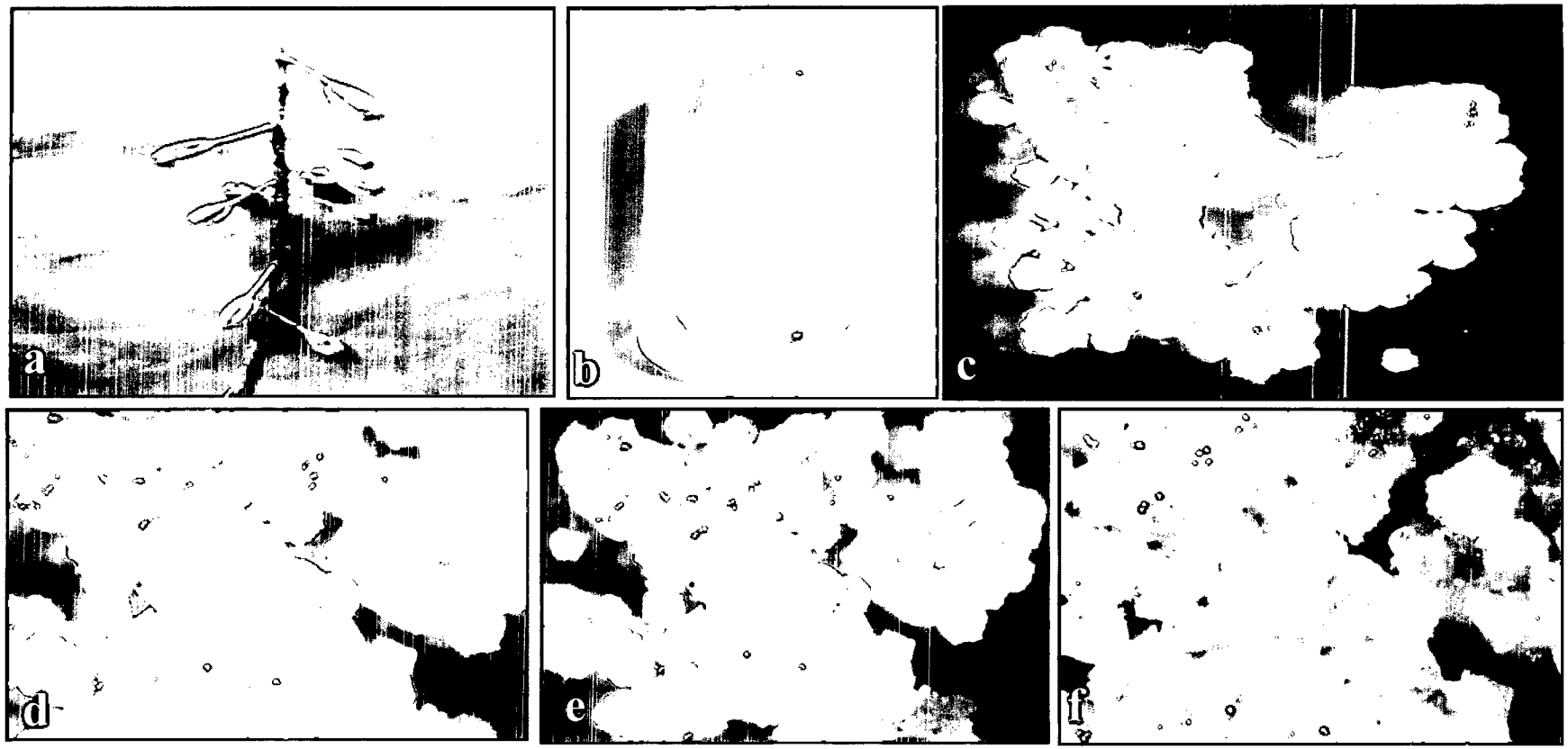


Plate 6. Callus formation from immature seeds of *Rhynchostylis retusa* cultured on VW medium supplemented with 15% coconut water. *a*. Immature capsules after 10 weeks after pollination. b. Callus developed from immature seeds. c-f. Closer view of callus.

Table 16. Effect of PGRs and growth adjunct on callus induction of immature seeds of *Rhynchostylis retusa*.

Medium	Media supplemented with/without growth regulators and growth adjunct	Responses of immature seeds in culture		
		Morphogenesis	Callus induction %	No. of days taken
I	VW	No response	0	-
II	VW + 2,4-D 0.5 mg l^{-1}	root formation	0	-
III	VW + NAA 0.5 mg l^{-1}	root formation	0	-
IV	VW + IAA 0.5 mg l^{-1}	No response	0	-
V	VW + CW 10%	Moderate quantity of calli (++)	72 ± 0.84	35
VI	VW + CW 15%	Large quantity of calli (+++)	80 ± 1.00	30
VII	VW + CW 20%	Small quantity of calli (+)	74 ± 0.55	32
VIII	MS	No response	0	-
IX	MS + 2,4-D 0.5 mg l^{-1}	root formation	0	-
X	MS + NAA 0.5 mg l^{-1}	root formation	0	-
XI	MS + IAA 0.5 mg l-1	No response	0	-
XII	MS + CW 10%	Moderate quantity of calli (++)	59 ± 0.81	37
XIII	MS + CW 15%	Small quantity of calli (+)	61.4 ± 0.43	34
XIV	MS + CW 20%	Moderate quantity of calli (++)	60.6 ± 1.32	35
XV	MI	No response	0	-
XVI	MI + 2,4-D 0.5 mg l^{-1}	root formation	0	-
XVII	MI + NAA 0.5 mg l^{-1}	root formation	0	-
XVIII	MI + IAA 0.5 mg l^{-1}	No response	0	-
XIX	MI + CW 10%	Small quantity of calli (+)	49.6 ± 0.59	39
XX	MI + CW 15%	Small quantity of calli (+)	53.4 ± 0.38	36
XXI	MI + CW 20%	Small quantity of calli (+)	50 ± 0.98	34

Data showing the mean of 10 replicates ± standard error (SE).

PGRs: Plant growth regulators, VW: Vacin and Went medium (1949), 2, 4-D: 2, 4-dichlorophenoxyacetic acid, NAA: 1-naphthaleneacetic acid, IAA: indole-3-acetic acid, CW: coconut water, MS: Murashige and Skoog medium (1962), MI: Mitra *et al.*, medium (1976).

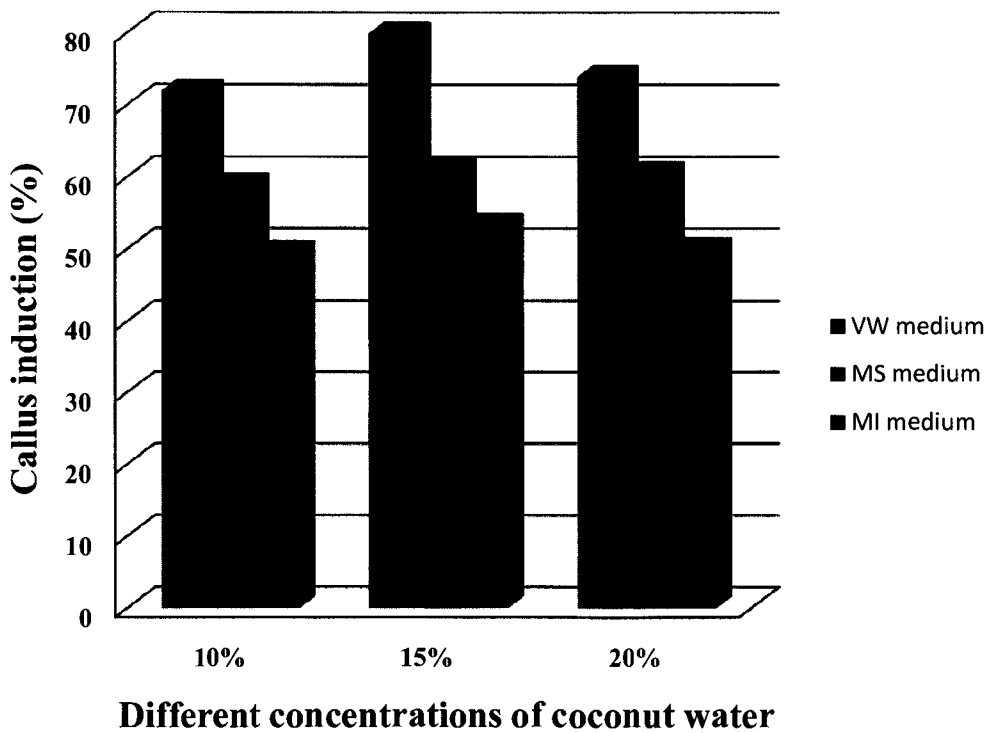
(10 to 20%) (Plate 6b-f) irrespective of basal medium used for the study. The callus was friable, opaque and creamish to light yellow in colour. The calli varied in size and were classified as small, medium and large (Table 16).

Basal media containing coconut water was most effective for callus induction. Among the basal media tried, VW medium was more effective than MS and MI media. 72-80% callus induction was noted for VW medium while, considerably lower percentage of callus induction was observed in the case of MS (59-61.4%) and MI (49.6-53.4%) medium (Fig. 6).

In case of VW medium supplemented with 10% coconut water 72% induction of callus was observed. It increased to maximum 80% with the increase in the concentration of coconut water to 15%. No increase in the percentage was observed in presence of increased coconut water concentration (20%). On the contrary, reduction in the callus induction was noticed (74%). Similar pattern of initial increase in callus induction upto 15% of coconut water and further decrease in the induction in presence of 20% of coconut water in the other two basal media (MS and MI) was observed. Among the different media tested, VW medium supplemented with 15% CW (Table 16, Medium VI) was found to be most effective for callus induction (Plate 6c-f).

Time taken for initiation of callus varied among the three basal media tested in the study. Early callus initiation was noticed in VW media i.e. 30 to 35 days after inoculation. In comparison to VW medium, slightly more time (34-37 days) was required for callus initiation in MS medium and MI medium (34-39 days). When compared to all other concentrations, 15% coconut water was found to be very effective in early initiation of callus in VW medium (30 days).

Fig. 6. Response of coconut water on callus induction from immature seeds of *Rhynchostylis retusa*.



4. 3. 2. 3. Regeneration

Calli of approx. 0.01 g (fresh weight) were cultured on MS medium supplemented with various combinations of growth regulators to test its regeneration ability (Table 17). Each treatment consisted of fifteen replicates. Number of Protocorm-like bodies (PLBs) were counted after two months of culture. Calli initially turned green and continued increasing in size (Plate 7a). Later, the callus differentiated into PLBs that were swollen at the base and pointed at the tip (Plate 7b-d).

MS medium with various concentrations and combinations of cytokinins and combinations of auxins and cytokinins were tried for regeneration ability from callus (Table 17; Fig. 7). In case of high concentrations of cytokinin and auxin combinations BA (5 mg l^{-1}) + NAA (5 mg l^{-1}); BA (5 mg l^{-1}) + NAA (2 mg l^{-1}), no response on PLB formations were observed. However, PLB formation was noticed in all the combinations of cytokinins. Among the different combinations tried, PLB formation was observed in the case of medium IV, V, VI, VII, VIII, IX, X and XI (Table 17).

PLB formation was observed in low concentrations of cytokinin and auxin combinations and also among cytokinin combinations. Low concentrations of cytokinin and auxin combinations were found to be better in terms of PLBs and plantlets formation. Average number of PLBs per callus in BA and NAA combination ranged from 5.33 to 15.83. Maximum number of PLBs (15.83 per callus) were noted in MS medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW. Number of PLB formation was low (10.4/callus) in presence of BA (2 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW while, least number of PLBs of 5.33 per callus were observed in presence of BA (2 mg l^{-1}) + NAA (2 mg l^{-1}) + 15% CW.

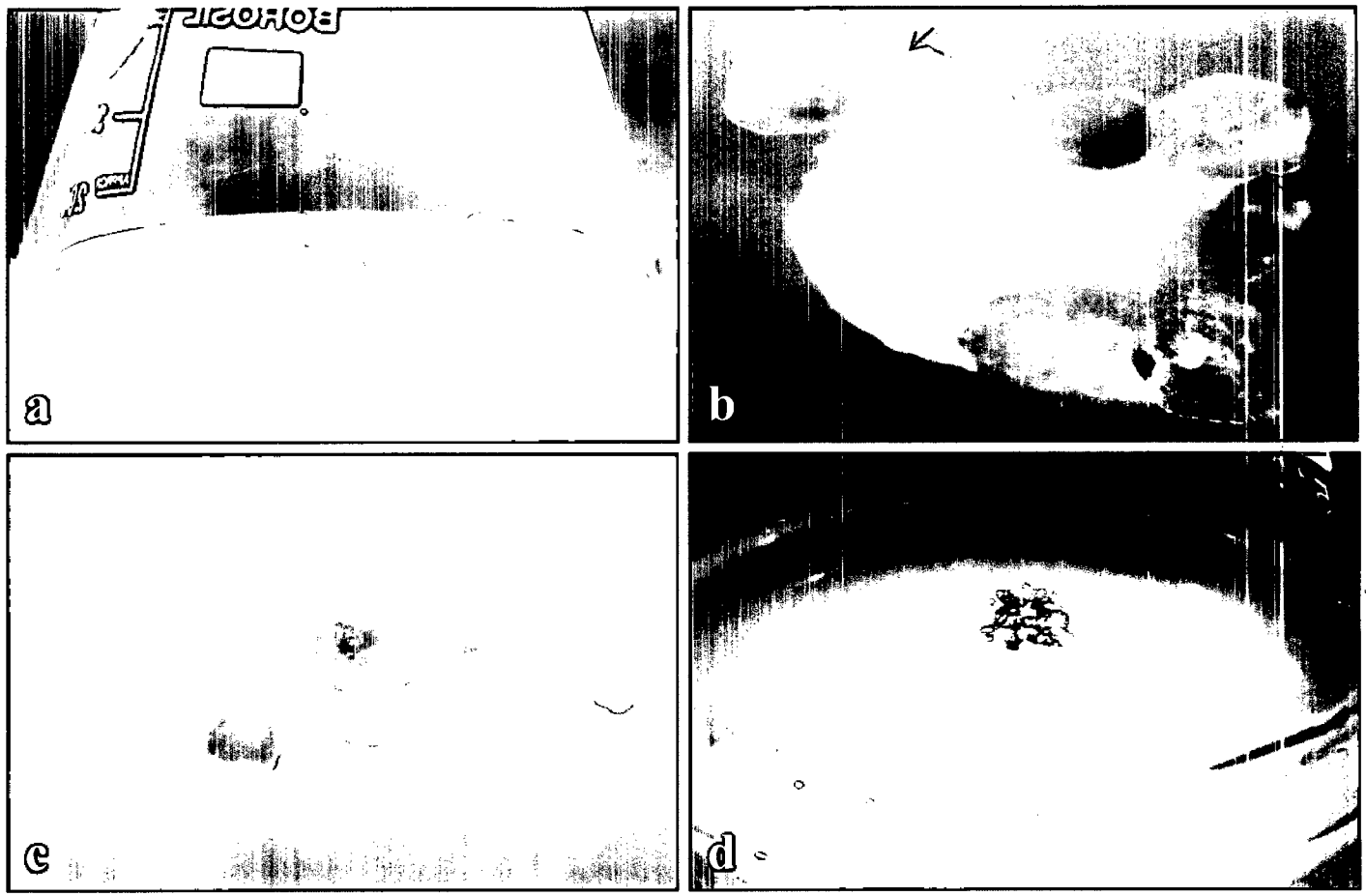


Plate 7. Development of PLBs from the callus of *Rhynchostylis retusa* on MS medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) and 15% coconut water. a. Broader view of PLB formation from the callus. b. Closer view early stage of PLB formation from the callus. c. Closer view of PLB formation. d. Conversion of PLBs into plantlets at early stage of development.

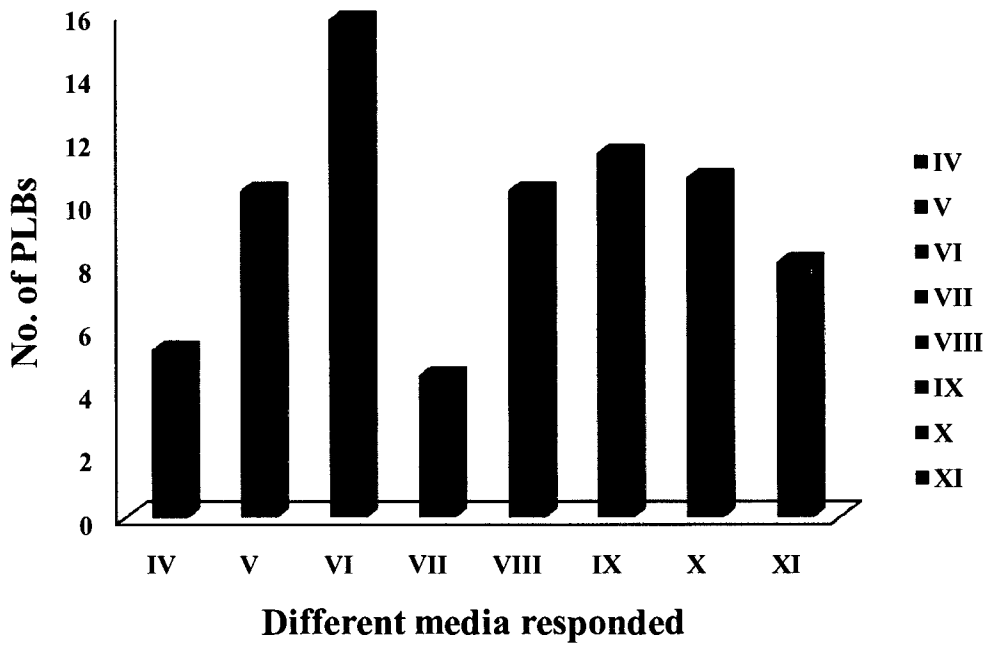
Table 17. Influence of growth regulators on development of protocorm-like bodies from immature seed callus of *Rhynchostylis retusa* on MS medium supplemented with 15% coconut water.

Medium	Treatments	Development of PLBs		Development of shoot buds and plantlets from PLBs
		No. of PLBs from each callus *	Type of response	
I	MS	No response		No response
II	BA 5 + NAA 5 mg l^{-1}	No response	-	No response
III	BA 5 + NAA 2 mg l^{-1}	No response	-	No response
IV	BA 2 + NAA 2 mg l^{-1}	5.33 ± 0.96	Greenish PLBs formed	No further growth
V	BA 2 + NAA 1 mg l^{-1}	10.4 ± 1.08	Green PLBs formed	Development of shoot buds from PLBs & plantlets formation (approx. 2-3 cm)
VI	BA 1 + NAA 1 mg l^{-1}	15.83 ± 0.81	Healthy and green PLBs formed	Development of shoot buds from PLBs & plantlets formation (approx. 2-3cm)
VII	BA 5 + KIN 5 mg l^{-1}	4.53 ± 0.65	Few green PLBs formed	No further growth
VIII	BA 5 + KIN 2 mg l^{-1}	10.4 ± 079	Green PLBs formed	No further growth
IX	BA 2 + KIN 2 mg l^{-1}	11.6 ± 0.46	Green PLBs formed	No further growth
X	BA 2 + KIN 1 mg l^{-1}	10.8 ± 1.32	Green PLBs formed	No further growth
XI	BA 1 + KIN 1 mg l^{-1}	8.13 ± 1.66	Few green PLBs formed	No further growth

* Mean number of PLBs per 0.01 g fresh weight of callus.
Data showing the mean of 15 replicates ± standard error (SE).

PGRs: Plant growth regulators, MS: Murashige and Skoog medium (1962), BA: 6-benzylamino purine: NAA: 1- naphthaleneacetic acid, KIN: kinetin.

Fig. 7. Response of cytokinin combinations and auxin and cytokinin combinations on PLB formation in *Rhynchostylis retusa* on MS medium supplemented with 15% coconut water.



IV. BA 2 (mg l^{-1}) + NAA 2 (mg l^{-1}); V. BA 2 (mg l^{-1}) + NAA 1 (mg l^{-1}); VI. BA 1 (mg l^{-1}) + NAA 1 (mg l^{-1}); VII. BA 5 (mg l^{-1}) + KIN 5 (mg l^{-1}); VIII. BA 5 (mg l^{-1}) + KIN 2 (mg l^{-1}); IX. BA 2 (mg l^{-1}) + KIN 2 (mg l^{-1}); X. BA 2 (mg l^{-1}) + KIN 1 (mg l^{-1}); XI. BA 1 (mg l^{-1}) + KIN 1 (mg l^{-1}).

The callus was sub-cultured on the medium containing combinations of BA and KIN produced PLBs ranging in number from (4.53 to11.6) per callus. High concentration of BA (5 mg l^{-1}) + KIN (5 mg l^{-1}) + 15% CW showed least no. of PLBs (4.53/callus). Maximum number of PLBs (11.6 per callus) was observed on medium supplemented with BA (2 mg l^{-1}) + KIN (2 mg l^{-1}) + 15% CW. Further decrease in the concentration of BA (2 mg l^{-1}) + KIN (1 mg l^{-1}) + 15% CW and BA (1 mg l^{-1}) + KIN (1 mg l^{-1}) + 15% CW, resulted in the reduction in the number of PLB formation to 10.8 and 8.13 per callus respectively.

Among the different media tried, MS basal medium with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW and MS basal medium with BA (2 mg l^{-1}) + KIN (2 mg l^{-1}) + 15% CW were most suitable for PLB formation from the callus. Maximum numbers of PLBs were observed in these media. After PLB formations, when they were sub-cultured on the same medium for further growth, it was observed that BA and NAA combinations were found to be better with respect to conversion of PLBs into plantlets. PLBs sub-cultured on MS basal medium with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW produced healthy plantlet after 8 to 10 weeks of culture (Plate 8a-d). However, PLBs formed on MS basal medium with the other different combinations of BA and KIN became brown and necrotic upon further sub-culturing on medium VII to XI (Table 17). Among the different combination tried for the conversion of PLBs into plantlets, VW basal medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW was the most suitable medium.

4. 3. 2. 4. Acclimatization

The percentage of survival of micropropagated plants is provided in Table 15 and Fig. 8. Each treatment consisted of 50 plants. Percentage survival ranged from 10 to 60%. Maximum survival percentage of 60% was recorded in the combinations of charcoal

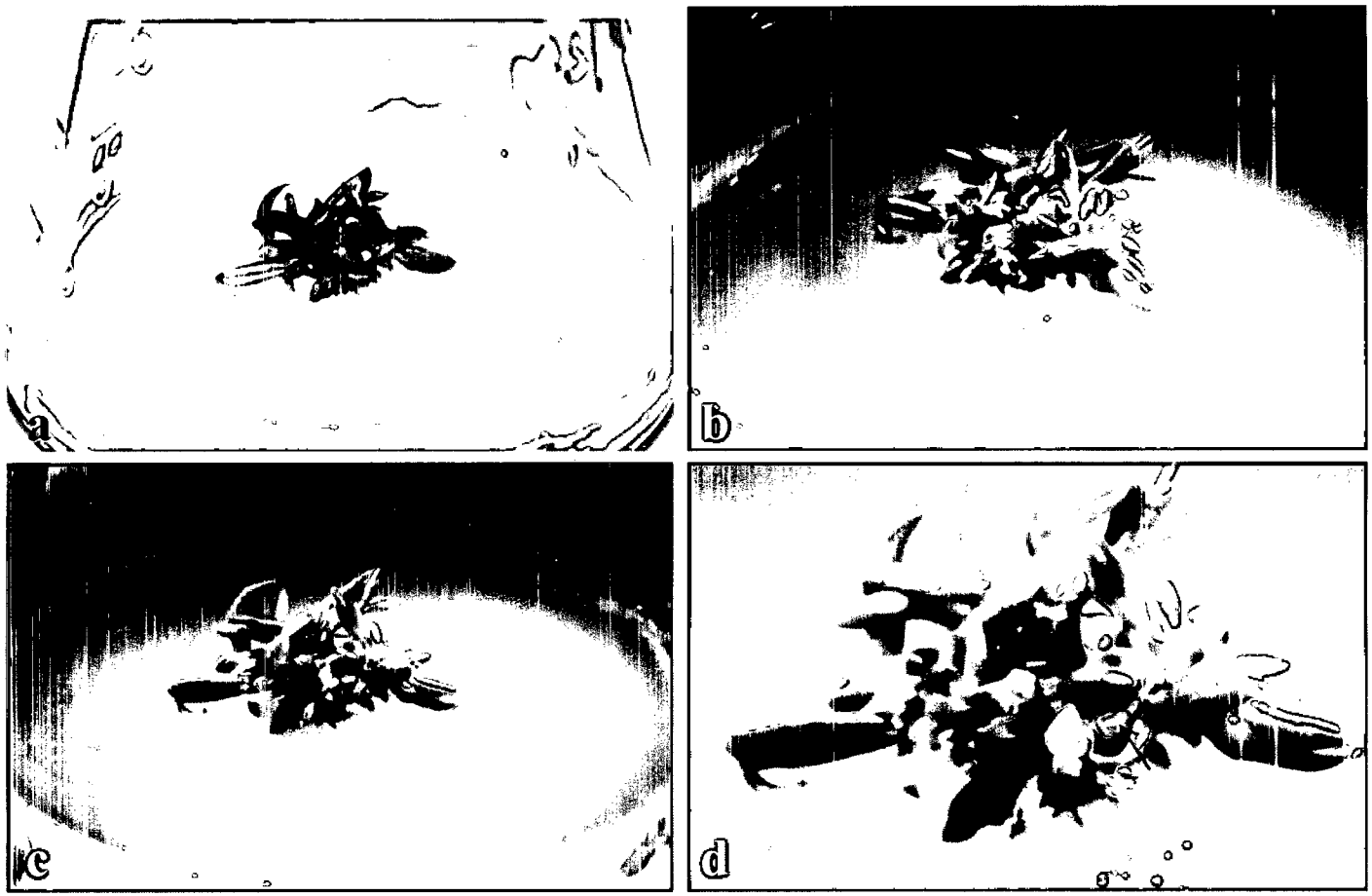
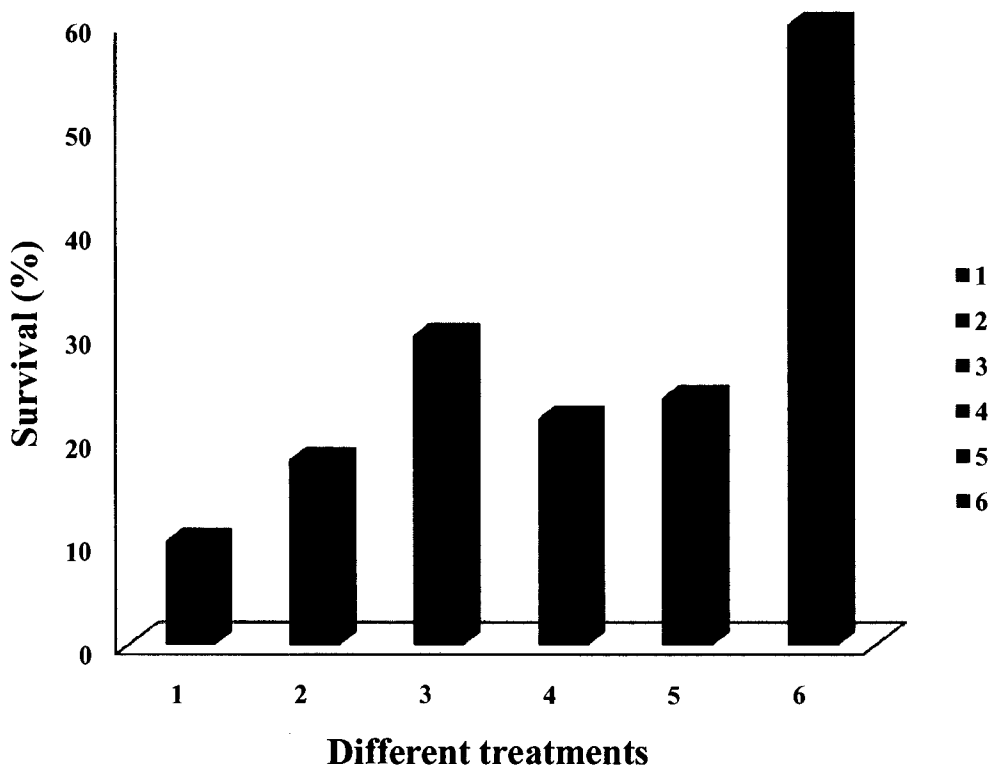


Plate 8. Development of plantlets from the PLBs in *Rhynchostylis retusa* cultured on MS medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) and 15% coconut water. a-c. Broader view showing plantlets developed from PLBs. d. Closer view of plantlets showing well developed roots.

Fig. 8. Percentage survival of plantlets of *Rhynchostylis retusa*.



1. Charcoal pieces (approx 2 cm); 2. Brick pieces (approx 2 cm); 3. Charcoal pieces + coconut husk; 4. Brick pieces + coconut husk; 5. Charcoal pieces + Brick pieces (1:1); 6. Charcoal pieces + Brick pieces (1:1) + coconut husk.

pieces, brick pieces (1:1) and coconut husk. Thirty percent survival was observed in the pot mixture of charcoal pieces and coconut husk. The lowest percentage (10%) survival was noticed when plantlets were grown in charcoal pieces alone. Regenerated plantlets responded well to watering and nutrient solution uptake during acclimatization process in the greenhouse (Plate 9a-d). Graphical representation of percentage survival of the micropropagated plants is given in Fig. 8.

4. 4. Molecular analysis and protein profiling

4. 4. 1. *Aerides maculosa*

4. 4. 1. 1. RAPD band pattern

Twenty RAPD primers obtained from Operon Technologies, USA (Table 18) were tested initially with randomly selected individuals from two populations. Thirteen primers gave consistent (when the profiles with these primers reproduced with at least three different preparation of the genomic DNA) profile. The details of different primer amplification are given in Table 18. Seven primers (OPA 05, OPA 06, OPA 07, OPD 04, OPD 06, OPD 10 and OPD 20) did not amplify the tested DNA. RAPD profiles of the different populations of this species are shown in Plate 10, 11 and 12. Total of 101 RAPD bands were generated, with the average of 7.76 bands per primer (Table 19). Out of which 94 bands with the mean of 7.23 per primer were polymorphic for all the populations. The percentage polymorphism across all the samples varied from 50 to 100% (average 90.45%). Polymorphic banding pattern of 100% was obtained using primers OPA 3, OPA 8, OPA 13, OPD 2, OPD 5, OPD 7 and OPD 8, while, lowest polymorphism was observed in OPA 14 primer (Table 19; Fig. 10). The number of amplified bands ranged from 12 (OPD 2) to 2 (OPA 14) (Fig. 9).

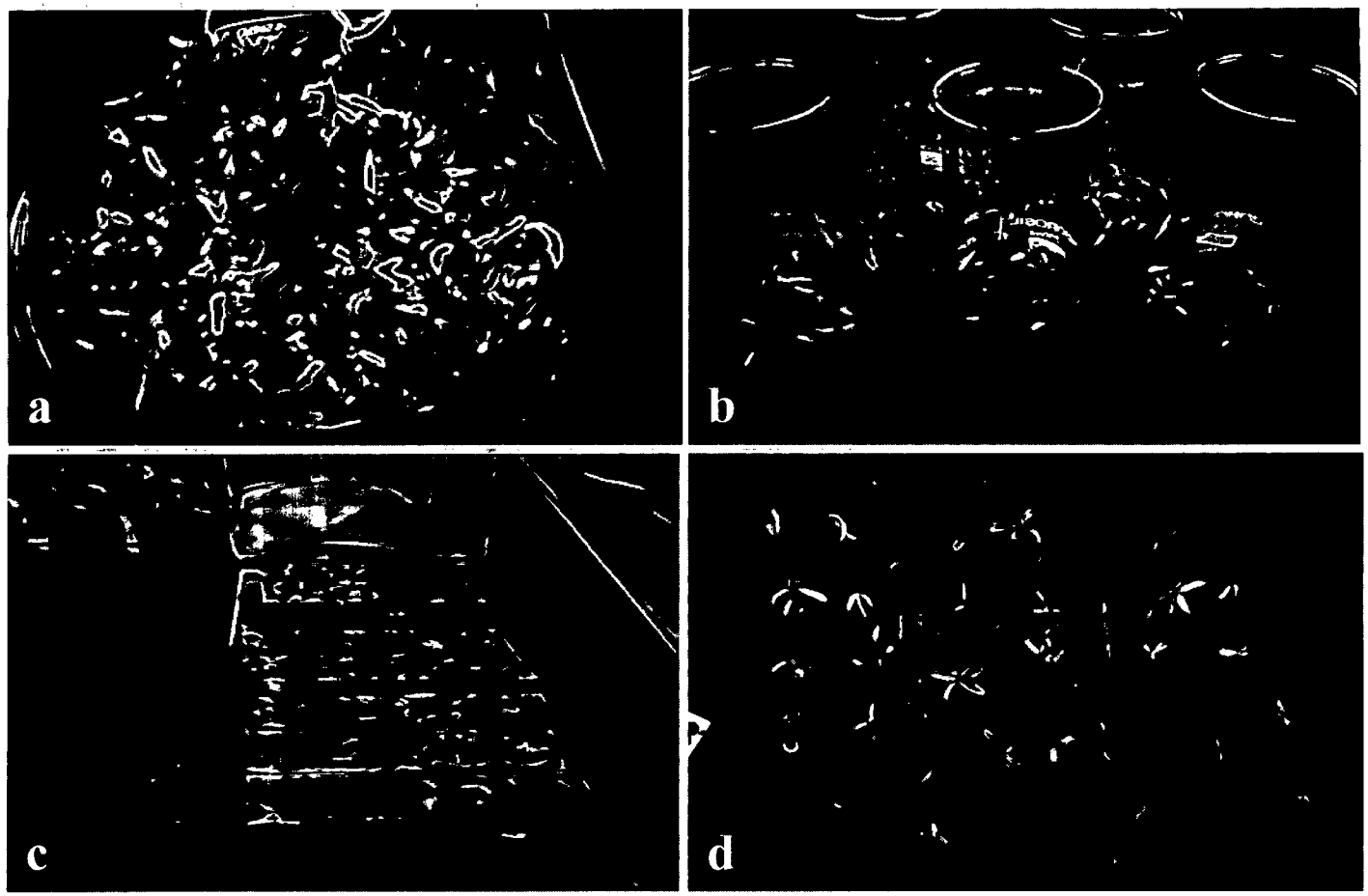


Plate 9. Acclimatization process of micropropagated plantlets of *Rhynchostylis retusa.* a. Well rooted plantlets. b. Well rooted plantlets planted on charcoal pieces for acclimatization in the culture room. c. Well rooted plantlets transferred to greenhouse in portray. d. Closer view of transplanted plantlets in portray.

Table 18. Primers used for RAPD analysis of *Aerides maculosa* with details of amplification.

Sl. No.	Primers	PO	PE	SA	VA	SH	LO	VG 1	VG 2
1	OPA 03	+	+	+	+	+	+	+	+
2	OPA 04	+	+	+	+	+	+	+	+
3	OPA 05	-	-	-	-	-	-	-	-
4	OPA 06	-	-	-	-	-	-	-	-
5	OPA 07	-	-	-	-	-	-	-	-
6	OPA 08	+	+	+	+	+	+	+	+
7	OPA 09	+	+	+	+	+	+	+	+
8	OPA 12	+	+	+	+	+	+	+	+
9	OPA 13	+	+	+	+	+	+	+	+
10	OPA 14	+	+	+	+	+	+	+	+
11	OPD 02	+	+	+	+	+	+	+	+
12	OPD 03	+	+	+	+	+	+	+	+
13	OPD 04	-	-	-	-	-	-	-	-
14	OPD 05	+	+	+	+	+	+	+	+
15	OPD 06	-	-	-	-	-	-	-	-
16	OPD 07	+	+	+	+	+	+	+	+
17	OPD 08	+	+	+	+	+	+	+	+
18	OPD 10	-	-	-	-	-	-	-	-
19	OPD 11	+	+	+	+	+	+	+	+
20	OPD 20	-	-	-	-	-	-	-	-

+ Amplified; - Not amplified

Different Populations: Ponsuli (PO); Pernem (PE); Salauli (SA); Valpoi (VA); Shiroda (SH); Loutulim (LO); Vagueri 1 (VG 1); Vagueri 2 (VG 2).

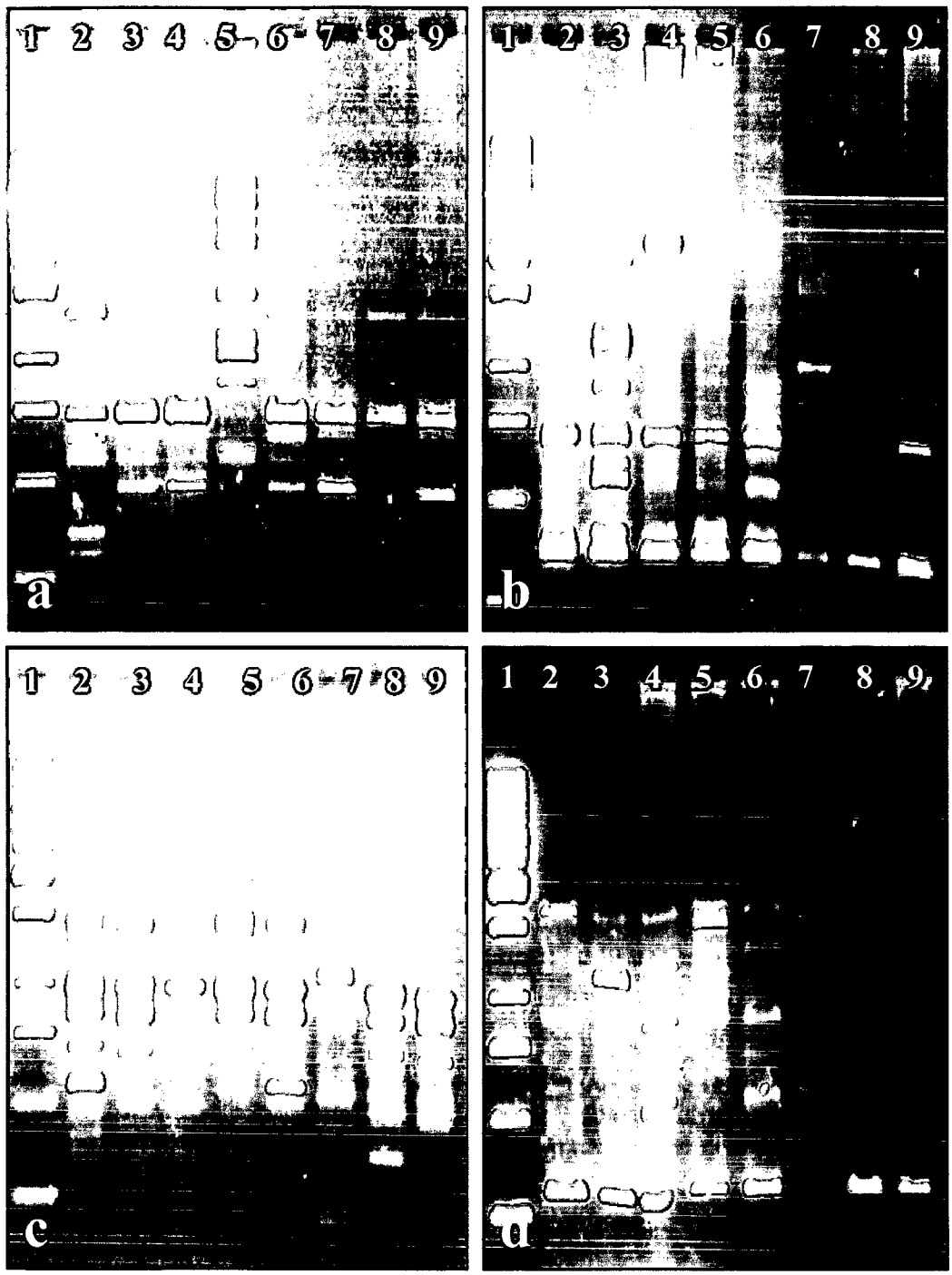


Plate 10. RAPD amplification profile of *Aerides maculosa* with different primers; a. Primer OPA 3; b. Primer OPA 8; c. Primer OPA 9; d. Primer OPA 12; Lane 1. Gene ruler^{TM} 1 kb DNA ladder; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1; 9. Vagueri 2.

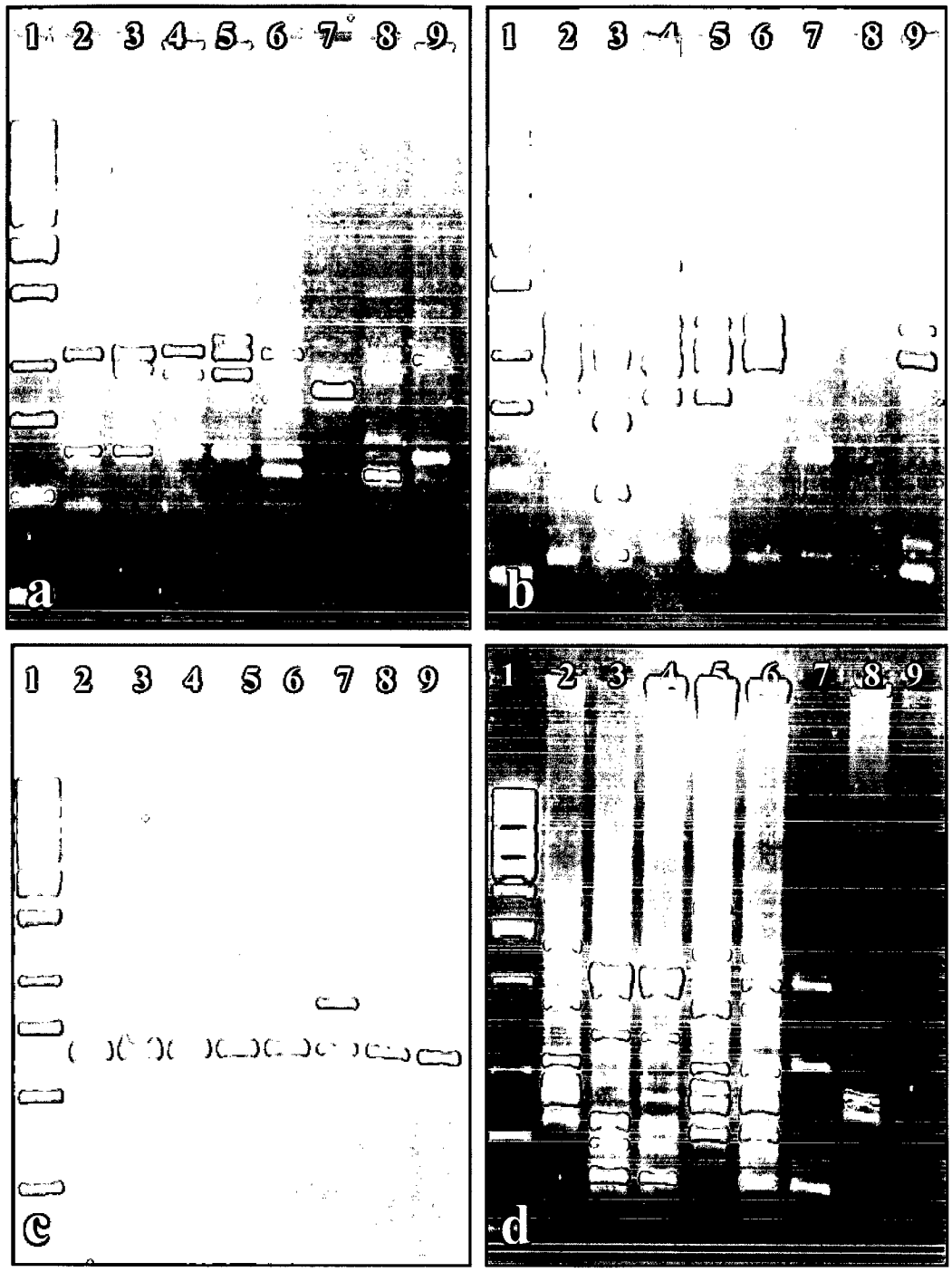


Plate 11. RAPD amplification profile of *Aerides maculosa* with different primers; a. Primer OPA 13; b. Primer OPA 4; c. Primer OPA 14; d. Primer OPD; 2; Lane 1. Gene ruler^{TM} 1 kb DNA ladder; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1; 9. Vagueri 2.

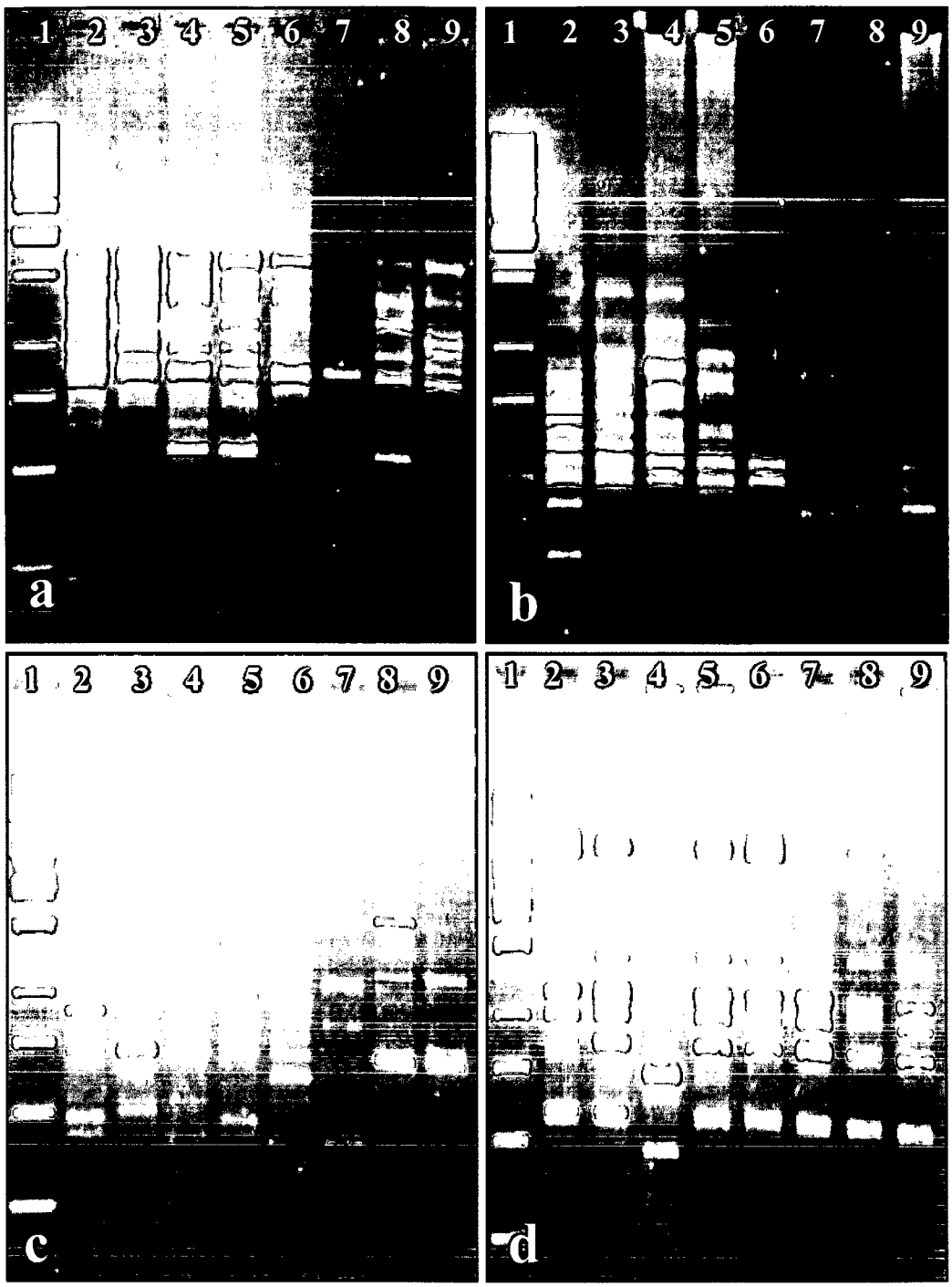


Plate 12. RAPD amplification profile of *Aerides maculosa* with different primers; a. Primer OPD 3; b. Primer OPD 5; c. Primer OPD 7; d. Primer OPD 8, Lane 1. Gene ruler^{TM} 1 kb DNA ladder; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1; 9. Vagueri 2.

Table 19. Amplified, polymorphic bands and percentage of polymorphism in RAPD analysis of *Aerides maculosa*.

Sl. No	Primers	No. of amplified bands	No. of polymorphic bands	Polymorphism %
1	OPA 03	7	7	100
2	OPA 04	8	7	87.5
3	OPA 08	8	8	100
4	OPA 09	9	7	77.0
5	OPA 12	7	6	85.7
6	OPA 13	7	7	100
7	OPA 14	2	1	50.0
8	OPD 02	12	12	100
9	OPD 03	8	7	87.5
10	OPD 05	10	10	100
11	OPD 07	6	6	100
12	OPD 08	9	9	100
13	OPD 11	8	**7**	87.5
	Total	**101**	**94**	
	Mean	**7.76**	**7.23**	**90.45**

Fig. 9. Amplified and polymorphic bands in RAPD analysis of *Aerides maculosa*.

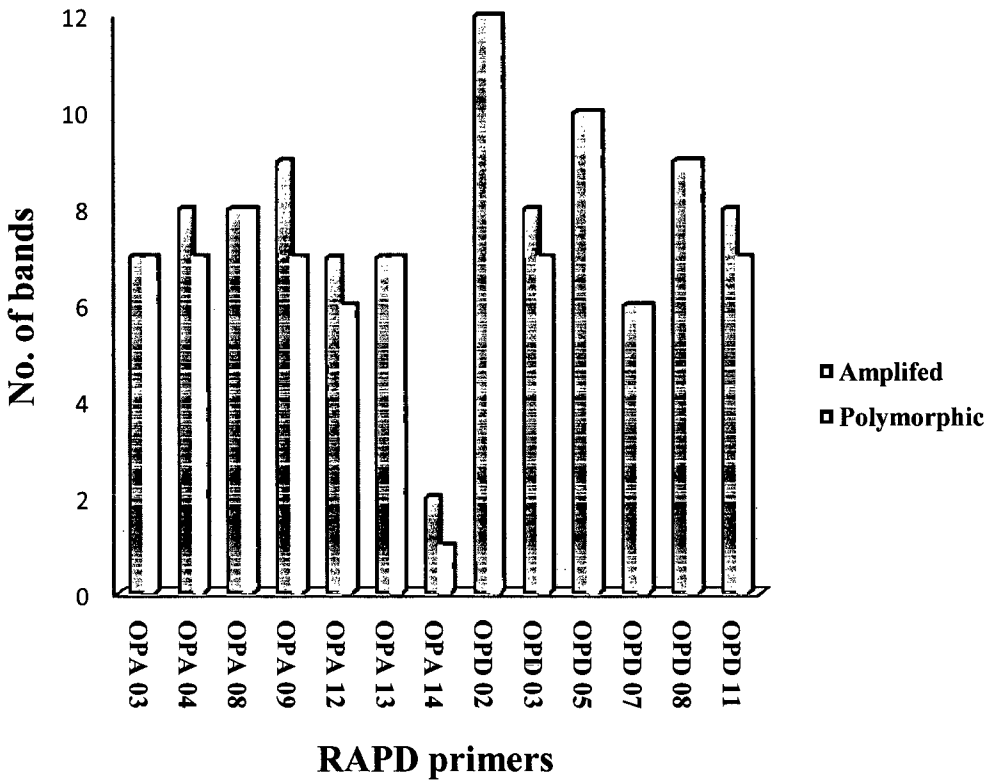
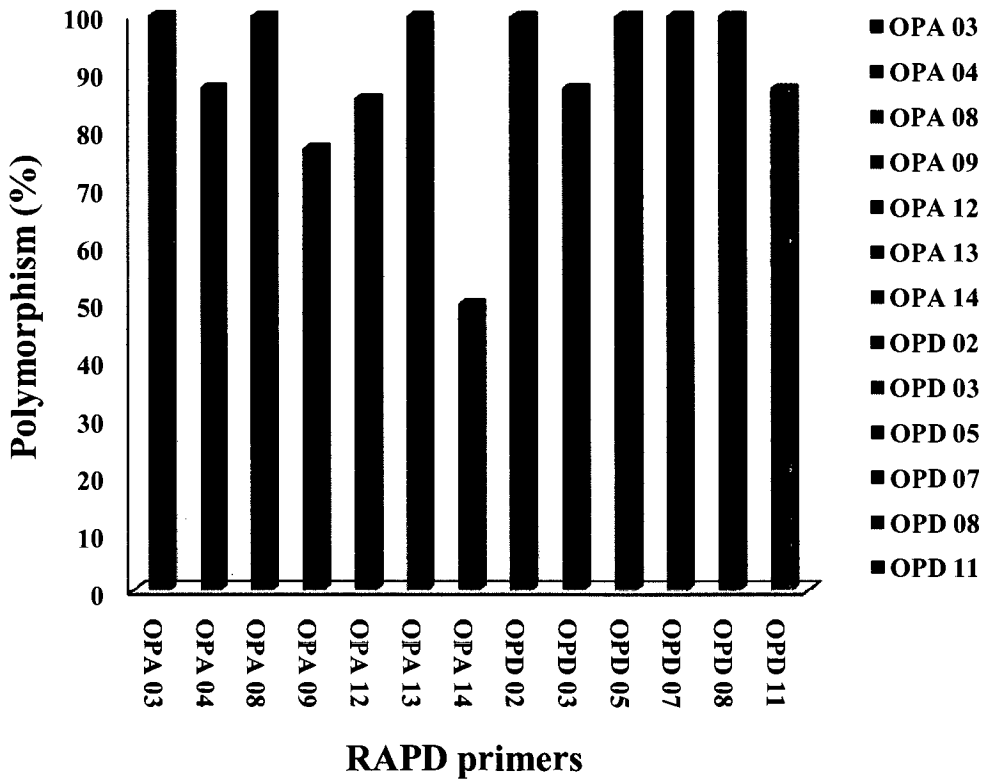


Fig. 10. Percentage polymorphism in RAPD analysis of *Aerides maculosa*.



4. 4. 1. 1. 1. Genetic identity and cluster analysis

Pair wise genetic similarities were computed from RAPD data using NTSYS-pc computer software (Rohlf, 1992). Value obtained for each populations are given in Table 20. Among all the populations, range of genetic identity coefficient was 0.465 to 0.762 with the average of 0.629. The highest genetic identity value (0.762) was observed for Shiroda-Pernem and Vagueri 1-Vaugeri 2 populations. While, the minimum value noted for Valpoi-Salauli and Lotulim-Salauli populations.

RAPD data of eight different populations of *Aerides maculosa* was used to generate dendrogram by using Unweighted Paired Group Method with Arithmetic Mean (UPGMA) method through the NTSYS-pc Version-2 computer software (Rohlf, 1992). Three major clusters were observed in the dendrogram (Fig. 11), which correspond to forest type of Goa and proximity of Western Ghats. The first cluster comprised of two populations belonging to open forest type (Pernem and Shiroda). Populations of dense forest hilly areas of Western Ghats (Vagueri 1 and Vagueri 2) were clustered together as the second cluster. While, third cluster comprised of Ponsuli and Valpoi populations (another dense forest type). However, remaining two populations out of eight namely Salauli and Loutulim did not cluster with other populations. On the contrary, these populations branched out separately.

4. 4. 1. 2. ISSR band pattern

ISSR analysis was carried out in seven populations collected from different regions of Goa. Out of 15 primers used for amplification of genomic DNA, seven primers did not amplify for any of the populations. The details of the primers which gave amplification are listed in Table 21. Only six primers showed clear and reproducible banding pattern. Amplification profile of amplified primers is shown in Plate 13 and 14. A total of 40 ISSR

Table 20. Genetic identity of *Aerides maculosa* populations based on RAPD primer amplification.

	Pernem	Ponsuli	Valpoi	Loutulim	Shiroda	Salauli	Vagueri 1	Vagueri 2
Pernem	---							
Ponsuli	0.653	---						
Valpoi	0.594	0.663	---					
Loutulim	0.653	0.604	0.623	---				
Shiroda	0.762	0.673	0.594	0.693	---			
Salauli	0.534	0.524	0.465	0.465	0.613	---		
Vaugeri 1	0.633	0.623	0.524	0.564	0.653	0.643	---	
Vaugeri 2	0.732	0.703	0.604	0.604	0.752	0.683	0.762	---

Fig. 11. Dendrogram of Nei's genetic identities between the populations of *Aerides maculosa* based on RAPD data.

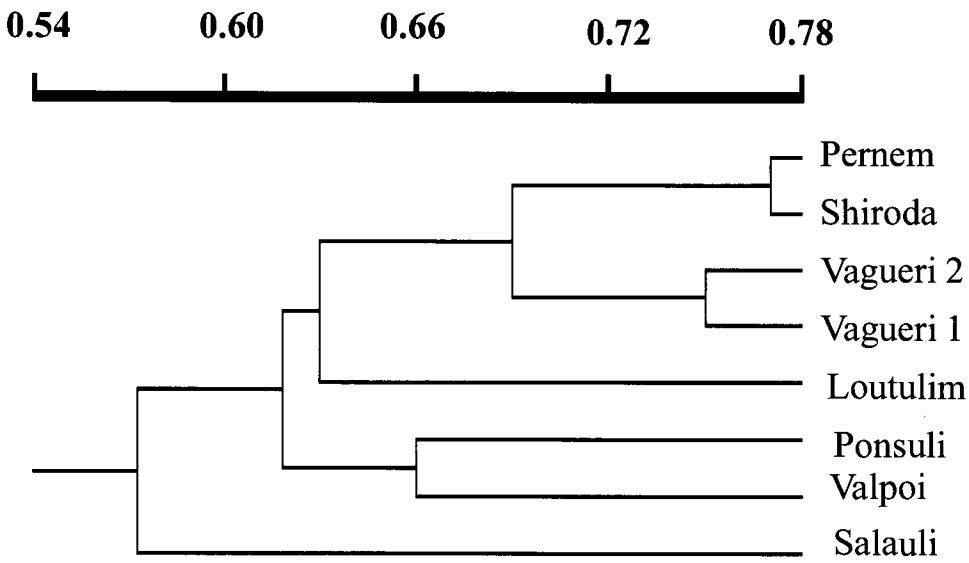


Table 21. Primers used for ISSR analysis of *Aerides maculosa* with details of amplification.

Sl. No.	Primers	PO	PE	SA	VA	SH	LO	VG 1	VG 2
1	HB 8	+	+	+	+	+	+	+	+
2	HB 9	+	+	+	+	+	+	+	+
3	HB 10	-	-	-	-	-	-	-	-
4	HB12	-	-	-	-	-	-	-	-
5	HB13	+	+	+	+	+	+	+	+
6	HB 14	+	+	+	+	+	+	+	+
7	HB15	+	+	+	+	+	+	+	+
8	814	-	-	-	-	-	-	-	-
9	814 A	-	-	-	-	-	-	-	-
10	844 B	-	-	-	-	-	-	-	-
11	P2	-	-	-	-	-	-	-	-
12	17898A	-	-	-	-	-	-	-	-
13	17898B	+	+	+	+	+	+	+	+
14	17899A	+	+	+	+	+	+	+	+
15	17899B	+	+	+	+	+	+	+	+

+ Amplified; - Not amplified

Different Populations: Ponsuli (PO); Pernem (PE); Salauli (SA); Valpoi (VA); Shiroda (SH); Loutulim (LO); Vagueri 1 (VG 1); Vagueri 2 (VG 2).

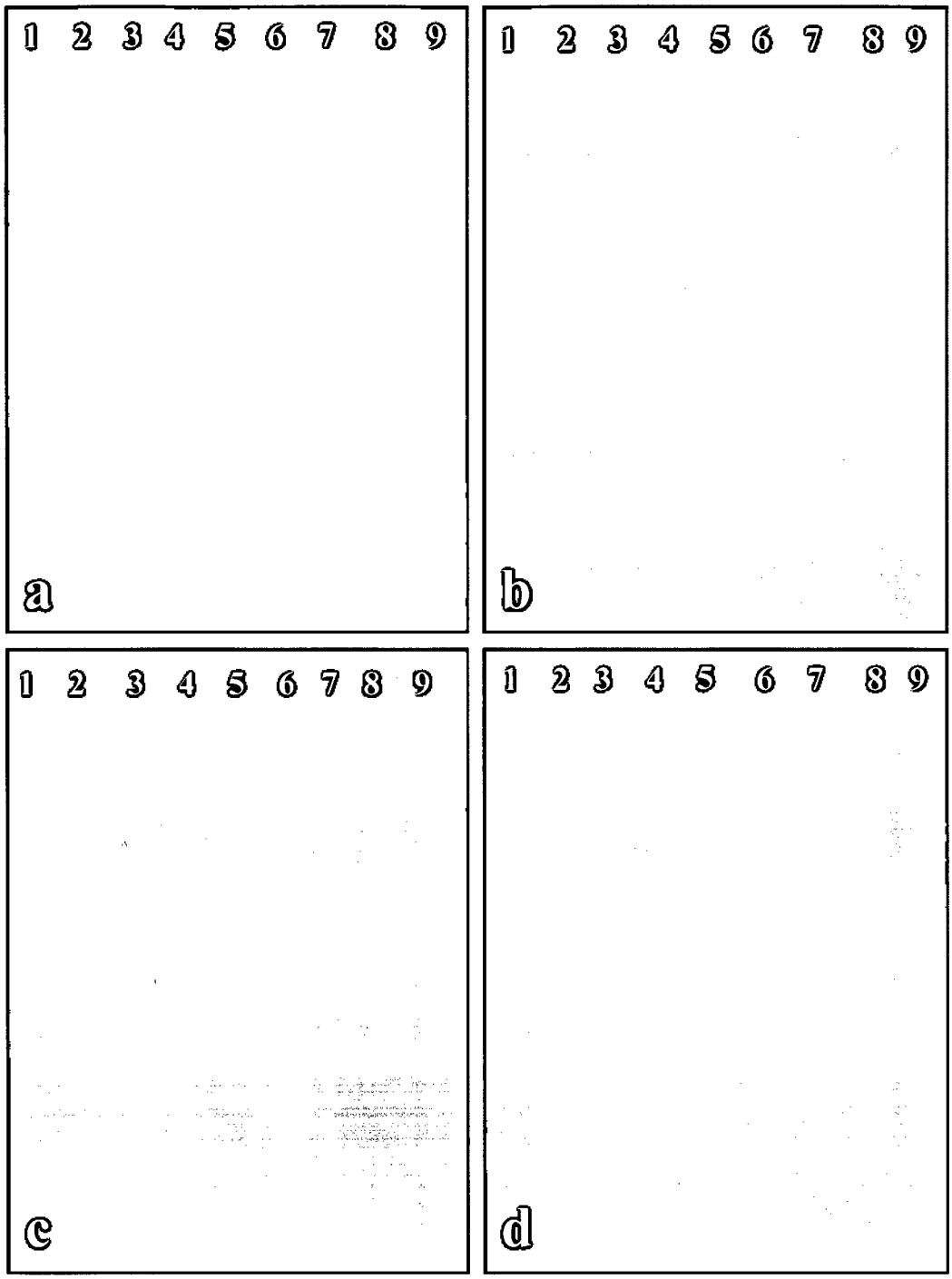


Plate 13. ISSR amplification profile of *Aerides maculosa* with different primers; a. Primer HB 8; b. Primer 17898 B; c. Primer 17899 A; d. Primer 17899 B; Lane 1. Gene ruler^{TM} 1 kb DNA ladder; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1; 9. Vagueri 2.

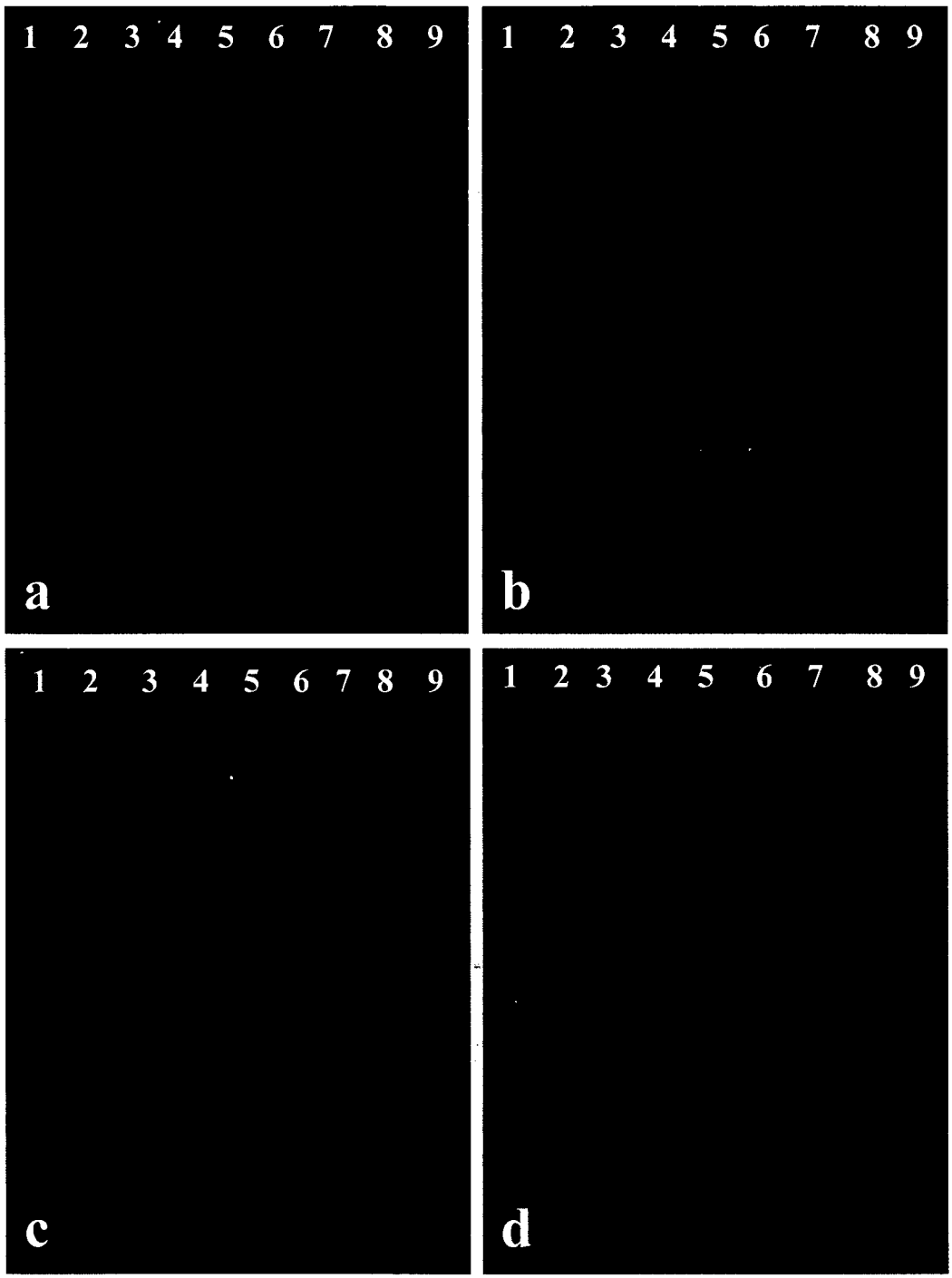


Plate 14. ISSR amplification profile of *Aerides maculosa* with different primers; a. Primer HB 9; b. Primer HB 14; c. Primer HB 13; d. Primer HB 15; Lane 1. Gene ruler^{TM} 1 kb DNA ladder; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1; 9. Vagueri 2.

bands were obtained from six different primers with an average of 6.66 bands per primer (Table 22). Of these thirty bands were polymorphic with an average of 5.0 bands per primer (Fig. 12). Polymorphism across all the samples varied from 50 (HB 13 and 17898 B) to 100% (HB 9) with an average of 72.85%. The percentage of polymorphism among the primers is shown in Fig. 13.

4. 4. 1. 2. 1. Genetic identity and cluster analysis

The ISSR data were used for the determination of genetic identity value in similar way as it was done in RAPD analysis. The identity values of all the populations are given in Table 23. The genetic identity value varied from 0.500 to 0.975, with the average of 0.704. Populations of hilly areas of Western Ghats (Vaugeri 1 and 2) shared maximum genetic identity (0.975). The lowest identity value (0.500) was observed between populations belonging to two different forest types i.e Shiroda (open type) and Vaugeri 1 (dense forest type).

Dendrogram was generated from ISSR data of eight populations of *Aerides maculosa* in the similar way as that of RAPD analysis. Dendrogram clearly revealed the formation of two major clusters (Fig. 14). In this case also the dendrogram generated from ISSR data revealed the clustering of populations based on forest type and proximity to Western Ghats. Populations of hilly areas of Western Ghats (Vagueri 1, Vagueri 2 and Valpoi) formed the first major cluster. Second major cluster comprised of Loutulim (non-forest type), Pernem (open-forest type) and Salauli (dense-forest type) populations. It was observed that populations of Ponsuli (dense-forest type) and Shiroda (open-forest type) remained separately branched.

Table 22. Amplified, polymorphic bands and percentage of polymorphism in ISSR analysis of *Aerides maculosa*.

Sl. No	Primers	No. of amplified bands	No. of polymorphic bands	Polymorphism %
1	17899A	7	6	85.71
2	17899B	7	5	71.4
3	HB 9	9	9	100.0
4	HB 8	5	4	80.0
5	HB 13	8	4	50.0
6	17898 B	4	2	50.0
	Total	**40**	**30**	-
	Mean	**6.66**	**5.0**	**72.85**

Fig. 12. Amplified and polymorphic bands in ISSR analysis of *Aerides maculosa.*

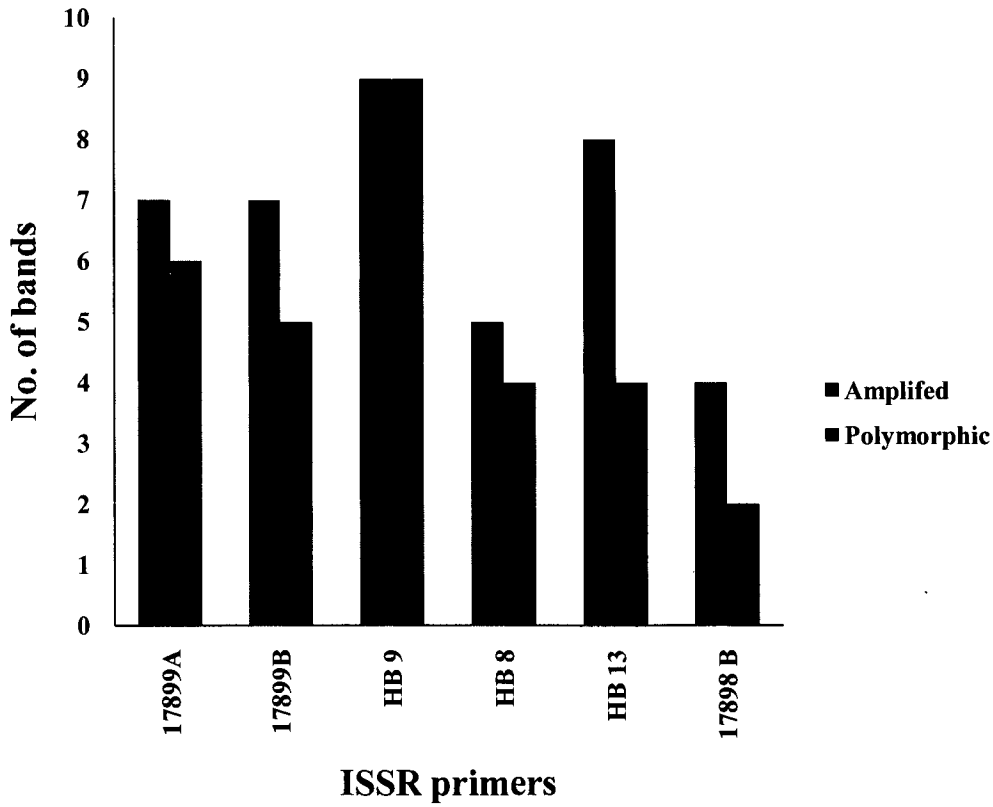


Fig. 13. Percentage polymorphism in ISSR analysis of *Aerides maculosa*.

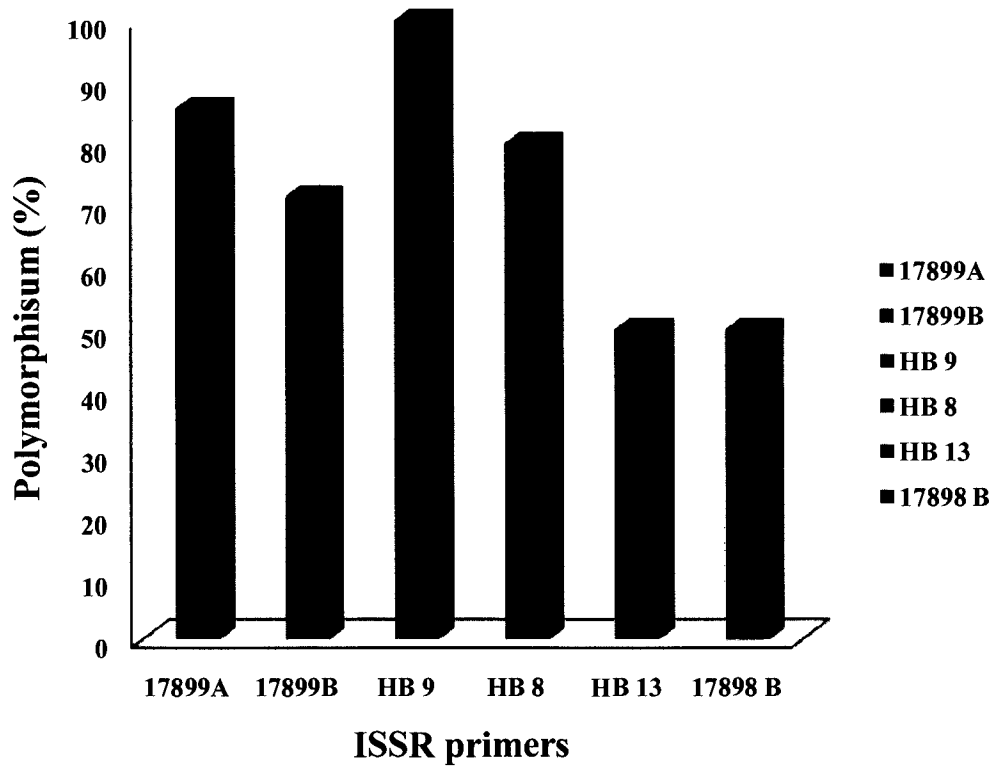
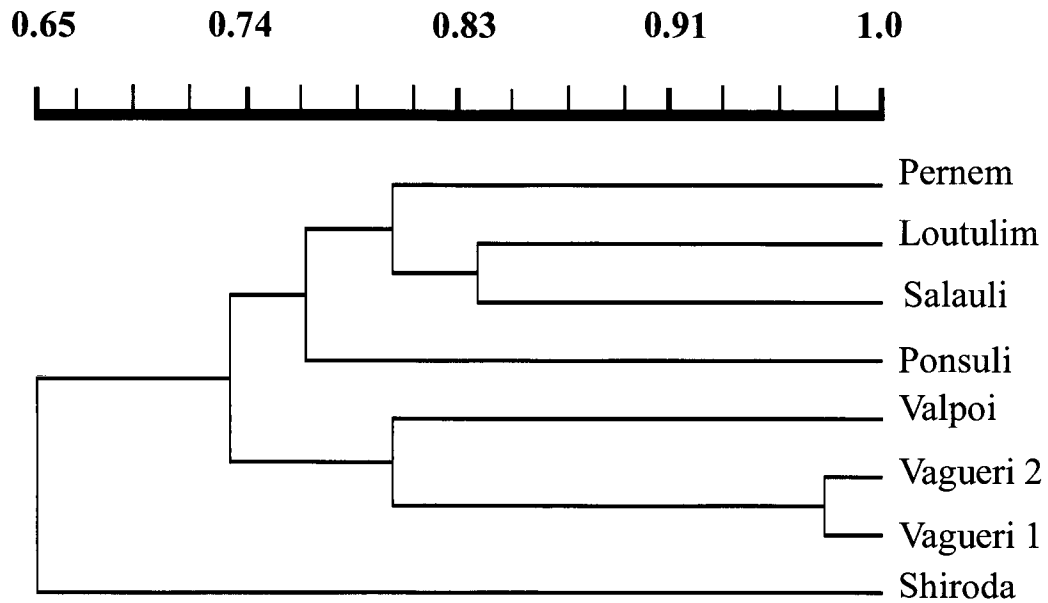


Table 23. Genetic identity of *Aerides maculosa* populations based on ISSR primer amplification.

	Pernem	Ponsuli	Valpoi	Loutulim	Shiroda	Salauli	Vagueri 1	Vagueri 2
Pernem	---							
Ponsuli	0.725	---						
Valpoi	0.675	0.700	---					
Loutulim	0.825	0.700	0.750	---				
Shiroda	0.575	0.700	0.550	0.600	---			
Salauli	0.800	0.775	0.725	0.825	0.675	---		
Vaugeri 1	0.725	0.600	0.750	0.700	0.500	0.775	---	
Vaugeri 2	0.750	0.625	0.775	0.725	0.475	0.750	0.975	---

Fig. 14. Dendrogram of Nei's genetic identities between the populations of *Aerides maculosa* based on ISSR data.



4. 4. 1. 3. Protein profile analysis using SDS-PAGE

The SDS-PAGE analysis of plant extract isolated at pH 7.5 showed distinct banding pattern with respect to different populations of *Aerides maculosa*. The protein profile of different populations is shown in Plate 15. In total, 3 to 9 bands per populations were detected. Total 42 bands were detected among all the populations with the average of 6 bands per population. Among these 27 bands were polymorphic with an average of 3.85 bands per population (Table 24). Maximum number of bands (9) were detected in Ponsuli population, while, minimum number of bands (3) were observed in Valpoi and Vagueri populations. Graphical representation of detected and polymorphic bands is given in Fig. 15. Percentage of polymorphism across all the populations ranged from 33.3 (Valpoi and Vagueri 1) to 77.7% (Ponsuli) (Fig. 16).

4. 4. 1. 3. 1. Genetic identity and cluster analysis

SDS-PAGE data obtained from seven populations of *Aerides maculosa* were used to compute the genetic identity, similar to that of RAPD analysis. The identity values for all the populations are given in Table 25. Genetic identity value among all the populations ranged from 0.222 to 0.980 with an average of 0.744. The maximum genetic identity value 0.980 was observed between Loutulim (non-forest type) and Shiroda (open-forest type) populations, while, the minimum identity value of 0.222 was noted among the populations of Ponsuli-Valpoi (both dense-forest type) and Ponsuli-Vagueri 1 populations (both dense-forest type).

Dendrogram was generated using SDS-PAGE data of seven populations (Fig. 17). In this case also clustering of populations was based on the forest types and proximity to Western Ghats similar to that of RAPD and ISSR analysis. Two major clusters were observed among the populations. Open and non-forest type populations (Pernem, Shiroda

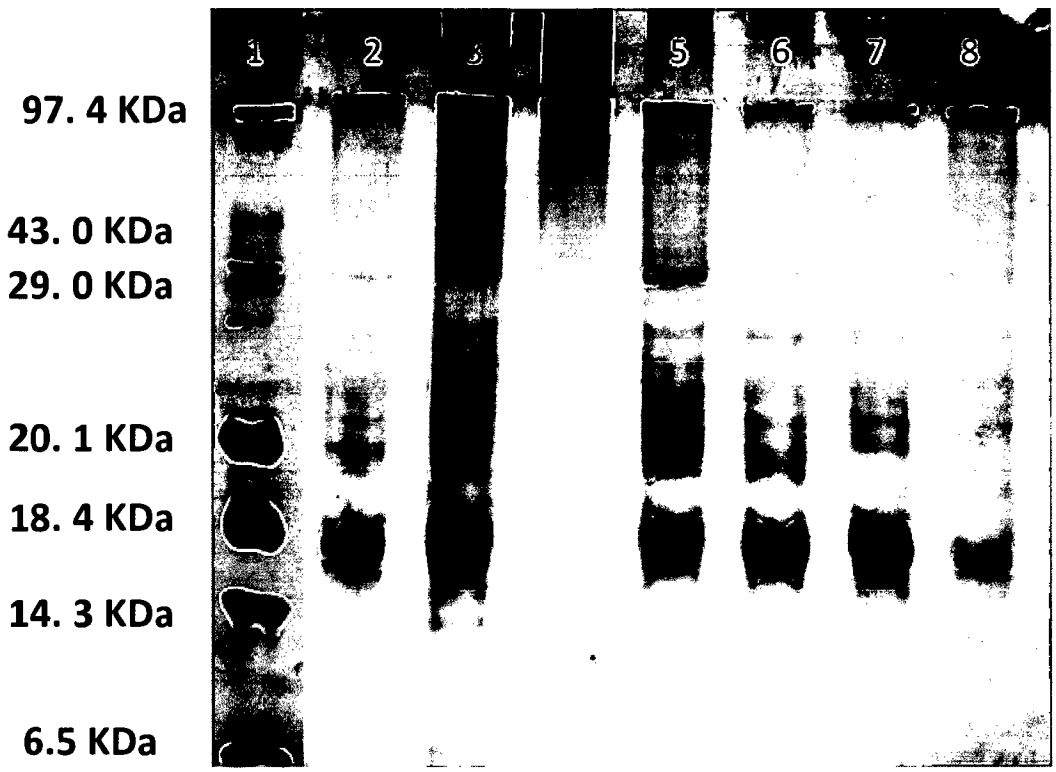
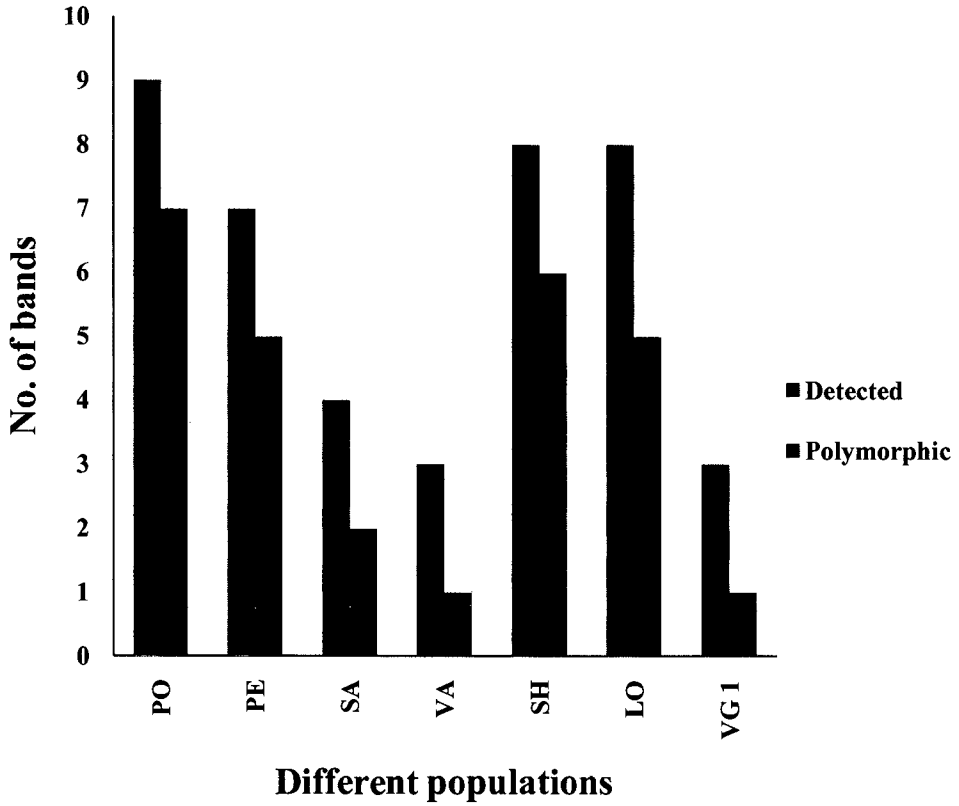


Plate 15. SDS-PAGE protein profile of *Aerides maculosa*; Lane 1. Molecular weight standard; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1.

Table 24. Detected, polymorphic bands and percentage of polymorphism in SDS-PAGE analysis of *Aerides maculosa*.

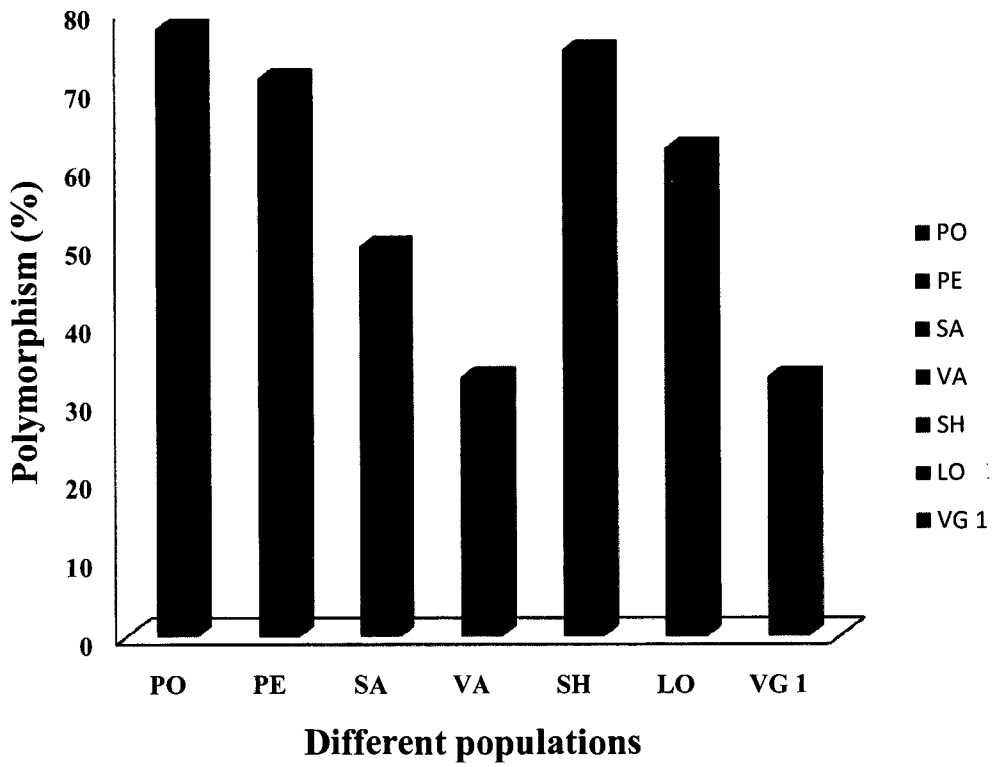
Sl. No.	Populations	No. of detected bands	No. of polymorphic bands	Polymorphism %
1	Ponsuli	9	7	77.7
2	Pernem	7	5	71.4
3	Salauli	4	2	50.0
4	Valpoi	3	1	33.3
5	Shiroda	8	6	75.0
6	Loutulim	8	5	62.5
7	Vagueri 1	3	1	33.3
	Total	**42**	**27**	-
	Mean	**6**	**3.85**	**57.6**

Fig. 15. Detected and polymorphic band in SDS-PAGE analysis of *Aerides maculosa.*



PO. Ponsuli; PE. Pernem; SA. Salauli; VA. Valpoi; SH. Shiroda; LO. Loutulim; VG 1. Vagueri 1.

Fig. 16. Percentage polymorphism in SDS-PAGE analysis of *Aerides maculosa*.

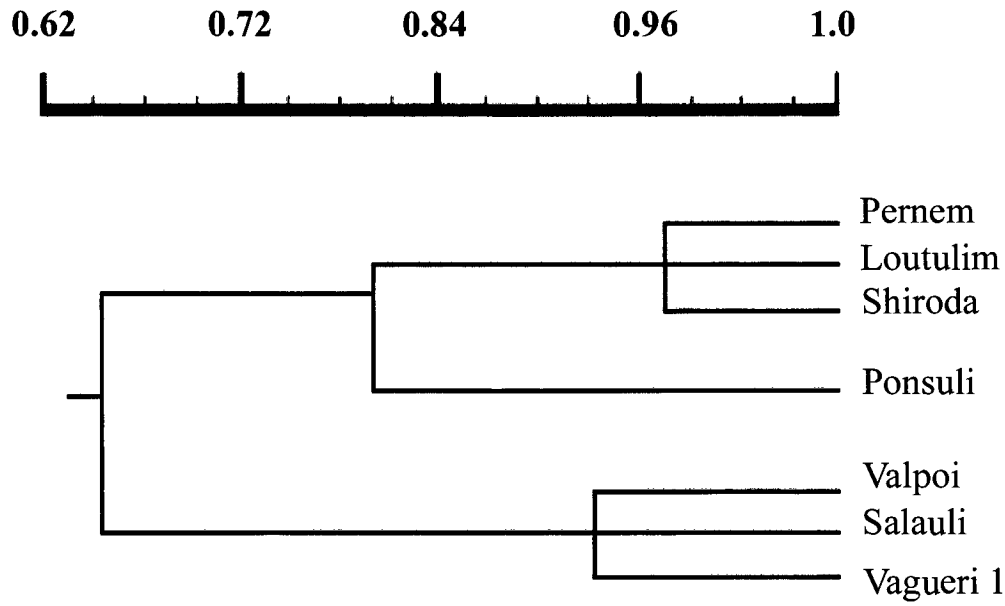


PO. Ponsuli; PE. Pernem; SA. Salauli; VA. Valpoi; SH. Shiroda; LO. Loutulim; VG 1. Vagueri 1.

Table 25. Genetic identity of *Aerides maculosa* populations based on SDS-PAGE analysis.

	Pernem	Ponsuli	Valpoi	Loutulim	Shiroda	Salauli	Vagueri 1
Pernem	---						
Ponsuli	0.798	---					
Valpoi	0.815	0.222	---				
Loutulim	0.920	0.916	0.656	---			
Shiroda	0.920	0.916	0.656	0.980	---		
Salauli	0.885	0.329	0.935	0.743	0.743	---	
Vaugeri 1	0.815	0.222	0.935	0.656	0.656	0.925	---

Fig. 17. Dendrogram of Nei's genetic identities between the populations of *Aerides maculosa* based on SDS-PAGE data.



and Loutulim) formed the first major cluster while, the second cluster consisted of Valpoi, Salauli and Vagueri 1 which belonged to dense forest type of hilly areas of Western Ghats.

4. 4. 2. *Rhynchostylis retusa*

4. 4. 2. 1. RAPD band pattern

A total of 20 decamer oligonucleotide primers were used for the investigation of seven populations of *Rhynchostylis retusa*. Out of twenty RAPD primers used to study the variation, 13 primers showed amplification in all the populations. The details of the primers and their amplification profiles are given in Table 26. Seven primers (OPA 05, OPA 06, OPA 07, OPD 04, OPD 06, OPD 10 and OPD 20) did not show any amplification for the tested DNA. RAPD profile of *Rhynchostylis retusa* is shown in Plate 16, 17, and 18. Thirteen primers chosen for the study generated 74 RAPD bands (Table 27). Of these, 57 were polymorphic with a mean of 4.38 per primer. For each primer, the number of bands ranged from 3 to 8, with an average of 5.69 (Fig. 18). OPD 2 primer recorded the maximum of 8 bands, while, minimum of 3 bands were recorded in OPA 8 primer. Graphical representation of percentage polymorphism among the different primers is given in Fig. 19. The percentage of polymorphism across all the population ranged between 33.3 to 100% with the average of 76.13%. Hundred percent polymorphic pattern was obtained using primers OPA 12, OPA 13 and OPD 11 (Table 27) while, lowest percentage of polymorphism (33.3%) was recorded in OPA 8 and OPD 5 primers.

4. 4. 2. 1. 1. Genetic identity and cluster analysis

RAPD data obtained from seven populations of *R. retusa* were used to compute genetic similarities using NTSYS-pc computer software (Rohlf, 1992). Identity values for each population are given in Table 28. Genetic identity among all the populations varied from 0.405 to 0.932 with an average of 0.685. Populations at similar altitude (Shiroda and

Table 26. Primers used for RAPD analysis of *Rhynchostylis retusa* with details of amplification.

Sl. No.	Primers	PO	PE	SA	VA	SH	LO	VG 1
1	OPA 03	+	+	+	+	+	+	+
2	OPA 04	+	+	+	+	+	+	+
3	OPA 05	-	-	-	-	-	-	-
4	OPA 06	-	-	-	-	-	-	-
5	OPA 07	-	-	-	-	-	-	-
6	OPA 08	+	+	+	+	+	+	+
7	OPA 09	+	+	+	+	+	+	+
8	OPA 12	+	+	+	+	+	+	+
9	OPA 13	+	+	+	+	+	+	+
10	OPA 14	+	+	+	+	+	+	+
11	OPD 02	+	+	+	+	+	+	+
12	OPD 03	+	+	+	+	+	+	+
13	OPD 04	-	-	-	-	-	-	-
14	OPD 05	+	+	+	+	+	+	+
15	OPD 06	-	-	-	-	-	-	-
16	OPD 07	+	+	+	+	+	+	+
17	OPD 08	+	+	+	+	+	+	+
18	OPD 10	-	-	-	-	-	-	-
19	OPD 11	+	+	+	+	+	+	+
20	OPD 20	-	-	-	-	-	-	-

+ Amplified; - Not amplified

Different Populations: Ponsuli (PO); Pernem (PE); Salauli (SA); Valpoi (VA); Shiroda (SH); Loutulim (LO); Vagueri 1 (VG 1).

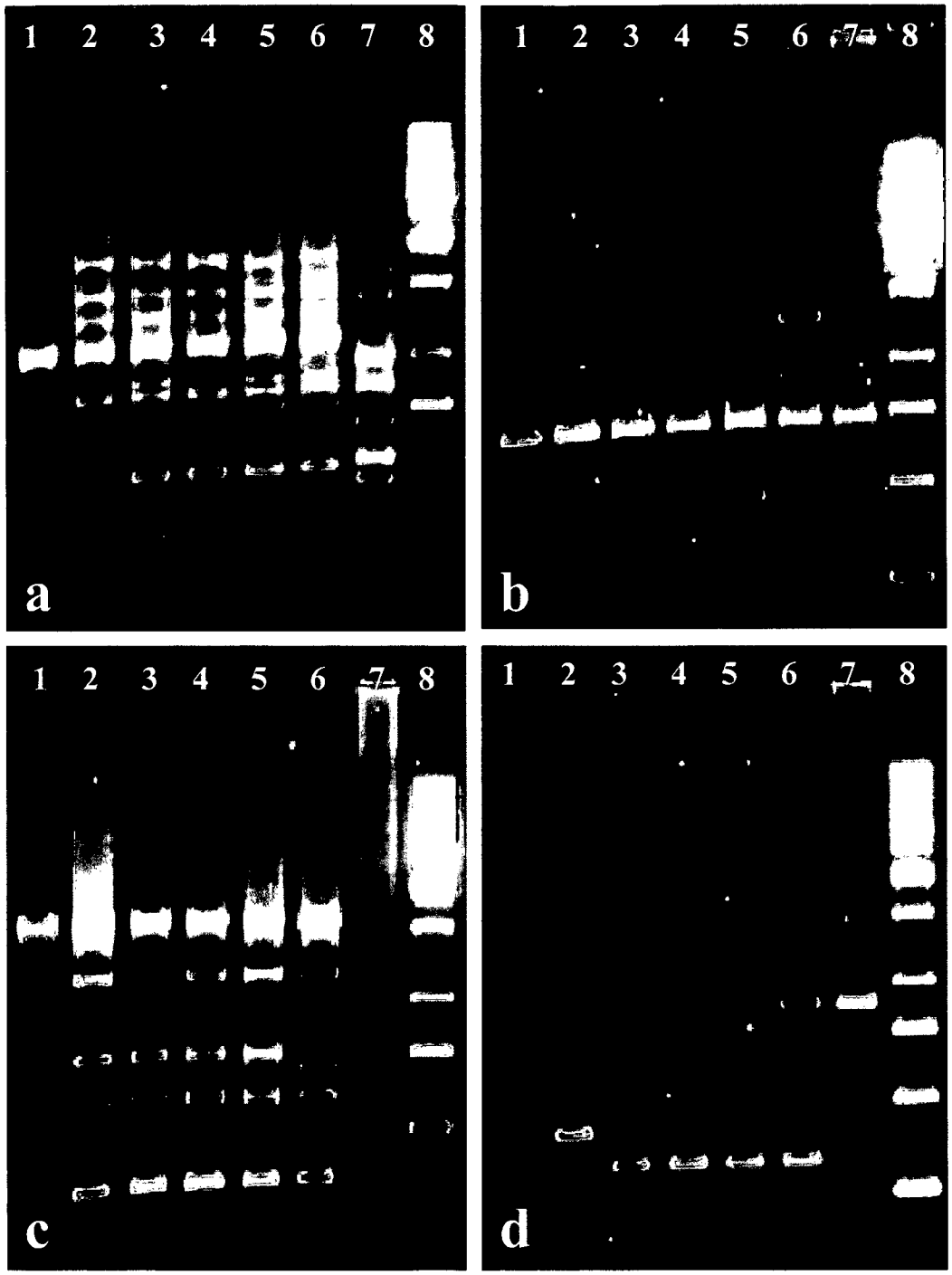


Plate 16. RAPD amplification profile of *Rhynchostylis retusa* with different primers; a. Primer OPA 3; b. Primer OPA 8; c. Primer OPA 9; d. Primer OPA 12; Lane 1. Pernem; 2. Ponsuli; 3. Salauli; 4. Valpoi; 5. Shiroda; 6. Loutulim; 7. Vagueri 1; 8. Gene ruler^{TM} 1 kb DNA ladder.

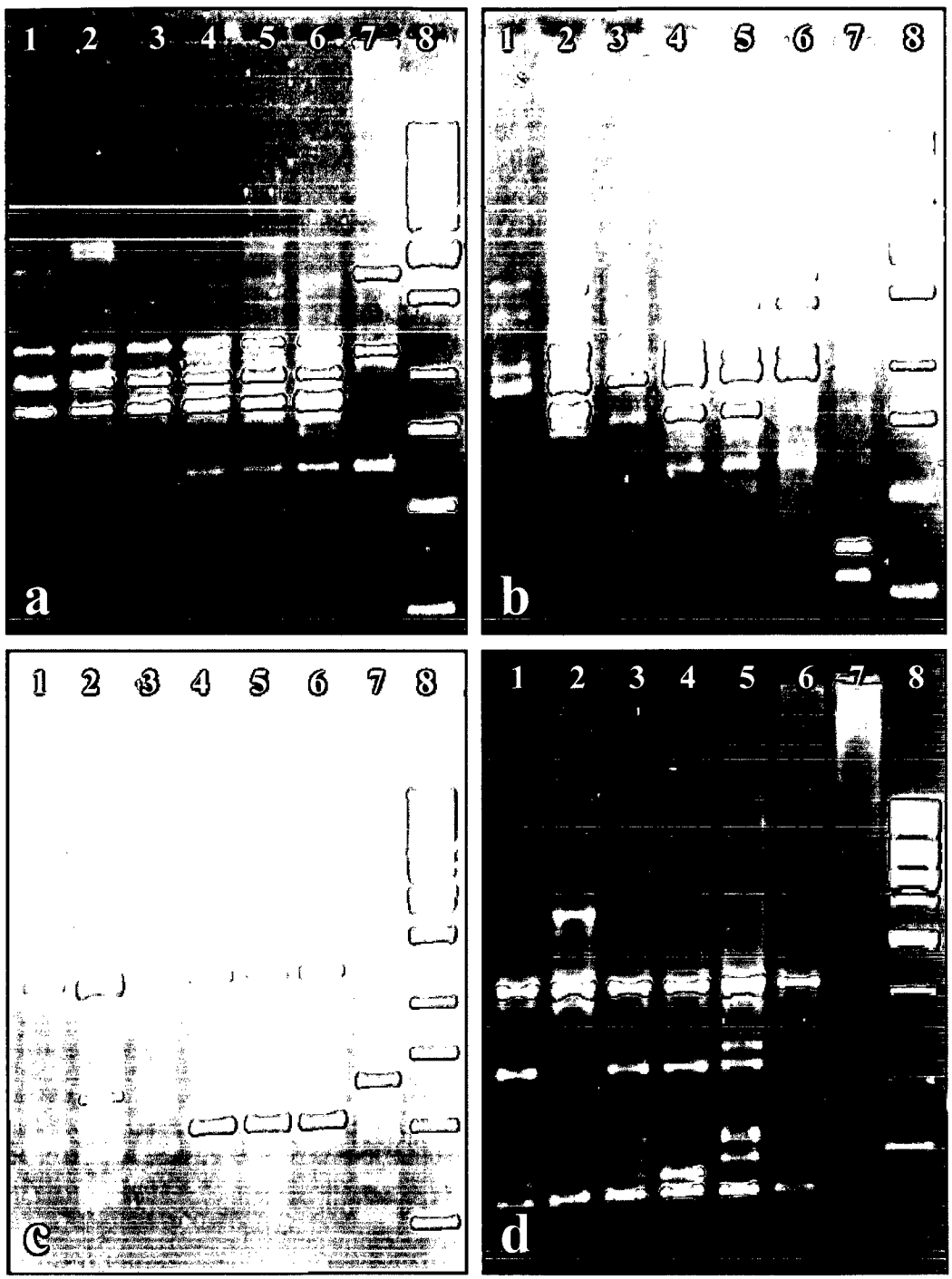


Plate 17. RAPD amplification profile of *Rhynchostylis retusa* with different primers; a. Primer OPA 13; b. Primer OPA 4; c. Primer OPA 14, d. Primer OPD 2; Lane 1. Pernem; 2. Ponsuli; 3. Salauli; 4. Valpoi; 5. Shiroda; 6. Loutulim; 7. Vagueri 1; 8. Gene ruler^{TM} 1 kb DNA ladder.

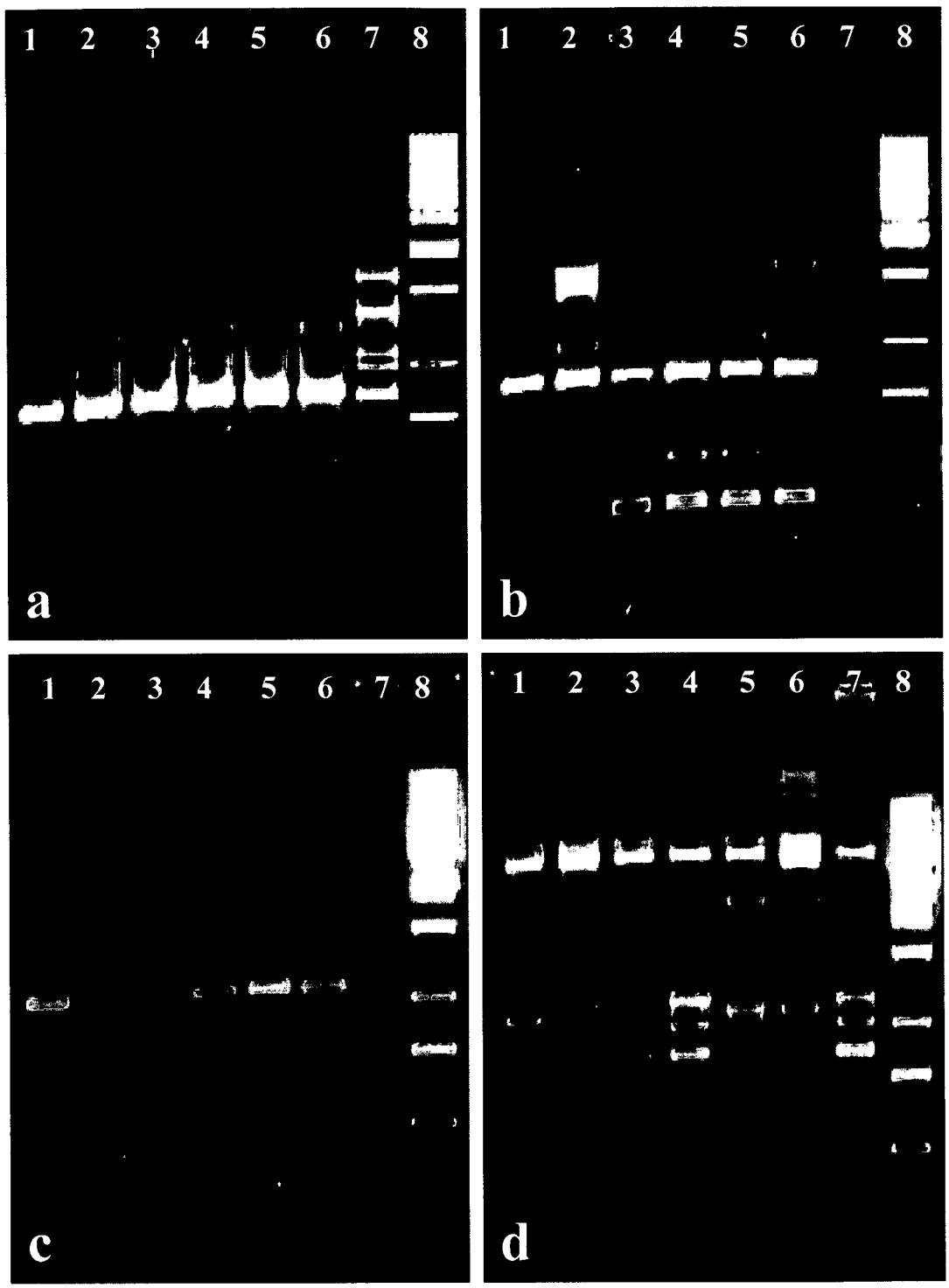


Plate 18. RAPD amplification profile of *Rhynchostylis retusa* with different primers; a. Primer OPD 3; b. Primer OPD 5; c. Primer OPD 7; d. Primer OPD 8; Lane 1. Pernem; 2. Ponsuli; 3. Salauli; 4. Valpoi; 5. Shiroda; 6. Loutulim; 7. Vagueri 1; 8. Gene ruler^{TM} 1 kb DNA ladder.

Table 27. Amplified bands, polymorphic bands and percentage of polymorphism in RAPD analysis of *Rhynchostylis retusa*.

Sl. No	Primers	No. of amplified bands	No. of polymorphic bands	Polymorphism %
1	OPA 03	7	5	71.4
2	OPA 04	6	4	66.6
3	OPA 08	3	1	33.3
4	OPA 09	7	5	71.0
5	OPA 12	4	4	100
6	OPA 13	6	5	83.3
7	OPA 14	4	4	100
8	OPD 02	8	7	87.5
9	OPD 03	5	4	80.0
10	OPD 05	6	2	33.3
11	OPD 07	5	4	80.0
12	OPD 08	6	5	83.3
13	OPD 11	7	7	100
	Total	**74**	**57**	-
	Mean	**5.69**	**4.38**	**76.13**

Fig. 18. Amplified and polymorphic bands in RAPD analysis of *Rhynchostylis retusa.*

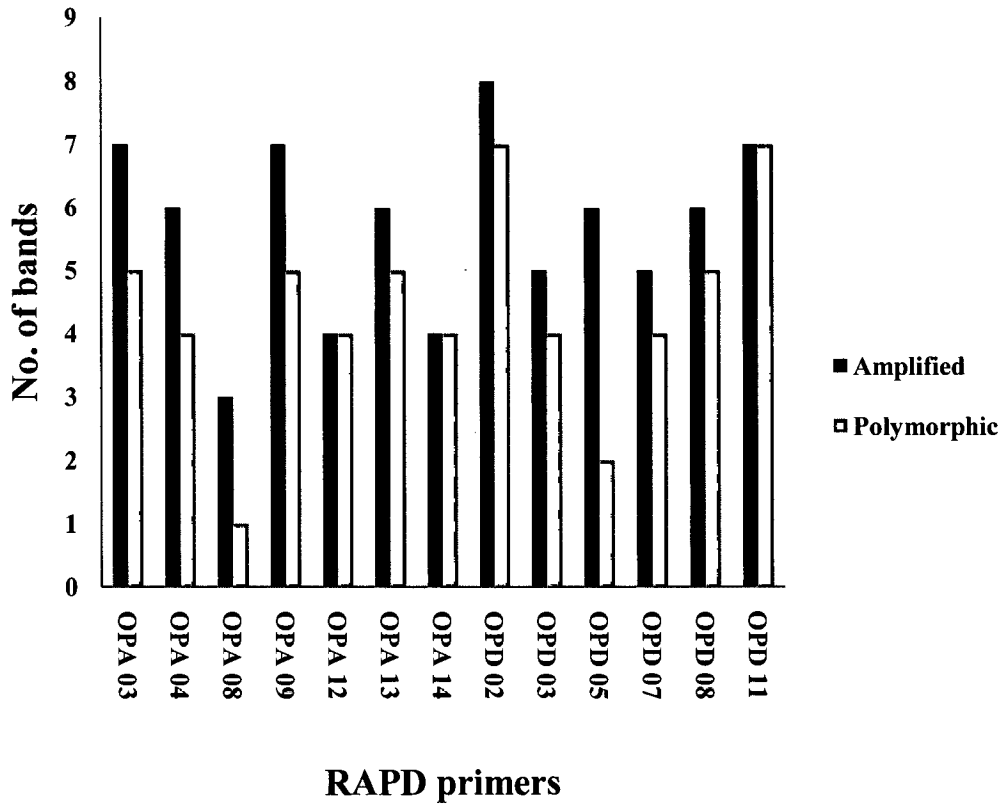


Fig. 19. Percentage polymorphism in RAPD analysis of *Rhynchostylis retusa*.

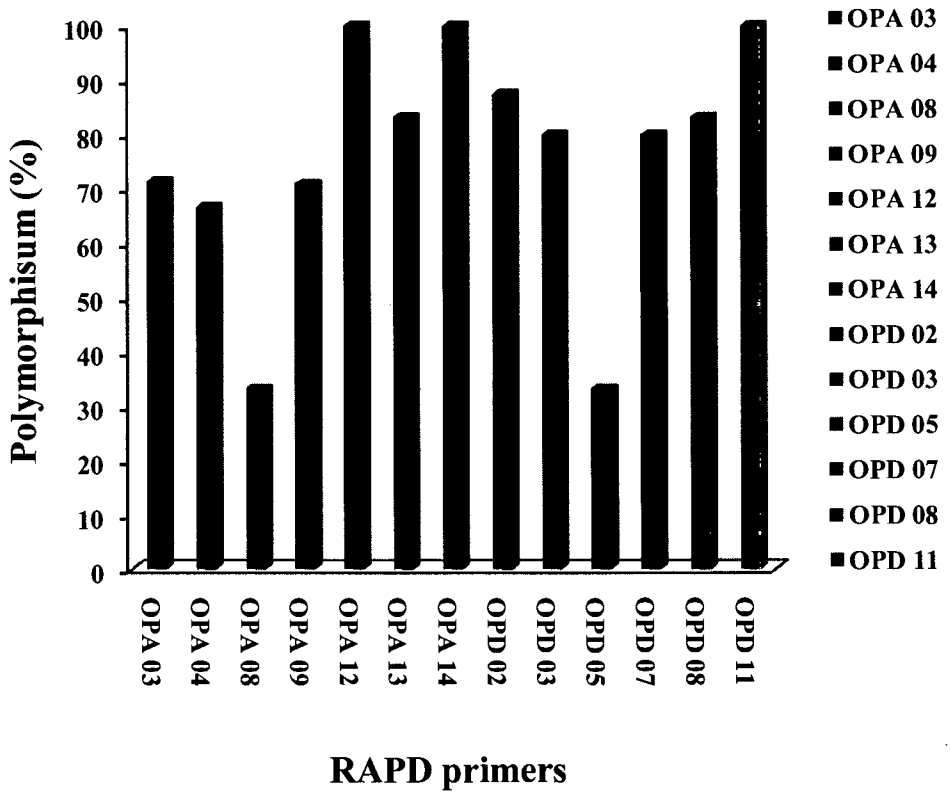


Table 28. Genetic identity of *Rhynchostylis retusa* populations based on RAPD analysis.

	Ponsuli	Pernem	Salauli	Valpoi	Shiroda	Loutulim	Vagueri 1
Ponsuli	---						
Pernem	0.702	---					
Salauli	0.702	0.851	---				
Valpoi	0.770	0.770	0.918	---			
Shiroda	0.743	0.797	0.864	0.864	---		
Loutulim	0.729	0.837	0.851	0.824	0.932	---	
Vaugeri 1	0.473	0.500	0.486	0.432	0.405	0.445	---

Loutulim) shared maximum genetic identity (0.932) while, populations of Shiroda and Vaugeri 1 shared the least identity (0.405). Difference in altitude was noticed in these populations.

NTSYS-pc Version-2 computer software (Rohlf, 1992) based on Unweighted Paired Group Method with Arithmetic Mean (UPGMA) method was used to generate dendrogram (Fig. 20) from the data obtained during the RAPD analysis. The dendrogram clearly revealed the formation of two major clusters. Almost similar altitude populations of Shiroda (102m) and Loutulim (103m) formed the first cluster, while, Salauli (76m) and Valpoi (71m) formed second cluster. Populations of Pernem, Ponsuli and Vaugeri 1 did not cluster with others. Among these, Vaugeri 1 population, located at a higher altitude (285m) compared to others, totally branched out separately.

4. 4. 2. 2. ISSR band pattern

Fifteen primers were initially screened using genomic DNA. Eight primers showed amplification for the tested DNA, among which seven primers produced clear and consistent banding pattern (Table 29). Amplification profile of different primers is given in Plate 19 and 20. Among the seven amplified primers, two primers (17898 B and HB 15) were monomorphic. The fingerprint pattern generated 30 bands with an average of 4.28 bands per primer. The number of amplified bands ranged from 2 (17898 B and HB 15) to 10 (HB 9) (Fig. 21). Sixteen polymorphic bands were produced from five primers with an average of 3.2 bands per primer (Table 30). The percentage of polymorphism across all the samples varied from 40 to 80% with the average of 62.6% (Fig. 22). The highest polymorphism of 80% was recorded in primer HB 13, while, the lowest in primer 17899A (40%).

Fig. 20. Dendrogram of Nei's genetic identities between the populations of *Rhynchostylis retusa* based on RAPD data.

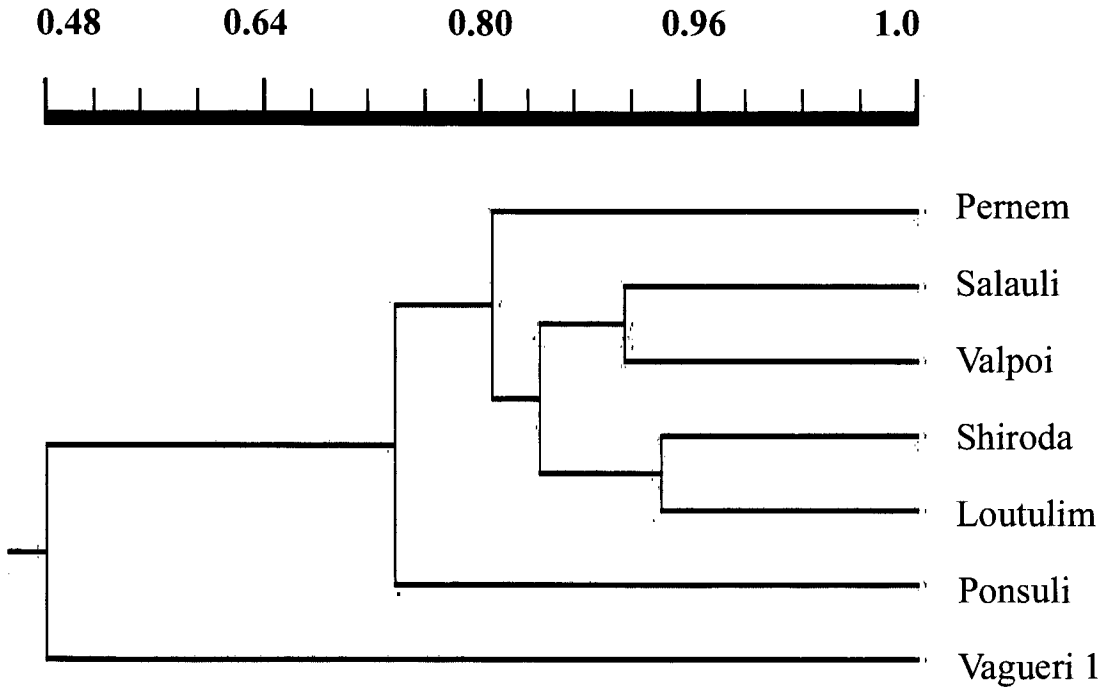


Table 29. Primers used for ISSR analysis of *Rhynchostylis retusa* with details of amplification.

Sl. No.	Primers	PO	PE	SA	VA	SH	LO	VG 1
1	HB 8	-	-	-	-	-	-	-
2	HB 9	+	+	+	+	+	+	+
3	HB 10	-	-	-	-	-	-	-
4	HB12	+	+	+	+	+	+	+
5	HB13	+	+	+	+	+	+	+
6	HB 14	-	-	-	-	-	-	-
7	HB15	+	+	+	+	+	+	+
8	814	-	-	-	-	-	-	-
9	814 A	-	-	-	-	-	-	-
10	844 B	-	-	-	-	-	-	-
11	P2	-	-	-	-	-	-	-
12	17898A	+	-	+	+	+	+	+
13	17898B	+	+	+	+	+	+	+
14	17899A	+	+	+	+	+	+	+
15	17899B	+	+	+	+	+	+	+

+ Amplified; - Not amplified

Different Populations: Ponsuli (PO); Pernem (PE); Salauli (SA); Valpoi (VA); Shiroda (SH); Loutulim (LO); Vagueri 1 (VG 1).

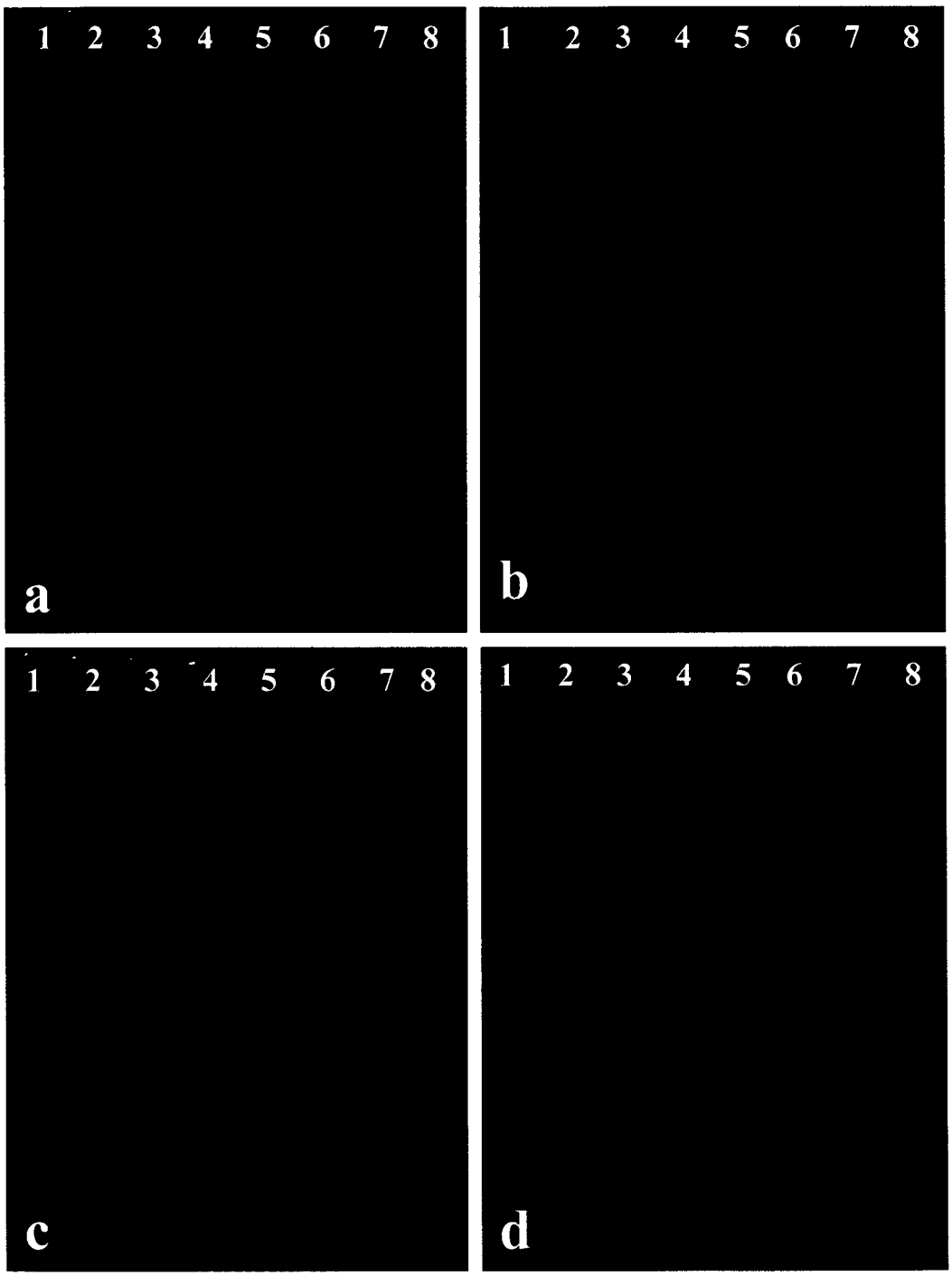


Plate 19. ISSR amplification profile of *Rhynchostylis retusa* with different primers; a. Primer 17899 A; b. Primer 17898 B; c. Primer 17898 A; d. Primer 17899 B; Lane 1. Pernem; 2. Ponsuli; 3. Salauli; 4. Valpoi; 5. Shiroda; 6. Loutulim; 7. Vagueri 1; 8. Gene ruler^{TM} 1 kb DNA ladder.

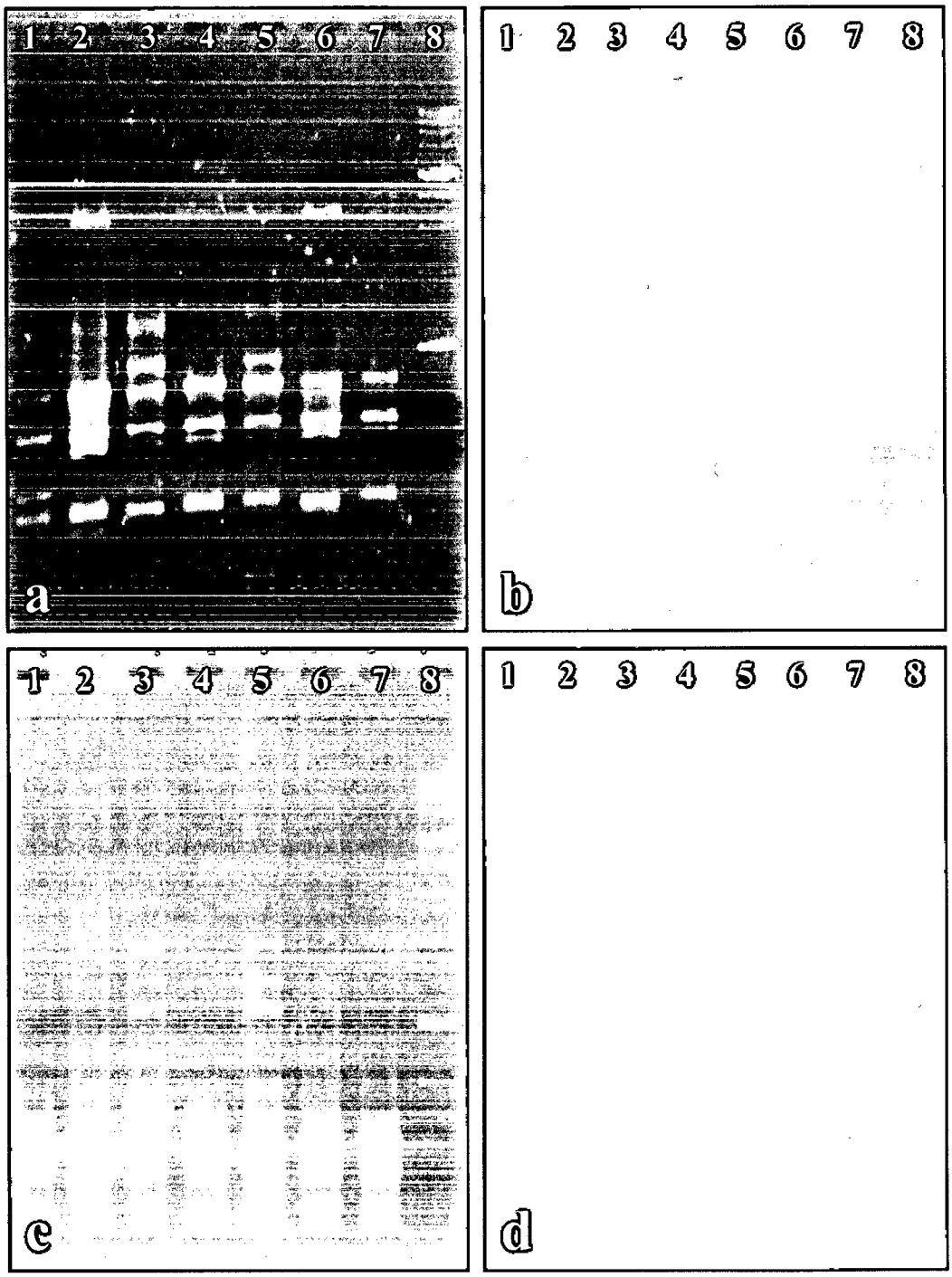


Plate 20. ISSR amplification profile of *Rhynchostylis retusa* with different primers; a. Primer HB 12; b. Primer HB 13; c. Primer HB 9; d. Primer HB 15; Lane 1. Pernem; 2. Ponsuli; 3. Salauli; 4. Valpoi; 5. Shiroda; 6. Loutulim; 7. Vagueri 1; 8. Gene ruler^{TM} 1 kb DNA ladder.

Fig. 21. Amplified and polymorphic bands in ISSR analysis of *Rhynchostylis retusa.*

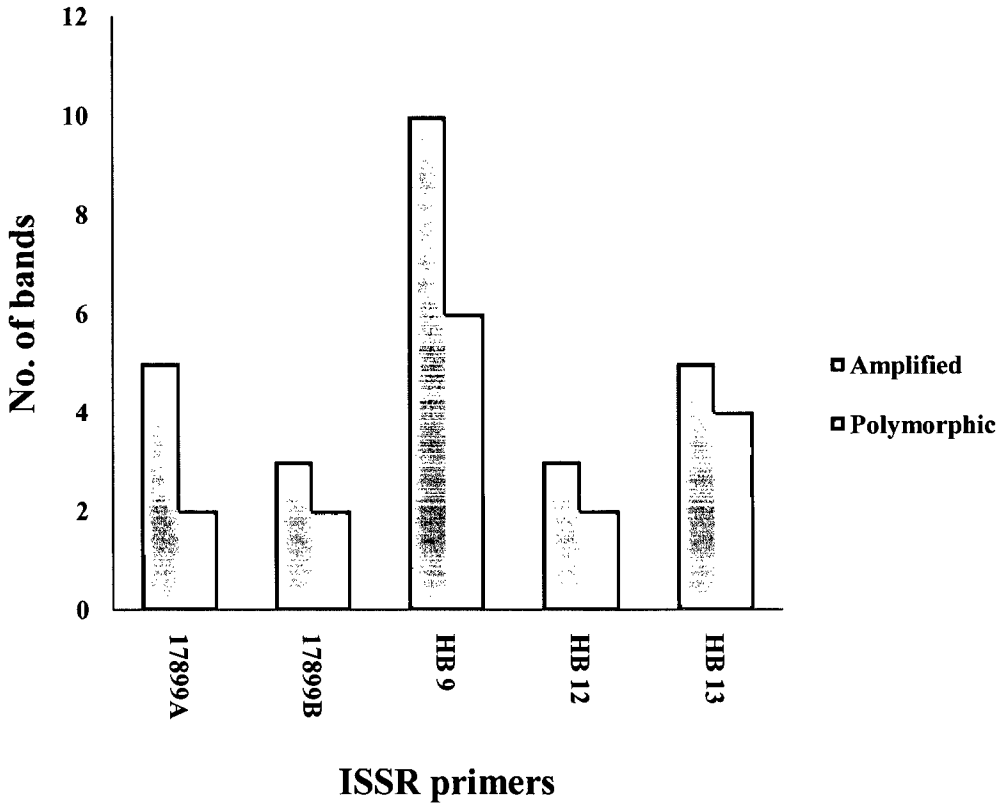
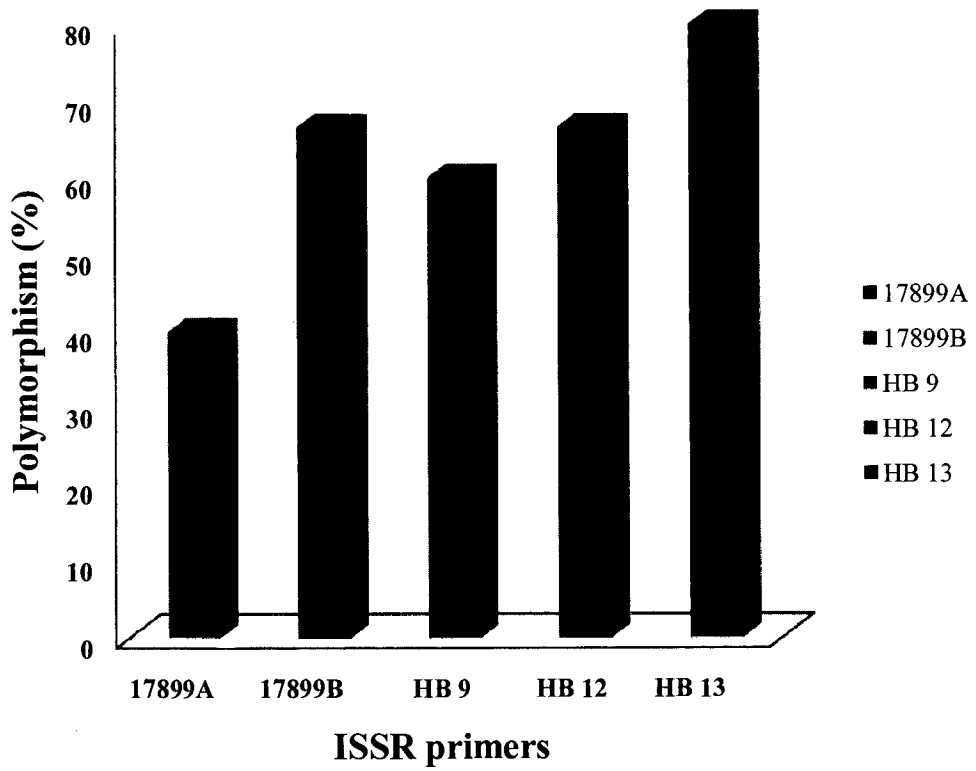


Table 30. Amplified bands, polymorphic bands and percentage polymorphism in ISSR analysis of *Rhynchostylis retusa.*

Sl. No.	Primers	No. of amplified bands	No. of polymorphic bands	Polymorphism %
1	17899A	5	2	40.0
2	17899B	3	2	66.6
3	HB 9	10	6	60.0
4	HB 12	3	2	66.6
5	HB 13	5	4	80.0
6	17898 B	2	-	-
7	HB 15	2	-	-
	Total	**30**	**16**	
	Mean	**4.28**	**3.2**	**62.6**

Fig. 22. Percentage polymorphism in ISSR analysis of *Rhynchostylis retusa*.



4. 4. 2. 2. 1. Genetic identity and cluster analysis

The identity values of seven populations determined by using ISSR data similar to that of RAPD analysis of *R. retusa* are given in Table 31. The identity values varied from 0.733 to 0.933 with the average of 0.844. Populations of Ponsuli and Valpoi shared the maximum identity (0.933) while, the lowest genetic identity value of 0.733 was obtained in Salauli and Loutulim populations.

Dendrogram (Fig. 23) was generated using ISSR data similar to that of RAPD analysis. The dendrogram generated revealed that the populations under study were grouped into three major clusters based on the geographical locations. Populations of Pernem and Loutulim formed the first cluster as they are located in the plains while, populations close to hilly areas i.e. Valpoi and Shiroda grouped together as a second cluster. Three populations of Ponsuli, Vagueri 1 and Salauli formed third cluster since they are occurring in the dense forests of the Western Ghats.

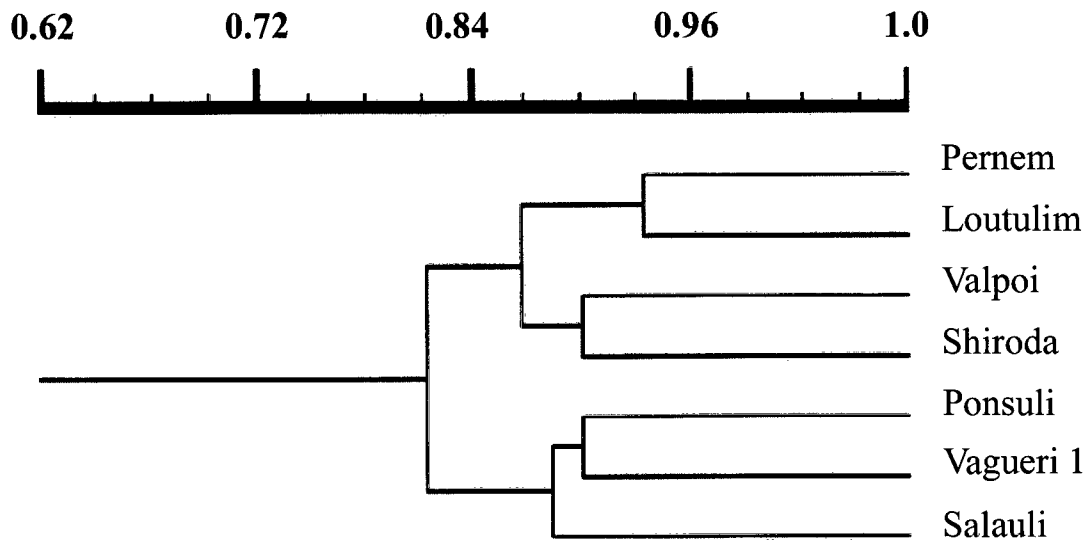
4. 4. 2. 3. Protein profile analysis using SDS-PAGE

Seven different populations of *R. retusa* were analysed using SDS-PAGE analysis (Plate 21). Total of 45 bands were detected in SDS-PAGE analysis with an average of 6.42 bands per population. Of these 32 bands with an average of 5.33 bands per population was polymorphic. Details of total and polymorphic bands are given in Table 32 and Fig. 24. Maximum number of bands (10) was recorded in the populations of Pernem and Salauli while, Ponsuli population recorded minimum number of bands (2). The percentage polymorphism across all the populations varied from 60 to 80% with an average of 72.5% (Fig. 25). Highest polymorphism of 80% was observed in three populations, namely, Pernem, Salauli and Shiroda. The lowest polymorphism of 60% was recorded in Valpoi and Loutulim populations.

Table 31. Genetic identity of *Rhynchostyis retusa* populations based on ISSR analysis.

	Ponsuli	Pernem	Salauli	Valpoi	Shiroda	Loutulim	Vagueri 1
Ponsuli	---						
Pernem	0.800	---					
Salauli	0.866	0.800	---				
Valpoi	0.933	0.800	0.866	---			
Shiroda	0.900	0.833	0.900	0.833	---		
Loutulim	0.866	0.866	0.733	0.800	0.833	---	
Vagueri1	0.833	0.900	0.766	0.900	0.800	0.900	---

Fig. 23. Dendrogram of Nei's genetic identities between the populations of *Rhynchostylis retusa* based on ISSR data.



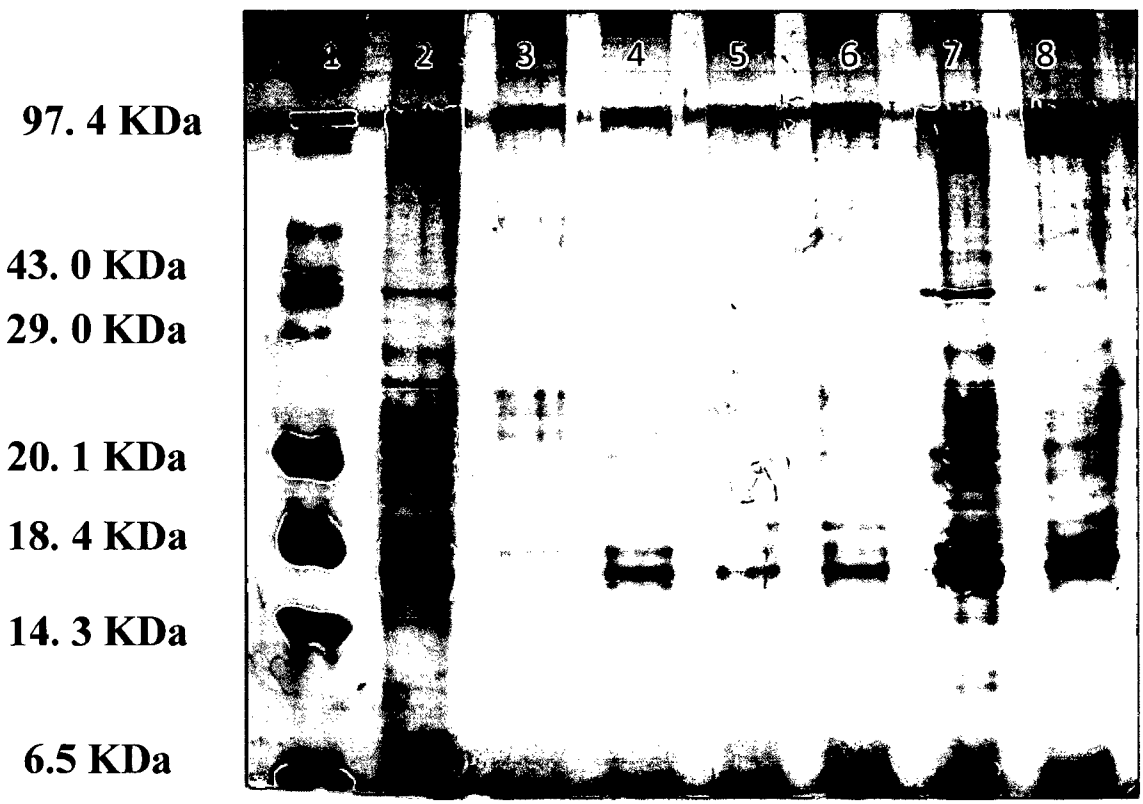
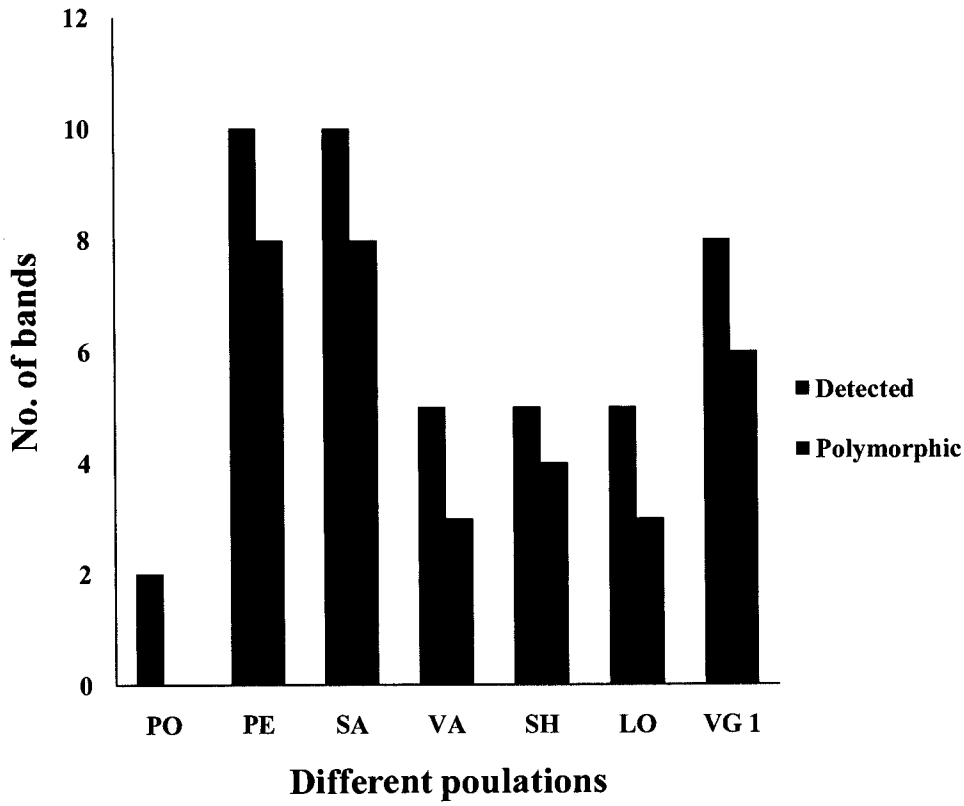


Plate 21. SDS- PAGE protein profile of *Rhynchostylis retusa*; Lane 1. Molecular weight standard; 2. Pernem; 3. Ponsuli; 4. Valpoi; 5. Loutulim; 6. Shiroda; 7. Salauli; 8. Vagueri 1.

Table 32. Detected, polymorphic bands and percentage of polymorphism in SDS-PAGE analysis of *Rhynchostylis retusa*.

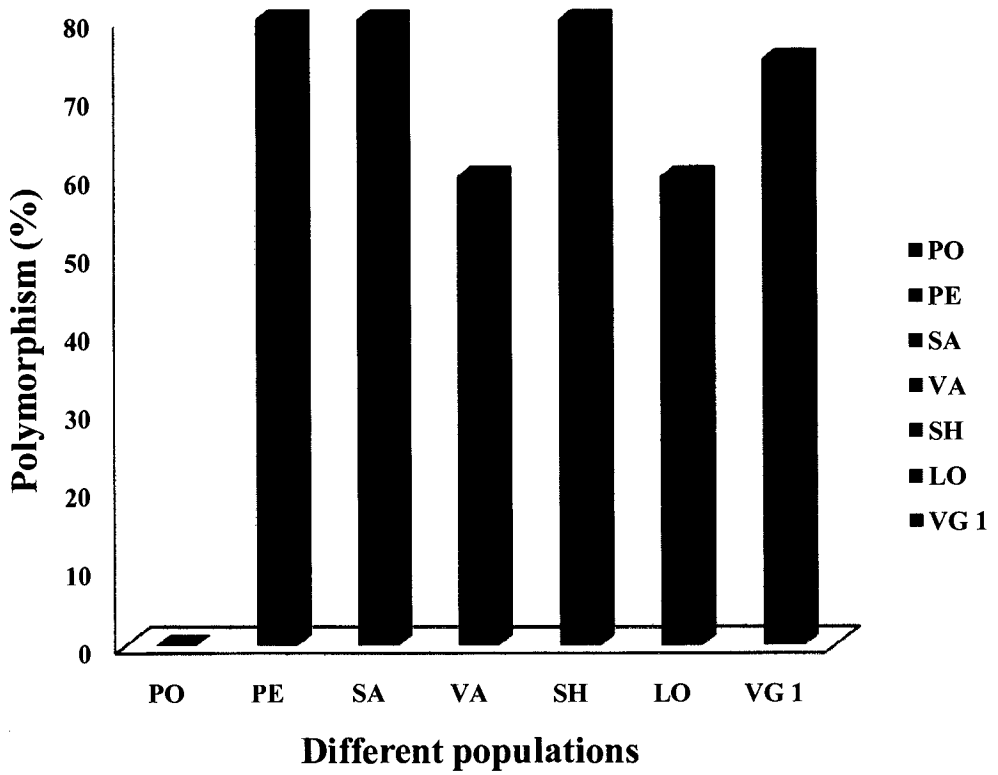
Sl. No.	Populations	No. of detected bands	No. of polymorphic bands	Polymorphism %
1	Ponsuli	2	-	-
2	Pernem	10	8	80.0
3	Salauli	10	8	80.0
4	Valpoi	5	3	60.0
5	Shiroda	5	4	80.0
6	Loutulim	5	3	60.0
7	Vagueri 1	8	6	75.0
	Total	**45**	**32**	**-**
	Mean	**6.42**	**5.33**	**72.5**

Fig. 24. Detected and polymorphic bands in SDS-PAGE analysis of *Rhynchostylis retusa.*



PO. Ponsuli; PE. Pernem; SA. Salauli; VA. Valpoi; SH. Shiroda; LO. Loutulim;VG 1. Vagueri 1.

Fig. 25. Percentage polymorphism in SDS-PAGE analysis of *Rhynchostylis retusa*.

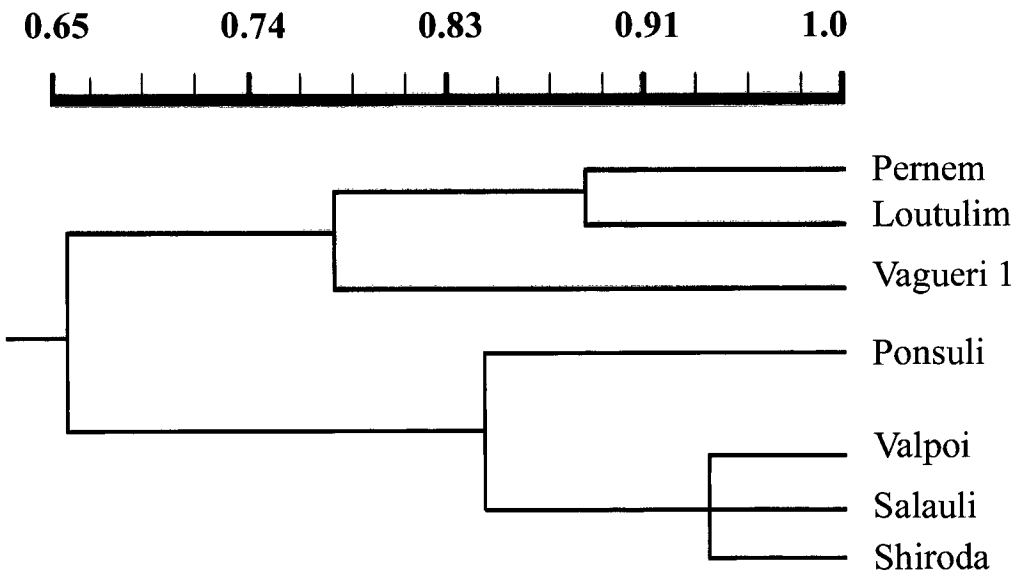


PO. Ponsuli; PE. Pernem; SA. Salauli; VA. Valpoi; SH. Shiroda; LO. Loutulim; VG 1. Vagueri 1.

Table 33. Genetic identity of *Rhynchostylis retusa* populations based on SDS-PAGE analysis.

	Pernem	Ponsuli	Valpoi	Loutulim	Shiroda	Salauli	Vagueri 1
Pernem	---						
Ponsuli	0.501	---					
Valpoi	0.702	0.912	---				
Loutulim	0.976	0.912	0.956	---			
Shiroda	0.702	0.912	0.956	0.945	---		
Salauli	0.702	0.501	0.702	0.702	0.702	---	
Vaugeri 1	0.951	0.828	0.957	0.957	0.957	0.951	---

Fig. 26. Dendrogram of Nei's genetic identities between the populations of *Rhynchostylis retusa* based on SDS-PAGE data.



4. 4. 2. 3. 1. Genetic identity and cluster analysis

SDS-PAGE data of seven populations were used to compute the genetic identity values, similar to that of RAPD analysis. The genetic identity values (Table 33) among all the populations ranged from 0.501 to 0.976 with an average of 0.839. Populations located in the plains (Pernem and Loutulim) shared the maximum genetic identity (0.976) while, population which is part of the Western Ghats (Ponsuli) and population located in the plains (Pernem) shared the least genetic identity value (0.501).

Dendrogram was generated by using SDS-PAGE data (Fig. 26). In this case, similar to ISSR analysis, populations were grouped on the basis of geographical locations. Two major clusters were observed comprising of three and four populations respectively. The first cluster was formed between populations of Valpoi, Salauli, Shiroda and Ponsuli. Valpoi and Shiroda populations were close to the Western Ghats, while, Salauli and Ponsuli were part of the Western Ghats. The second cluster comprised of the populations of Pernem, Loutulim and Vagueri 1. Pernem and Loutulim populations were located in the plains, however, grouping of Vagueri population could not be explained.

DISCUSSION

5. DISCUSSION

5. 1. Collection, Documentation and Taxonomy

Orchidaceae is the largest and most diverse family of flowering plants, consisting of 30,000-35,000 species belonging to 600-800 genera (Freudenstein and Rasmussen, 1999; Hajra, 2001; Singh *et al.*, 2007). It is estimated that India has about 1141 species of orchids belonging to 166 genera (Bhanwra *et al.*, 2006). In the present study, altogether 49 locations in three talukas of Western Ghat regions of Goa were surveyed for the collection of orchid species (Table 1; Fig. 1). The identified specimens belong to 20 genera and 26 species. However, Rao (1986) reported 18 genera and 29 species covering entire Goa, Diu, Daman, Dadra and Nagarhaveli regions. Among the collected orchids, *Dendrobium* and *Habenaria* genera were found to be with three species each (Table 12).

Among the above collected orchids, *Aerides maculosa* and *Rhynchostylis retusa* were selected for micropropagation studies due to their attractive colour and long inflorescences. *A. maculosa* is one of the most important epiphytic orchids, valued for its beautiful inflorescence/flowers (Murthy and Pyati, 2001). This species is endemic to South India and its natural populations are declining due to overexploitation (Rao, 1998). *R. retusa,* another epiphytic orchid seen in the forests of Western Ghats having beautiful flowers arranged in racemose inflorescence, considered as one of the important Indian ornamental orchids (Vij *et al.*, 1984; Thomas and Michael, 2007). Most of the orchids have been over-exploited by horticulturists and traders due to their great diversity in structure, colour and longevity of flowers (Xiaohong *et al.*, 2007).

5. 2. Micropropagation

In spite of a very large number of seeds produced by the orchids in nature, only 0.2-0.3% germinates in nature (Singh, 1992). Hence, the development of efficient protocols for *in vitro* mass multiplication is essential for the conservation and commercial utilization of orchid species.

5. 2. 1. Explants

Production of orchids through tissue culture forms the basis for the entire horticultural and floricultural trade (Yan *et al.*, 2006). Asymbiotic seed germination has emerged as an important tool for propagating a large number of orchids and hybrids (Arditti *et al.*, 1982). Germination of immature seeds of orchids in, *in vitro* is referred to as ovule/embryo/green pod/green fruit culture (Sagawa, 1963). Green pod culture technique has been considered as one of the major advancement in orchid seed culture (Sharma *et al.*, 2005). In this technique, immature seeds from the green capsules, prior to dehiscence are cultured *in vitro* on a medium (Sharma *et al.*, 2005). Immature seeds are removed from the pod, which have progressed 1/2 to 2/3 in their development from fertilization to maturity (De *et al.*, 2006).

In the present study, immature seeds were obtained from the capsules of *A. maculosa* (Plate 1a) and *R. retusa* (Plate 6a). Seeds were placed on VW medium supplemented with various plant growth regulators and different concentrations of coconut water for callus induction (Tables 13, 16). Immature seeds were scooped out from the capsule 5-10 weeks after pollination (WAP) for *A. maculosa* and 10-20 WAP in case of *R. retusa* for *in vitro* culture.

The advantages of using immature seeds from green capsules for micropropagation have been reported by several workers, since it is easy to surface-sterilize (Van Waes and Debergh, 1986; Yam and Weatherhead, 1988; Mitchell, 1989), increased rate of seed germination, early start by immature seeds, immature seed culture helps in getting seedlings from wide crosses where embryos in mature seeds often get aborted and loss of seeds by sudden natural dehiscence (De *et al.*, 2006). Temjensangba and Deb (2005) used immature seeds (16-18 weeks after pollination) of *Arachnis labrosa,* an epiphytic orchid, to develop protocol for regeneration and mass multiplication. Several other workers established protocol for mass multiplication of different orchids using immature seeds (Nagaraju and Mani, 2005; Chen *et al.*, 2005; Kalimuthu *et al.*, 2007; Thomas and Michael, 2007).

Plant regeneration using different explants such as leaf (Vij *et al.*, 1984) and immature seeds (Thomas and Michael, 2007) have been established in *R. retusa* while, in *A. maculosa* plant regeneration were obtained using leaf segments as explant (Murthy and Pyati, 2001). However, there are no reports available on callus induction and regeneration of plantlets from the callus via protocorm-like bodies (PLBs) for the plant chosen in the present study. It was also reported that in orchids, the regeneration of plantlets from the callus usually occurred through PLB formation involving somatic embryogenesis (Begum *et al.* 1994; Chang and Chang 1998). Hence, attempt has been made in the present study for the callus induction using immature seeds of these orchid species and also to develop and standardize the protocol for *in vitro* regeneration and mass multiplication via PLB formation.

5. 2. 2. Induction of callus

In orchids, *in vitro* propagation involving an intermediary callus phase is considered as difficult morphogenetic pathway. Limited success has been reported on callus culture in orchids, this is mainly due to the limited growth and high necrosis of callus (Kerbauy, 1984).

In the present study, coconut water was more effective for callus induction than 2,4-D, NAA or IAA. Callus formation was observed in all the three basal media in presence of coconut water. However, better response was noted in the case of VW medium in presence of coconut water. Percentage callus induction in VW media ranged from 77 to 81% (*A. maculosa*, Fig. 3) and 72 to 80% (*R. retusa,* Fig. 6), which was much higher than the other media tested. Amongst the coconut water treatments, VW media supplemented with coconut water of 15%v/v was found to be most effective for callus induction in both species (Tables 13, 16). Large quantities of soft, friable and yellowish green callus were produced on VW medium supplemented with 15% coconut water. However, callus induction in the absence of PGRs was reported earlier in *Dendrobium fimbriatum* (Mitra *et al.*, 1976; Roy and Banerjee, 2003) and *Dendrobium chrysotoxum* (Roy *et al.*, 2007).

Ishii *et al.* (1998) produced large number of calli in orchid *Phalaenopsis* when cultured on VW medium supplemented with CW (20%). Coconut water contains considerable amount of auxins and cytokinins (Arditti and Ernst, 1993) and is required for the maintenance of callus (Chen and Chang, 2000; Lin *et al.*, 2000; Lee and Lee, 2003; Huan *et al.* 2004; Huan and Tanaka, 2004; Zhao *et al.*, 2008). Therefore, logical explanation for induction of callus in coconut water treatment would be presence of auxins and cytokinins.

It has been reported that necrosis is a major problem faced during the callus culture of orchids (Lu, 2004; Wu *et al.*, 2004; Roy *et al.*, 2007). Absence of essentially required plant growth regulators during callus phase usually leads to necrosis (Roy *et al.*, 2007). However, complete absence of necrosis in *Dendrobium fimbriatum* was reported by Roy and Banerjee (2003). Similarly, in the present study we observed absence of necrosis in all the treatments used for callus induction.

These results suggest that the callus induction in *A. maculosa* and *R. retusa* are influenced by the coconut water in the medium and optimum concentration is essential for maximum amount of callus formation.

5. 2. 3. Regeneration

Vij and Kaur (1992) reported plant regeneration from callus culture in orchids via PLB formation which is an intermediary somatic embryo phase. This type of morphogenetic development has been reported in a number of orchid species by various workers (Colli and Kerbauy, 1993; Chen and Chang, 2000; Wu *et al.*, 2004; Roy *et al.*, 2007; Zhao *et al.*, 2008).

It was reported in orchids that the differentiation of PLBs from callus occur in two different ways. In some orchid species, PLB formation requires exogenous plant growth hormones (Lee and Lee, 2003; Lu, 2004; Wu *et al.*, 2004; Zhao *et al.*, 2008) while, in some species PLB differentiation from the callus occurs in the absence of plant growth hormones (Roy and Banerjee, 2003; Huan *et al.*, 2004).

Anjum *et al.* (2006) reported that cytokinin and auxin ratio plays a vital role in the differentiation of callus into PLBs. In the present study, we observed that the callus differentiation into PLBs depends on concentration of cytokinins and auxins used.

In *Aerides maculosa* regeneration ability of the induced callus was tested on VW medium supplemented with 15% CW in presence of different growth regulators (Table 14). More number of protocorm-like bodies (average 13.93/callus) were produced by the medium containing BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) while, less number of PLBs (average 5.33/callus) was produced by the medium containing BA (2 mg l^{-1}) + NAA (1 mg l^{-1}). Poor results were observed in the medium containing BA (2 mg l^{-1}) + NAA (2 mg l^{-1}) (Table 14, Medium VI, IV and V).

Reduction in number of PLB formation was observed in cytokinin combinations similar to that of auxin and cytokinin combinations. Maximum number of PLBs per callus (11.17) was observed on medium containing BA (1 mg l^{-1}) + KIN (1 mg l^{-1}). No significant difference in number of PLBs formation was observed in medium containing BA (2 mg l^{-1}) + KIN (1 mg l^{-1}) (10.77/callus) and BA (2 mg l^{-1}) + KIN (2 mg l^{-1}) (9.3/callus). However, less number of PLBs (average 5.17/callus) was produced on medium containing BA (5 mg l^{-1}) + KIN (2 mg l^{-1}) (Table 14, Medium VIII, IX, X and XI).

In *R. retusa,* the induced callus was tested on MS medium supplemented with 15% CW in presence of different growth regulators for its regeneration potential (Table 17). The study resulted in similar observation to that of *A. maculosa.* The best response of PLB formation (15.83 PLBs/callus) was observed in case of medium containing BA (1 mg l^{-1}) + NAA (1 mg l^{-1}). Less number of PLBs were produced (average 10.4/callus) on a medium

containing BA (2 mg l^{-1}) + NAA (1 mg l^{-1}) while, reduction in number of PLBs formation (average 5.33/callus) was observed in the presence of BA (2 mg l^{-1}) + NAA (2 mg l^{-1}). In case of medium containing higher concentration of BA (5 mg l^{-1}) along with NAA (2-5 mg l^{-1}), PLB formation was not observed.

MS medium supplemented with BA and KIN combination (BA 2 mg l^{-1} + KIN 2 mg l^{-1}), produced more number of PLBs (average 11.6/callus). It was followed by 2 mg l^{-1} BA and KIN 1 mg l^{-1} (average 10.8/callus) while, less number of PLB formation (average 8.13/callus) was observed on a medium containing BA (1 mg l^{-1}) + KIN (1 mg l^{-1}). On contrary to higher concentration of BA and NAA which did not produce any PLBs, BA (5 mg l^{-1}) + KIN (5 mg l^{-1}) and BA (5 mg l^{-1}) + KIN (2 mg l^{-1}) produced PLBs of 4.53 and 10.4/callus respectively (Table 17, Medium VII to XI).

Requirement of auxins and cytokinins for callus differentiation into PLBs are well known and reported in number of species: *Cymbidium ensifolium* (Chang and Chang, 1998; Chang and Chang, 2000), *Paphiopedilum* hybrid (Lin *et al.*, 2000), *Dendrobium fimbriatum* (Roy and Banerjee, 2003), *Pleione formosana* (Lu, 2004), *Cymbidium* (Huan and Tanaka, 2004; Huan *et al.*, 2004), *Vanilla planifolia* (Janarthanam and Seshadri, 2008).

Similarly, in the present study we report best response in BA and NAA combinations in both the species. In *Aerides maculosa* maximum number of PLBs (13.93) per cultured callus was produced on VW medium containing BA (1 mg l^{-1}) and NAA (1 mg l^{-1}) (Table 14; Fig. 4; Plate 3b-d). Similarly, in *R. retusa,* PLB formation was maximum (15.83 PLBs per callus) on MS medium supplemented with 1mg l^{-1} each of BA and NAA (Table 17; Fig. 7; Plate 7b-d). The synergistic effect observed in the combinations of BA

(1 mg l^{-1}) and NAA (1 mg l^{-1}) to form maximum number of PLBs in both the species agreed to other orchid species studied. It was reported that the leaf callus of *Vanilla planifolia* transferred to MS basal medium supplemented with 13.32 μM BAP and 13.43 μM NAA showed greatest number shoot regeneration (Janarthanam and Seshadri, 2008).

The synergistic action of auxin and cytokinin for direct PLB formation of was also reported in *R. retusa* using juvenile leaf segment (Vij *et al.*, 1984). Similarly, Seeni and Latha (2000) reported maximum number of PLB formation in presence of BA (8.8 μM) and NAA (4.1 μM) from the seedling leaves of *Vanda coerulea*. The combination of BA 1 mg l^{-1} with an auxin NAA 0.5 mg l^{-1} was found to be more effective in regeneration of *Dendrobium firmbriatum* via PLB formation (Roy and Banerjee, 2003).

In the present study, it was observed that PLBs obtained on medium containing BA and NAA combinations developed into well rooted plantlets after 8-10 weeks in the same media for both the species (Table 14, Medium IV; Table 17, Medium IV). Similarly, development of plantlets after PLB formation from callus was reported earlier in other orchid species such as *Dendrobium fimbriatum* (Roy and Banerjee, 2003), *Cymbidium* (Huan and Tanaka, 2004; Huan *et al.*, 2004) and *Dendrobium candidum* (Zhao *et al.*, 2008).

In the present study, coconut water was used for PLB formation, differentiation and plantlet development. Beneficial effect of coconut water in the form of sugar, natural cytokinins and vitamins promotes PLBs formation, differentiation and plantlet formation (Lawrence and Arditti, 1964; McIntyre *et al.*, 1974; Matthews and Rao, 1980; Sarma, 2002; Sharma *et al.*, 2005; De *et al.*, 2006).

5. 2. 4. Acclimatization

Well developed plants with 3-4 leaves and 1-2 roots were used for the acclimatization process. In the present study, regenerated plants were acclimatized to an 'intermediate stage' between *in vitro* and *ex vitro*. The technique of intermediate stage consists of keeping the well developed plantlets on the layer of charcoal pieces (about 5 to 7 cm thick) in conical flask with 10 ml of 1/10 diluted Vacin and Went medium plugged with cotton plugs inside the culture room and later keeping the conical flask open without cotton plug. Batty *et al.* (2001, 2004) reported that the beneficial effect of 'intermediate stage' for maintaining high humidity for plantlets survival in Australian terrestrial orchids, *Caladenia arenicola, Diuris magnifica, D. micrantha, Pterostylis sanginea* and *Thelymitra manginiorum* and Zettler *et al.* (2005) reported the similar observation in *Platanthera leucophaea*. Requirement of pre-hardening medium containing paclobutrazol (0.25 mg l^{-1}) and activated charcoal (1.5 mg l^{-1}) for the period of two months was reported in *Zygopetalum intermedium* (Nagaraju and Mani, 2005). The plantlet were successfully transplanted in greenhouse condition and 60% of plantlets survived after 3 months of transplantation in the both species in the present study (Plates 5, 9). Similarly, 60% of survival rate was reported in the case of *Arachnis labrosa* (Temjensangba and Deb, 2005). Maximum survival rate was observed in the combinations of charcoal pieces, brick pieces and coconut husk. This was mainly due to retention of optimum moisture, better drainage and good aeration which provided suitable conditions for establishment and growth of plantlets (Nagaraju and Mani, 2005). As precautionary measures, plants were sprayed with 0.1% bavistin and 2 ppm of Ceftriaxone as antibiotic once in 15 days to prevent fungal and bacterial infection. Similar to our treatment *in vitro* plantlets of *Zygopetalum intermedium* was given bavistin (0.2%) treatment for 1 h before acclimatization (Nagaraju and Mani, 2005).

The present study shows that the calli of *A. maculosa* and *R. retusa* can be induced from immature seeds on media containing coconut water. PLB formation from the calli needs synergistic combination of BA and NAA and presence of coconut water while, development of plantlet from PLB does not require presence of any growth regulator. This provides an efficient *in vitro* multiplication method for *A. maculousm* and *R. retusa* from immature seeds. This callus-embryoid-plant regeneration system opens up new avenue for *in vitro* clonal mass multiplication of these potential ornamental orchid species.

5. 3. Molecular analysis

An understanding of the level of genetic variation within and among populations is essential for developing appropriate conservation and sustainable utilization strategies (Frankham *et al.*, 2002). Loss of genetic variation is a major problem in conservation biology since it can prevent a species from responding to natural selection which limits evolutionary potential of the species (Qamaruz-Zaman *et al.*, 1998). The plants of wide geographical locations and out crossing plants are believed to be with high genetic diversity (Hamrick and Godt, 1990). It was also reported that high genetic diversity in natural populations is an indication of out breeding and evolutionary potential of particular species (Falk and Holsinger, 1991).

In the recent years, various markers have been used to measure genetic diversity which includes random amplified polymorphic DNA (RAPD) as in *Goodyera procera* (Wong and Sun, 1999), *Paphiopedilum micranthum* (Li *et al.*, 2002), *Phalaenopsis* (Goh *et al.*, 2005) and *Vanilla planifolia* (Minoo *et al.*, 2008); inter-simple sequence repeats (ISSR) as in *Cymbidium goeringii* (Xiaohong *et al.*, 2007; Wang *et al.*, 2008) and enzyme polymorphism as in *Amitostigma gracile* (Chung and Park, 2008). Sodium dodecyl

sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles have also been used as a marker to study genetic variation in *Brachiaria* (Neto *et al.*, 2002), Seabuckthorn (Ahmad *et al.*, 2003), *Brassica rapa* (Mukhlesur *et al.*, 2004), *Fagopyrum* (Rout and Chrungoo, 2007) and *Phaseolus vulgaris* (Igrejas *et al.*, 2009).

5. 3. 1. Genetic variation Study in *Aerides maculosa*

In this work, we compared the applicability of RAPD, ISSR and SDS-PAGE protein profile to characterize the population of *A. maculosa*. At the RAPD level, higher genetic variation of 90.45% polymorphism was detected (Table 19). The results are consistent with other comparative studies that reported more variation at RAPD level. Wong and Sun (1999) found higher variation in *Goodyera procera* by using RAPD (P=97%) in comparison with isozyme analysis. The average genetic identities between the populations of *A. maculosa* were lower (0.629) (Table 20) than the average reported for conspecific plant populations (0.956) (Gottlieb, 1981). The pattern of genetic variation in *A. maculosa* was also more comparable to that of predominant outcrosses species studied (Hamrick and Godt, 1996). Nybom (2004) demonstrated that life history traits, breeding systems and successional status had a significant impact on genetic diversity based on the RAPD studies. The possible explanations for high genetic diversity revealed in *A. maculosa* is that, it may be due to high inherent variability of the ancestral species, therefore, the current high genetic diversity possibly reflect the historical pattern of genetic variation in *A. maculosa.* However, relatively low genetic variation was also revealed by RAPD in other orchid species such as *Eulophia sinensis* (P=73.3%) (Sun and Wong, 2001), *Paphiopedilum micranthum* (P=73.3%) (Li *et al.*, 2002) and *Changnienia amoena* (P=76.5%) (Li and Ge, 2006).

ISSR technique is a very useful molecular tool for studying population genetics in broad range of plant species, as well as it helps in understanding the genetic variability among the populations due to its high sensitivity (Zietkiewicz *et al.*, 1994; Jarne and Lagoda, 1996; Fang and Roose, 1997; Raina *et al.*, 2001). This is the first report of the use of ISSR markers for analyzing the genetic variation and determination of genetic relationship in *A. maculosa*. In comparison with RAPD, ISSR markers showed lower genetic variation of 72.85% polymorphism (Tables 19, 22). Smith *et al.* (2002) reported very low intra-population polymorphism for a terrestrial woodland orchid *Tipularia discolor* in eastern United States based on ISSR markers with polymorphism ranging from 0.00 to 18.2%. Based on ISSR analysis, Wu *et al.* (2006) observed lower level of genetic variation (35.71%) in the cultivated populations of *Gastrodia elata*, a rare and endangered orchid. Genetic identity values based on ISSR (0.704) also confirms the RAPD estimates (0.629) (Table 23).

Use of SDS-PAGE protein profile to analyze the genetic variation of *A. maculosa* is also the first report. In comparison with RAPD and ISSR levels of genetic variations, much lower level of polymorphism (57.6 %) was recorded (Tables 19, 22, 24). Compared to this result, lower level of genetic variations of 31.3% was reported in *Brassica rapa* (Mukhlesur *et al.*, 2004). Use of SDS-PAGE protein profile analysis has been reported to study the genetic variations in different plant species such as *Brachiaria* (Neto *et al.*, 2002), *Vigna angularis* (Takehisa *et al.*, 2001) and *Oryza sativa* (Asghar *et al.*, 2004). Average genetic identity values of SDS-PAGE protein profile estimated to be 0.771 which also corroborates the RAPD and ISSR estimates (Table 25).

In the present study, among the three estimates i.e. polymorphism (P), polymorphic bands (PB) and genetic identities (GI), two estimates (P and PB) based on RAPD are much higher than the ISSR and SDS-PAGE protein profile. This particular observation is in agreement with the general findings by Nybom and Bartish (2000) and Nybom (2004). Theoretically, ISSR markers are considered superior to RAPD (Qian *et al.*, 2001). Lower levels of variation detected with ISSR markers may indicate that the ISSR loci are relatively more conserved in *A. maculosa* (Aga *et al.*, 2005).

5. 3. 1. 1. Genetic relationship between the populations of *Aerides maculosa*

The cluster analysis of *A. maculosa* based on RAPD, ISSR and SDS-PAGE protein profile analysis revealed that the populations were grouped on the basis of proximity to the Western Ghats and forest types.

The detailed cluster analysis using RAPD is given in Fig. 11. Populations of Pernem and Shiroda formed first cluster since, they belongs to open forest types. The populations of Vagueri 1 and 2 are closely related as they are dense forests of hilly areas of Western Ghats. Loutulim, which belong to non-forest type are branched out separately. Populations of dense forest areas (Ponsuli and Valpoi) are closely related. While, Salauli is another dense forest type have totally branched out separately from the other populations studied.

The dendrogram constructed showed the relationship of the different population based on the ISSR data (Fig. 14). Populations of Vagueri 1, 2 and Valpoi were clustered together as they are dense forest types of hilly areas of Western Ghats. While, populations belongs to open-forest type (Pernem) and non-forest type (Loutulim) were grouped together, however, grouping of dense-forest type (Salauli) with the open and non-forest

type could not be explained. Another open-forest type population (Shiroda) was totally branched out separately.

Dendrogram generated based on SDS-PAGE protein profile data revealed two major clusters based on the forest type and proximity to Western Ghats (Fig. 17). The first cluster comprised of populations of open-forest type (Pernem and Shiroda) together with non-forest type (Loutulim). While, populations of Vagueri 1, Salauli and Valpoi were closer as they belongs to dense-forest of hilly areas of Western Ghats. Ponsuli branched out separately since it belongs to dense-forest type.

The forest types that predominantly persist in Goa are dense and open type forest, inhabited by a number of terrestrial and epiphytic orchids (Rao, 1986; FSI, 1997). Grouping of open forest populations (Pernem, Shiroda and Loutulim) and dense forest of hilly area of Western Ghats (Vagueri 1, 2, Valpoi, Salauli and Ponsuli) shows that genetic structure of *A. maculosa* is independent of physical distance among the population sites (Fig. 2). Murren (2003) studied in *Catasetum viridiflavum* an epiphytic orchid the amount of genetic variation between island and mainland sites of the populations using allozyme to examine the differences in genetic structure associated with these two forest types. It was found that the physically isolated patches of this orchid comprised a single larger genetic population which was independent of physical distance among the sites.

The results obtained during the present study revealed high genetic variability and grouping of populations based on forest types and proximity to Western Ghats, which suggests that *A. maculosa* possesses high inherent variability and can reproduce across the geographical scale.

5. 3. 2. Genetic variation study in *Rhynchostylis retusa*

The present work evaluates the genetic variation, genetic distance and relationship among the populations of *R. retusa* using RAPD, ISSR and SDS-PAGE protein profile markers.The results indicated that the percentage of RAPD polymorphism (76.13%) was higher than that of ISSR analysis (62.6%) and SDS-PAGE protein profile (72.5%) (Tables 27, 30, 32). Similar to that of *A. maculosa*, *R. retusa* also showed higher genetic variation in RAPD, compared to ISSR and SDS-PAGE protein profile. Higher polymorphism observed using RAPD marker agrees with the finding reported in many other studies. Wong and Sun (1999) found higher genetic variation using RAPD (P=97%) in comparison with isozyme in *Goodyera procera*. Similarly, using allozyme and RAPD, Wallace (2002) reported higher polymorphism in *Platanthera leucophaea.*

The present study is the first report of the use of ISSR markers in measuring genetic variation and determination of genetic relationships in *R. retusa* populations. During the study 30 bands were detected using 8 primers, about 4.28 bands per primer. Furthermore average of 62.6% polymorphism was revealed across all the populations showing considerably low level of polymorphism compared to RAPD (76.13%) (Tables 27, 30). Similarly, Smith *et al.* (2002) reported very low intra-population polymorphism for a terrestrial woodland orchid *Tipularia discolor* in eastern United States based on ISSR markers, with polymorphism ranging from 0.00 to 18.2%.

Further, use of SDS-PAGE protein marker was also the first report to analyze the genetic variation and to determine the relationship among the populations. Higher level of polymorphism (72.5%) was detected compared to ISSR, but was lower to RAPD (76.13%)

analysis (Tables 27, 30, 32). It was observed that 32 bands with an average of 5.33 bands per population were detected.

The present study compares the applicability of RAPD, ISSR and SDS-PAGE protein profile to characterize the populations of *Rhynchostylis retusa*. The results suggest that RAPD markers are superior to ISSR and SDS-PAGE protein profile in revealing more polymorphic bands. Similar to *A. maculosa*, higher genetic variation was observed in RAPD may be due to high inherent variability of the ancestral species. Secondly, greater genetic variation in RAPD marker may be attributed to the fact that many loci are amplified, providing a genome-wide survey (Williams *et al.*+, 1990). However, lower levels of genetic variation detected with ISSR markers indicates that the ISSR loci may be relatively more conserved in *R. retusa* (Aga *et al.*, 2005).

5. 3. 2. 1.Genetic relationship between the populations *Rhynchostylis retusa*

The present study compared the applicability of RAPD, ISSR and SDS-PAGE protein profile analysis to characterize the populations of *R. retusa*. The dendrogram generated by RAPD agrees better with the altitude of the population under study. The altitudes of the sample collection sites widely varied between the populations and it ranged from 10 to 295 meters (Table 7). Interestingly, almost similar altitude populations of Salauli (76m) and Valpoi (71m) clustered together, while, Shiroda (102m), Loutulim (103m) formed second cluster. The populations of Pernem (15m) and Ponsuli (10m) branched out separately from the major two clusters. In addition, population of Vagueri 1 has totally branched out from all other populations. The altitude of this population is much higher (295m) in comparison with all other populations and this may be the reason why the Vagueri 1 population branched out separately. It was interesting to note that the clustering

was observed based on the altitude irrespective of physical distance between the populations.

Relatively low genetic identity of Vagueri 1 population with other populations signifies its isolation due to higher altitude (Table 7). Genetically distinct isolated populations were also reported in *Paphiopedilum micranthum* from Southwestern China (Li *et al.*, 2002) and also in the case of *Laelia speciosa*, an endangered orchid from Mexico using isozyme studies (Diaz and Oyama, 2007). The distinctiveness of Vagueri population may represent unique evolutionary process.

The highest genetic distance was observed between populations of Vagueri 1 and Shiroda. These two populations are from different regions indicating that geographical isolation is one of the important factors for the observed genetic distance. Shiroda and Loutulim populations have the closest genetic identities as they were ecologically and geographically close to each other (Fig. 20) while, Salauli and Valpoi populations coming under Western Ghats belt region of Goa state shares next highest genetic identities. Ponsuli and Pernem populations were close to each other as they are confined to coastal area and also at low altitude level (Table 7).

Dendrogram generated by ISSR marker agrees with the geographical location of the populations (Fig. 23). It was observed that the populations located in the plains were grouped together (Pernem and Loutulim) forming the first cluster. Populations close to the hilly areas of Western Ghats i.e. Valpoi and Shiroda formed the second major cluster. However, Ponsuli, Salauli and Vagueri 1 populations formed the separate cluster since they are the parts of Western Ghats of Goa region.

Population of Ponsuli and Valpoi shared the maximum identity as these populations remain as a part of hilly areas surrounding the Western Ghat of Goa region. It was also obvious that the populations located in two different regions i.e. plains (Loutulim) and hilly part of Western Ghats (Salauli) shared the lowest genetic identity.

Similar to ISSR analysis dendrogram generated in using SDS-PAGE protein profile also agrees with the geographical locations (Fig. 26). Four populations namely, Valpoi, Salauli, Shiroda and Ponsuli formed the first major cluster. Two populations namely, Valpoi and Shiroda were close to the Western Ghats whereas Salauli and Ponsuli were part of the Western Ghats. Populations located in the plains (Pernem and Loutulim) along with Vagueri 1 were grouped together as the second cluster. Clustering of Vagueri 1 with Pernem and Loutulim populations could not be explained. Populations located in the plains (Pernem and Loutulim) shared the maximum genetic identity (0.976) while, population which is part of Western Ghats (Ponsuli) and population located in the plains (Pernem) shared the least genetic identity (0.501).

The combined ISSR and RAPD data of *A. maculosa* obtained from the same localities as that of *R. retusa* clearly separated populations of hilly and forest areas from the remaining areas. It showed that pattern of gene flow is more or less identical as both are epiphytic and are from the same localities (Parab *et al.*, 2008; Parab and Krishnan, 2008).

Goa is a small state with an area of 3702 sq. km. and having length of 105 kms and width of 60 km. Goa is a major iron ore exporting state and over 60% of country's iron ore export is from Goa. The mining belt of Goa covers approximately 700 sq. km. and it is mostly concentrated in four talukas namely, Bicholim of North Goa district and Salcete,

Sanguem and Quepem of South Goa district. The major threat to orchid flora in the state of Goa is deforestation through felling of forest trees and mining of valuable economic resource such as iron ore. In case of epiphytic orchids the loss of the host tree species are bound to result in the elimination of species.

The present study reveals that within the small region taken up for study, higher genetic variability exists amongst the populations of *Aerides maculosa* and *Rhynchostylis retusa*. Information on genetic diversity will contribute to a better understanding of the genetic profile that can be used to develop strategies for its conservation and sustainable utilization and also forms starting point for future research on the population and evolutionary genetics of these species.

CONCLUSIONS

CONCLUSIONS

Goa being in the mining belt, the collection and documentation of orchid species belongs to 20 genera and 26 species is important from the conservation point of view. Since Goa is continuously under the influence of environmental pollution and degradation of forest, development of *in vitro* propagation methods will contribute to the conservation and commercialization of orchids. The present study also proves that higher genetic variability exist among the populations of these species. To maintain this diversity, exploitation of wild populations from the hands of collector's should be stopped to ensure the continuous survival of these species. The present *in vitro* micropropagation protocol developed will contribute enormously towards their conservation.

SUMMARY

During this study, field survey and collection trips were made to Western Ghats talukas of Goa namely Sattari, Sanguem and Canacona for the collection of orchid species. Identified orchids belonged to 20 genera and 26 species. Among the collected orchids, two species namely, *Rhynchostylis retusa* (L.) Bl. and *Aerides maculosa* Lindl. were selected for micropropagation, biochemical and molecular studies due to their attractive flower colour and long inflorescences.

In vitro propagation protocol was developed for these species. The callus embryoid plant regeneration was developed using immature seeds. Immature capsules of 5-10 weeks after pollination (*Aerides maculosa*) and 10-20 weeks after pollination (*Rhynchostylis retusa*) were used for callus induction. In both the species best response of callus induction was observed on Vacin and Went medium supplemented with 15% coconut water.

In *A. maculosa*, regeneration ability of the induced callus were evaluated by sub culturing on VW medium while, in the case of *R. retusa* it was evaluated on MS medium. These basal medium was supplemented with various concentrations and combinations of cytokinins and auxin and cytokinin combinations. In *A. maculosa*, maximum number of PLBs of 13.93 per callus was noted on VW medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW. Among cytokinin combinations BA (1 mg l^{-1}) + KIN (1 mg l^{-1}) + 15% CW produced maximum number of PLBs (11.17 per callus). Produced PLBs got converted into plantlets after 8 to 10 weeks of culture only in the case of auxin and cytokinin combinations. Better response was observed in case of BA (1 mg l^{-1}) + NAA (1mg l^{-1}) + 15% CW. While, PLBs formed on VW medium with different combinations of cytokinin alone became brown and necrotic upon sub-culturing.

In *R. retusa*, better response of PLB formation from the callus was observed on the MS medium. Maximum number of PLBs (15.83 per callus) were noted in MS medium supplemented with BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW. Maximum PLBs (11.6 per callus) was observed among the cytokinin combinations in presence of BA (2 mg l^{-1}) + KIN (2 mg l^{-1}) + 15% CW. Better response for the conversion of PLBs to plantlet was observed in case of auxin and cytokinin combinations. Combination of BA (1 mg l^{-1}) + NAA (1 mg l^{-1}) + 15% CW was recorded better response. No response of PLB conversion to plantlet was observed in BA and KIN combination, on the contrary PLBs become brown and necrotic.

In vitro regenerated plantlets of both the species were acclimatized to green house conditions. The maximum survival rate of 60% was noted in the combination of charcoal pieces, brick pieces (1:1) and coconut husk.

In the present work, three different markers such as RAPD, ISSR and SDS protein profile was used to characterize the populations of both the species. Twenty RAPD and fifteen ISSR primers obtained from Operon Technology, USA were tested during this investigation.

In *A. maculosa* thirteen RAPD and six ISSR primers produced clear and reproducible banding pattern. The percentage of polymorphism (average 90.45%) was reported in RAPD analysis while, 72.85% was observed in ISSR analysis. SDS protein analysis resulted in 57.6% polymorphism. Among the populations genetic identity co-efficient ranged from 0.465 to 0.762, 0.500 to 0.975 and 0.220 to 0.980 in RAPD, ISSR and SDS protein profile respectively. Dendrogram constructed on the basis of all the three

markers revealed that the populations were clustered together based on the forest type of Goa and proximity to the Western Ghats.

In *R. retusa*, thirteen RAPD and seven ISSR primers gave clear and consistent banding pattern. The average percentage of polymorphism was recorded as 76.13% in RAPD, 62.65% in ISSR and 72.5% in SDS protein analysis. Genetic identity varied from 0.405 to 0.932, 0.733 to 0.933 and 0.501 to 0.976 among all the populations in RAPD, ISSR and SDS protein analysis respectively. Dendrogram generated on the basis of RAPD analysis revealed that similar altitude populations were clustered together. While in case of ISSR and SDS protein analysis it was observed that the populations were grouped on the basis of geographical location.

The present study showed that the calli could be induced from immature seeds of *Aerides maculousm* and *Rhynchostylis retusa* on Vacin and Went medium in presence of coconut water. The formation of PLBs from the embryogenic calli needs synergistic combination of BA and NAA in presence of coconut water while, development of plantlet from PLB does not require any additional growth regulators. The present investigation of plant regeneration and mass multiplication via callus-embryoid-plant regeneration system may provide an alternative system which may ease the pressure off the waning wild populations.

Molecular and biochemical studies revealed that within the small region taken up for study, higher genetic variability exists amongst the populations of *Aerides maculosa* and *Rynchostylis retusa*. This information will contribute to a better understanding of the genetic profile that can be used to develop strategies for its conservation and sustainable utilization. It also forms a starting point for future research on the population and evolutionary genetics of these species.

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ssessment of genetic variation among populations of *Rhynchostylis retusa*, an epiphytic orchid from Goa, India using ISSR and RAPD markers

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Rhynchostylis retusa (L.) Bl., a monopodial epiphytic orchid species with attractive flowers arranged in racemose nflorescence, ranks among the important Indian ornamental orchids. Comparative population studies using PCR based narkers, RAPD and ISSR, were performed to assess the genetic diversity of the wild orchid. Among the 35 primers tested, 3 RAPD and 7 ISSR primers were selected for the analysis. In total, 74 RAPD and 30 ISSR fragments were generated. High level of polymorphism was recorded in RAPD (76.13%) than ISSR (62.6%). In case of RAPD, Nei's average genetic dentities value for different populations of *R. retusa* ranged from 0.405 to 0.932. While for ISSR, it ranged from 0.733 to 0.933. The results of the present study can be seen as starting point for future research on the population and evolutionary genetics of this species.

Keywords: *Rhynchostylis retusa*, RAPD, ISSR, genetic variation

duction

chidaceae is one of the largest families of ring plants[1] and orchids are marketed as both and cut flower. The largest exporters of potted ls are Taiwan, Thailand, the United Kingdom, Japan, New Zealand and Brazil, while the t importer of potted orchids is the United [2]. Orchids are currently the second most ole potted crop in the United States with a total sale value of $144 million[3]. In India, however, ulture is picking up as an industry with a turn of about, $ 47 million[4].

nchostylis retusa (L.) Bl. is a monopodial, ytic orchid species with beautiful flowers ged in racemose inflorescence, which rank the s among the important Indian ornamental ls (Fig. 1). Despite the tremendous diversity the family[5], very little is known about the e diversity within natural populations. Habitat ction, macroclimatic changes and shifting ation deplete orchid populations[6]. Many natural ations have been destroyed or restricted to r size for habitat destruction and fragmentation d by various developmental activities. erious effect of habitat destruction and over exploitation of wild orchids has been reported from different part of world[7-9]. Comparative population studies using DNA based marker systems are emphasized to collect information on the level and pattern of genetic diversity of wild orchids, which is the first step to facilitate their conservation. Knowledge about genetic diversity is considered the baseline for conservation[10].

To study the genetic variation in various plant species, a number of DNA based reliable marker systems have been developed. These markers have been used widely, either alone or in tandem with morphological markers, to obtain more consistent information on the existing genetic diversity with a number of species groups. The techniques such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) are used frequently to study plant diversity. RAPD and ISSR are two simple and quick techniques, where the former detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequence[11] and the latter permits detection of polymorphism in inter-microsatellite loci using a primer designed from dinucleotide or trinucleotide simple sequence repeats[12-13].

The present study is aimed to explore the extent of genetic variation among the wild populations of *R.*

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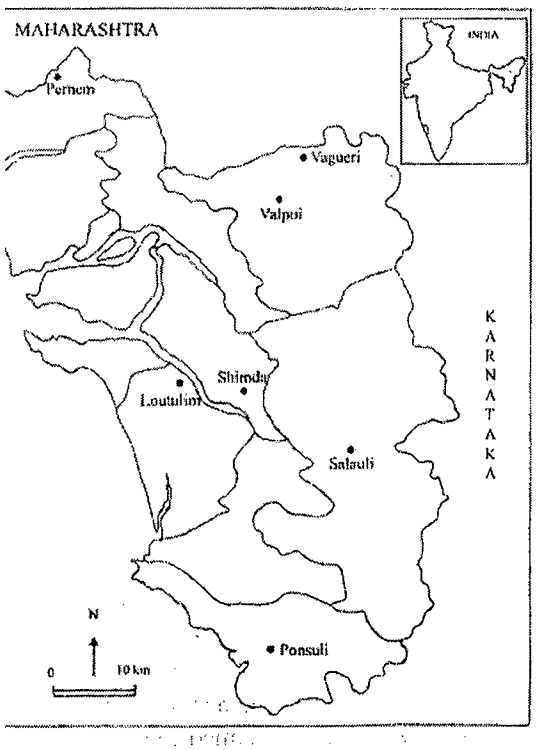
which would help in better understanding of : profile that can be used to develop strategies conservation and sustainable utilization.

ials and Methods

aterial

f samples of *R. retusa* representing seven nt natural populations in Goa were collected. were stored at 4°C in zip-lock plastic bags till

Fig. 1—*R. retusa* in flowering



—Map of Goa showing collection site of different ions

they were processed for DNA isolation. The locations from where the leaf samples are collected are illustrated in Fig. 2. List of populations, their altitude and other details are given in the Table 1.

Genomic DNA Extraction

DNA was isolated using a modified CTAB (cetyl trimethyl ammonium bromide) method[14]. For each accession, about 5 g of bulked leaf tissue from 50 plants was ground to a fine powder using liquid nitrogen, which was then suspended in 20 mL of extraction buffer (20 m*M* EDTA at *p*H 8.0, 100 m*M* Tris-HCl at *p*H 8.0, 1.5 *M* NaCl, 2% CTAB and 1% β-mercaptoethanol). The suspension was mixed well, incubated at 60°C for 45 min, followed by chloroform:isoamyl alcohol (24:1) extraction and precipitation with 0.6 volume of isopropanol at –20°C for 1 h. The DNA was pelleted down by centrifugation at 12,000 rpm for 10 min and was then suspended in TE buffer (10 m*M* Tris-HCl and 1 m*M* EDTA, *p*H 8.0). The DNA was purified from RNA and proteins by standard procedures[15], and its concentration was estimated by agarose gel electrophoresis and staining with ethidium bromide.

RAPD Amplification

Twenty RAPD primers obtained from Operon Technologies, USA were tested initially with randomly selected individuals from two populations. Thirteen primers that showed clear and reproducible results were chosen for the study. PCR amplification was performed in a 25 µL-reaction volume containing 50 ng of genomic DNA, 2.5 µL of 10-X Taq assay buffer (100 m*M* Tris-HCl, *p*H 8.3; 20 m*M* $MgCl_2$; 500 m*M* KCl; 0.1 % gelatin), 2.5 µL of 2.5 m*M* dNTPs, 15 ng of primers and 0.5 units of Taq polymerase (Bangalore Genei).

The mixture was subjected to PCR on Perkin-Elmer 480 thermal cycler programmed for an initial step of 3 min at 94°C, followed by 40 cycles of 94°C

Table 1—Collection sites of *R. retusa* populations

Populations	Latitude	Longitude	Altitude (m)
Ponsuli	14° 59' 56.9"N	74° 02' 34.6" E	10
Pernem	15° 43' 31.6"N	73° 47' 47.2" E	15
Salauli	15° 09' 28.01"N	74° 13' 47.7" E	76
Valpoi	15° 33' 26.01" N	74° 04' 36.01" E	71
Shiroda	15° 27' 31.01" N	73° 59' 55.6"E	102
Loutulim	15° 23' 06.6" N	73° 56' 22.5" E	103
Vagueri	15° 34' 59.8" N	74° 05' 47.6" E	295

n, 37°C for 1 min and 72°C for 2 min and 10
or final extension step at 72°C. The
ation products were analysed on 1.4%
gels with mol wt marker (1 Kb Marker,
ler™, MBI Fermentas, USA). The gel was
with ethidium bromide, visualized under
let light and photographed using gel
ntation system (Amersham Pharmcia Biotech
age master).

plification

n ISSR primers from Operon Technologies,
ia, USA was initially tested. Out of 15
7 showed clear and reproducible bands and
re used during the study. The amplification
formed in a 25 µL reaction volume containing
genomic DNA, 2.5 µL of 2.5 m*M* dNTP's, 25
of primer and 0.6 units of Taq polymerase.
enaturation was for 5 min at 94°C, followed
cles of 1 min at 94°C, annealing temperatures
or different primers (Table 2) and 2 min at
n additional cycle of 10 min at 72°C was used
ner extension. The PCR products were
l on 1.2% agarose gels and stained with
n bromide, photographed under ultraviolet
using gel documentation system. Mol wt was
d by using 1 Kb DNA ladder (GeneRuler™).

lysis

and RAPD amplified fragments named by
code and mol wt (bp) were scored as band
(1) or absent (0) for each DNA sample.
gram and genetic distance was generated by
imple matching coefficient) the POPGENE
6.

ng the total primers tested, 13 RAPD and 7
rimers that showed clear and reproducible
pattern are given in Fig. 3 and Tables 3

List of ISSR primers and their annealing temperatures

SR Primers	Annealing temperatures
B 10, HB12, HB13, HB 14, B15	39° C for 30 sec
898A, 17898B, 17899A, 899B	44° C for 30 sec
B 8, HB 9	46° C for 30 sec.
4, 814 A	53° C for 1 min
4 B; P2	57° C for 30 sec

RAPD Banding Pattern

The thirteen primers were chosen to generate 74 RAPD fragments, an average of 5.69 bands per primers, of which 57 bands are polymorphic for all the populations. The percentage polymorphism across all the samples varied from 33.3 to 100% (average 76.13%). The polymorphism was the highest in OPA-12, 14 and OPD 11 primers, while it was the lowest in

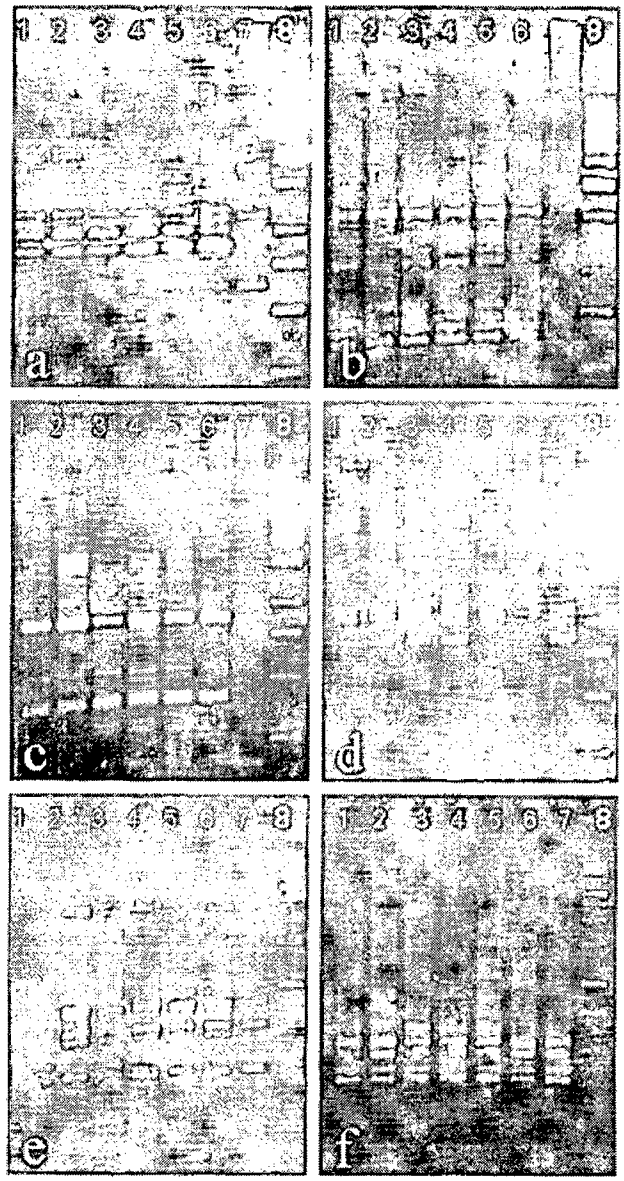


Fig. 3—RAPD and ISSR amplification profile of *R. retusa* with different primers. RAPD primers (a-d): a. Primer OPA 13, b. OPD 2, c. OPA 9, d. OPD 8; Lines (1-8): 1. Pernem, 2. Ponsuli, 3. Salauli, 4. Valpoi, 5. Shiroda, 6. Loutulim, 7. Vagueri, 8. Gene ruler™ 1 Kb DNA ladder, ISSR primers (e-f.): e. HB 12, f. HB 14; Lines (1-8): 1. Pernem, 2. Ponsuli, 3. Valpoi, 4. Loutulim, 5. Shiroda, 6. Salauli, 7. Vagueri, 8. Gene ruler™ 1 Kb DNA ladder.

Table 3—Primer sequences, amplified bands, polymorphic bands and percentage polymorphism in RAPD analysis.

	Primer	Sequence 5' to 3'	Amplified bands	Polymorphic bands	% polymorphism
	OPA 03	AGTCAGCCAC	7	5	71.4
	OPA 04	AATCGGGCTG	6	4	66.6
	OPA 08	GTGACGTAGG	3	1	33.3
	OPA 09	GGGTAACGCC	7	5	71.0
	OPA 12	TCGGCGATAG	4	4	100
	OPA 13	CAGCACCCAC	6	5	83.3
	OPA 14	TCTGTGCTGG	4	4	100
	OPD 02	GGACCCAACC	8	7	87.5
	OPD 03	GTCGCCGTCA	5	4	80.0
	OPD 05	TGAGCGGACA	6	2	33.3
	OPD 07	TTGGCACGGG	5	4	80.0
	OPD 08	GTGTGCCCCA	6	5	83.3
	OPD 11	AGCGCCATTG	7	7	100
l			74	57	
n			5.69	4.38	76.13

Table 4—Primer sequences, amplified bands, polymorphic bands and percentage polymorphism in ISSR analysis

o.	Primer	Sequence 5' to 3'	Amplified bands	Polymorphic band	% polymorphism
	17899A	CACACACACACAAC	5	2	40.0
	17899B	CACACACACACAGG	3	2	66.6
	HB 9	GTGTGTGTGTGTGG	10	6	60.0
	HB 12	CACCACCACGC	3	2	66.6
	HB 13	GAGGAGGAGGC	5	4	80.0
	17898 B	CACACACACACAGT	2	-	-
	HB 15	GTGGTGGTGGC	2	-	-
al			30	16	
an			4.28	3.2	62.6

Table 5—Genetic identity of *Rhynchostylis retusa* populations based on RAPD primer amplification

Ponsuli	Pernem	Salauli	Valpoi	Shiroda	Loutulim	Vagueri
-	-					
0.702	0.851	-				
0.770	0.770	0.918	-			
0.743	0.797	0.864	0.864	-		
0.729	0.837	0.851	0.824	0.932	-	
0.473	0.500	0.486	0.432	0.405	0.445	-

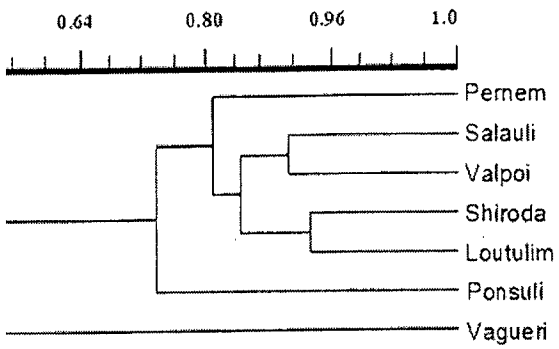
and OPA-8 primers. Nei's average genetic value ranged from 0.405 to 0.932 with the of 0.709 for different population compared 5). The dendrogram (Fig. 4) was constructed n simple matching coefficients taking into the presence or absence of bands. These four populations forming two clusters. The ıster comprised of two populations from and Valpoi at similarity of 90%. The second at similarity of 94% consists of populations iroda and Loutulim. However, the other three ons Pernem, Ponsuli and Vagueri formed nal taxonomic units.

ISSR Banding Pattern

The seven ISSR primers tested, generated total 30 RAPD fragments, an average of 4.28 bands per primer. Sixteen bands, out of 30 bands, were polymorphic with the mean of 3.2 bands per primer (Table 4). The percentage of polymorphism across all the samples varies from 40 to 80% (average 62.6%). The polymorphism was the highest in primer HB 13, while it was the lowest in primer 17899A. The ISSR derived data were used to calculate the genetic identities (Table 6). The genetic similarity co-efficient varied from 0.733 to 0.933 with the average of 0.844. Simple matching coefficient method was used to

Table 6—Genetic identity of *R. retusa* populations based on ISSR primer amplification

	Ponsuli	Pernem	Salaulim	Sattari	Shiroda	Loutulim	Vagueri
	0.800	-					
	0.866	0.800	-				
	0.933	0.800	0.866	-			
	0.900	0.833	0.900	0.833	-		
m	0.866	0.866	0.733	0.800	0.833	-	
i	0.833	0.900	0.766	0.900	0.800	0.900	-



—Dendrogram of Nei's genetic identities between the ions of *R. retusa* based on RAPD data

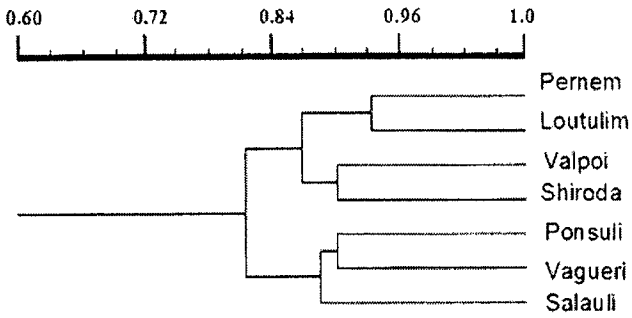


Fig. 5—Dendrogram of Nei's genetic identities between the populations of *R. retusa* based on ISSR data

uct dendrogram (Fig. 5). The populations under were grouped into 3 groups. The first cluster d between Pernem and Loutulim populations at similarity. The populations from Valpoi and da formed the second cluster at 90% similarity. the populations of Ponsuli, Vagueri and Salauli d the third cluster.

ssion

pulation genetics of large number of plant s were studied using RAPD[17-19]. The level of ic diversity of populations as well as the degree ne differentiation between the populations is tant for genetic conservation[20]. The maintenance netic diversity is considered crucial for long-term al and the evolutionary response of population pt to the changes in the environment[21,22].

the present study, the applicability of ISSR and) was compared as genetic marker to cterize the population of *R. retusa*. The results te that the percentage of RAPD polymorphic (76.13%) was higher than that of ISSR analysis %). The mean number of amplified RAPD bands .69 bands per primer, which was more than that SR (4.28). The results also suggest that RAPD rs were superior to ISSR markers in the ity of revealing more polymorphic bands. ar results were obtained for *Citrus* sp. which showed RAPD PCR had a higher level of variations than ISSR[23]; whereas these two markers produced similar level of polymorphism in *Phaseolus vulgaris*[24].

In the present study, it is obvious that the dendrogram based on RAPD marker (Fig. 4) is not in accord with the dendrogram based on ISSR marker (Fig. 5). The dendrogram generated by RAPD matrix agrees better with the altitude of the populations (Fig. 4). The altitudes of the sample collection sites were widely varying for population to population and it ranges from 10 to 295 m (Table 1). Interestingly, almost similar altitude populations from Salauli (76 m) and Valpoi (71 m) formed one cluster, while Shiroda (102 m) and Loutulim (103 m) formed another. The populations from Pernem (15 m) and Ponsuli (10 m) formed out separate branches from the major two clusters. In addition, population from Vagueri (295 m) had a totally separate branch from all other populations since the altitude of this population is much higher in comparison with all other populations. Relatively low genetic identity of Vagueri population with other populations signifies its isolation due to higher altitude (Table 1). Genetically distinct isolated populations were also reported in *Laelia speciosa*, an endangered orchid from Mexico, using isozyme studies[25]. The distinctiveness of Vagueri population may represent

ue evolutionary process. Similar observation was rted in another orchid species, *Paphiopedilum anthum* from South-western China[26].

ighest genetic distance was observed between ılations of Vagueri and Shiroda. These two ılations come from different regions indicating geographical isolation is one of the important ors for the observed genetic distance. Shiroda and tulim populations have the closest genetic tities as they are morphologically, ecologically geographically close to each other (Fig. 2). While uli and Valpoi populations coming under Western ts belt region of Goa state share the next highest etic identities. Ponsuli and Pernem populations e close to each other as they are confined to tal area and also at low altitude level (Table 1).

endrogram generated by ISSR matrix agree with geographical location of the populations. Pernem Loutulim populations share the highest genetic larity, as they are in plains. Populations close to hilly areas, i.e. Valpoi and Shiroda, shared 90% larity. Ponsuli, Salauli and Vagueri populations ed a separate cluster. Salauli and Vaugeri were ıped together since they are almost in the parts of stern Ghats of Goa region, while closeness of suli population could not be explained. Combined R and RAPD data of *Aerides maculosum*, another hytic orchid from the same localities as that of *R. sa*, clearly separated the populations of hilly and st areas from the remaining areas. It shows that ern of gene flow is more or less identical as both epiphytic and are from the same localities[19].

he present study reveals that within the small on taken up for study, higher genetic variability ts among the populations of *R. retusa*.

nowledgement

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rt Communication

R and RAPD Markers Assessed Genetic Variation of *Aerides culosum* – an Epiphytic Orchid from Goa, India

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***s maculosum* Lindl is one of the most important orchids valued for its beautiful inflorescence/flowers. The present aimed to understand the level of genetic variation among the populations of *A. maculosum* using RAPD and ISSR 's. Among the 35 primers tested, 13 RAPD and 6 ISSR primers were selected for the analysis. Total of 101 RAPD and 40 ragments were generated. High level of polymorphism was recorded in RAPD (90.45%) compared to ISSR (72.85 %). verage genetic identity values for different populations of *A. maculosum*- ranged from 0.465 to 0.762 (RAPD), while for : ranged from 0.475 to 0.975. The present study provides important insights about genetic variation in *A. masculosum* ay facilitates the conservation and management of this species.**

ords: Aerides maculosum, RAPD, ISSR, genetic variation.

s one of the biodiversity hotspots (1) contributing to rld's biological resources from the long stretches of rn Ghats, the greater Himalayan range on the rn Plains and Western Ghats. Western Ghats in India ds over 0.14 million km^2 in the five states i.e. astra, Goa, Karnataka, Kerala and Tamil Nadu. The rn Ghats lying in Goa covers about 600 km^2. The extends in the form of an arc for a length of 125 km orth to South. Over the entire length of the Western the widest belt of forest is around Goa. The forest that predominantly persist in Goa are dense and ype forest, inhabited by a number of terrestrial and tic orchids (*Aerides maculosum*) (2). Epiphytic s are largely tropical and subtropical in distribution.

ıe major threat to orchid flora in the state of Goa is station and mining of valuable economic resources ıs bauxite and graphite etc. In case of epiphytic s, the loss of host tree species are bound to result in nination of species. In view of this, the conservation res are necessary to protect the orchid flora in the Understanding of the genetic profile of a particular s is important to devise conservation strategies.

veral molecular markers were successfully develop- ing the last two decades to study plant diversity. isozymes and restriction fragment length polymorphism served as reliable markers for genetic analysis of plant species. But, PCR based techniques such as Random Amplified Polymorphism DNA (RAPD) (3) and Inter Simple Sequence Repeats (ISSR) (4) developed in recent years are being extensively used for DNA finger printing (5), population genetics (6) and phylogenetic studies (7). Till date there is no information available on genetic diversity of *A. maculosum*. Therefore, the present study aimed to understand the level of genetic variation among the population of *A. maculosum* using RAPD and ISSR markers.

Leaf samples of *A. maculosum* representing eight different natural populations in Goa were collected. Leaves were stored at 4°C in zip-lock plastic bags till they were processed for DNA isolation. DNA was isolated using a modified CTAB (cetyl trimethyl ammonium bromide) method. For each accession, about 5 g of bulked leaf tissue from 50 plants was ground to a fine powder using liquid nitrogen which was then, suspended in 20 ml of extraction buffer (20 mM EDTA at pH 8.0, 100 mM Tris-Cl at pH 8.0, 1.5 M NaCl, 2% CTAB and 1% β-mercaptoethanol). The suspension was mixed well, incubated at 60°C for 45 min, followed by chloroform:isoamyl alcohol (24:1) extraction and precipitation with 0.6 volume of isopropanol at -20°C for 1 h. The DNA was pelleted down by centrifugation at 12,000 rpm for 10 min and was then suspended in TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0). The DNA

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purified from RNA and proteins by standard procedures its concentration was estimated by agarose gel trophoresis and staining with ethidium bromide.

Twenty RAPD primers obtained from Operon Technogies, USA were tested initially with random selected viduals from two populations. Thirteen primers that wed clear and reproducible results were chosen for ner study. PCR amplification was performed in a 25 µl tion volume containing 50 ng of genomic DNA, 2.5µl 0 x *Taq* assay buffer (100 mM Tris-Cl, pH 8.3; 20 mM Cl_2; 500 mM KCl; 0.1 % gelatin), 2.5 µl of 2.5 mM dNTPs, g of primers and 0.5 units of *Taq* polymerase (Bangalore ei, India). The mixture was subjected to PCR on Perkiner 480 thermal cycler programmed for an initial step of in at 94°C, followed by 40 cycles of 94°C for 1 min, C for 1 min and 72°C for 2 min and 10 min final extension at 72°C. The amplification products were analysed on % agarose gel with molecular weight marker (1kb ker, GeneRuler, MBI Fermentas, USA). Gel was trophoresed at 50 V for 4 h. The gel was stained with dium bromide and visualized under ultraviolet light and tographed using gel documentation system ersham Pharmcia Biotech VDS Image master).

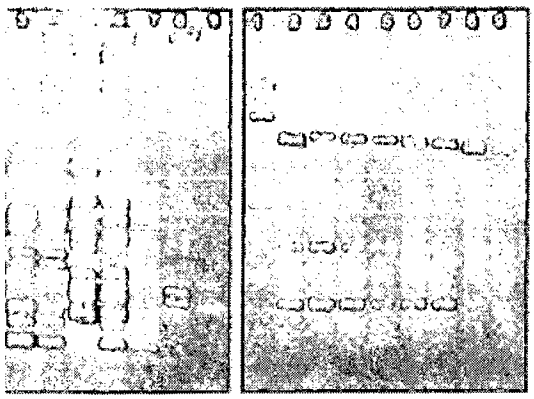
Fifteen ISSR primers from Operon Technologies, fornia, USA, was initially tested and seven primers, ch showed clear and reproducible bands, were used. amplification was performed in a 25 µl reaction volume aining 50 ng of genomic DNA, 2.5µl of 2.5 mM dNTPs, mol μl^{-1} of primer and 0.6 units of *Taq* polymerase. al denaturation was for 5 min at 94°C, followed by 35 es of 1 min at 94°C, for annealing temperatures 39-C depending upon the *Tm* of each primer, and primer nsion at 2 min at 72°C. The PCR products were lyzed on 1.4 % agarose gels, electrophoresed at 50 V 4 h and stained with ethidium bromide, photographed er ultraviolet light by using gel documentation system. ecular weight was estimated by using 1kb DNA ladder l Fermentas, USA). Each informative RAPD and ISSR d was scored independently as 1 for presence and 0 absence. Percentage of polymorphism was calculated he proportion of polymorphic bands over the total ber of bands. Dendrogram was generated by UPGMA hod using the NTsys-pc version computer software (8).

Among the total primers tested for molecular analysis . *maculosum* 100% polymorphic pattern was obtained 7 RAPD primers (OPA 3, OPA 8, OPA 13, OPD 2, OPD 5, OPD 7 and OPD 8) and one ISSR primer (HB 09). Total 13 RAPD and 6 ISSR primers produced a clear and reproducible banding pattern (Table 1). The 13 RAPD primers produced 101 fragments with an average of 7.76 bands per primer. Out of which 94 bands with the mean of 7.23 per primer were polymorphic for all the populations. The percentage polymorphism across all the samples varied from 50 to 100% (average 93.09 %). The polymorphism was higher with OPA 3, OPA 8, OPA 13, OPD 2, OPD 5, OPD 7 and OPD 8, while lowest in OPA 14 primer. Nei's average genetic identity value for different population ranged from 0.465 to 0.762 with the average of 0.629. An example of banding pattern obtained with RAPD and ISSR marker is given in Fig. 1.

In this study, total of 6 primers produced 40 ISSR fragments, with an average of 6.66 bands per primer. Thirty bands, out of 40 were polymorphic with the mean of 5 bands per primer. The percentage of polymorphism across all the samples varied from 50 to 100 (average 75 %). The polymorphism was highest in HB 9, while lowest in HB-13 and 17898-B. ISSR derived data have been used to calculate the genetic identities. The genetic similarity coefficient varied from 0.475 to 0.975 with the average of 0.704.

Table 1. Primer sequences, polymorphic bands, and percentage polymorphism in RAPD and ISSR analysis

Sr. No	Primers	Sequence 5′ to 3′	Polymorphic band	% Polymorphism
RAPD				
1.	OPA 03	AGTCAGCCAC	7	100
2.	OPA 04	AATCGGGCTG	7	87.5
3.	OPA 08	GTGACGTAGG	8	100
4.	OPA 09	GGGTAACGCC	7	77
5.	OPA 12	TCGGCGATAG	6	85.7
6.	OPA 13	CAGCACCCAC	7	100
7.	OPA 14	TCTGTGCTGG	1	50
8.	OPD 02	GGACCCAACC	12	100
9.	OPD 03	GTCGCCGTCA	7	87.5
10.	OPD 05	TGAGCGGACA	10	100
11.	OPD 07	TTGGCACGGG	6	100
12.	OPD 08	GTGTGCCCCA	9	100
13.	OPD 11	AGCGCCATTG	7	87.5
ISSR				
1.	17899A	CACACACACACAAC	6	85.71
2.	17899B	CACACACACACAGG	5	71.40
3.	HB 9	GTGTGTGTGTGTGG	9	100
4.	HB 8	GAGAGAGAGAGAGG	4	80
5.	HB 13	GAGGAGGAGGC	4	50
6.	17898 B	CACACACACACAGT	2	50

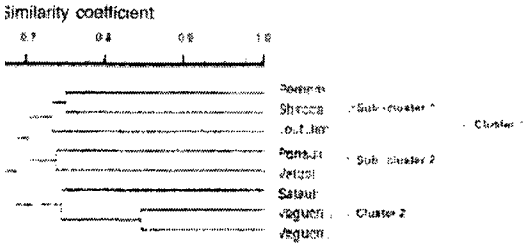


RAPD and ISSR amplification profile of *Aerides maculosum*. PD primer OPD 2, and (**b**) ISSR primer HB 14. Lanes 1: uler™ 1 kb DNA ladder, 2, Pernem, 3, Ponsuli, 4, Valpoi, 5, n, 6, Shiroda, 7, Salauli, 8, Vagueri 1, 9, Vagueri 2.

endrogram constructed with the combined data from IAPD and ISSR markers formed two major clusters 2). First cluster comprising of populations from m, Shiroda, Loutulim, Ponsuli and Valpoi with two usters and the second cluster comprising of popu- from Salauli and Vagueri.

this study, we compared the applicability of RAPD SR as genetic marker to characterize the population *naculosum*. The results indicated that the percentage orphism in RAPD was higher than that of ISSR. Mean er of polymorphic bands per primer was higher in (7.23) than in case of ISSR (5.0). ISSR markers are ered superior to RAPD in terms of their reproducibili- efferent amplifications (9). In our experiment RAPD rs were superior to ISSR marker in terms of revealing polymorphism. Lower levels of variation detected SSR markers may indicate that the ISSR loci are ely more conserved in *A. maculosum* (10).

ne dendrogram revealed that the populations were ed on the basis of proximity to the Western Ghats



. Dendrogram of Nei's genetic identities between the ions of *Aerides maculosum* based on RAPD and ISSR s.

and forest types (Fig. 2). The first cluster is characterized by the populations that are away from the Western Ghats and the sub-clusters within that by the forest type. The populations from Shiroda and Pernem (sub-cluster1) belong to open forest type and Loutulim belong to non-forest type. The populations from Ponsuli and Valpoi (sub-cluster 2) are from dense forest areas. The populations from Salauli, Vagueri (1 & 2) are from the dense forests of hilly areas of Western Ghats and they form the second major cluster. Among them, two populations from Vagueri form a small cluster. Thus, the gene flow among the studied populations could be correlated with factors such as hilly areas, forest types and proximity to either of them.

The results of this study would help in better understanding of the genetic profile that can be used to develop strategies for conservation and sustainable utilization of epiphytic orchid. Also this study forms a starting point for future research on the population and evolutionary genetics of this species.

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