DIVERSITY AMONG THE WILD POPULATION OF *AERIDES MULTIFLORUM* FROM KANGRA VALLEY

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CERTIFICATE

I hereby certify that the work which is being presented in this thesis entitled "**Diversity among the wild populations of *Aerides multiflorum* from Kangra Valley**" by me in partial fulfillment of requirements for the award of degree of **Master of Technology in Biotechnology** from **Thapar University, Patiala,** is an authentic record of my study carried under the guidance and supervision of Dr. Anil Kumar (Associate Professor), DBT, Thapar University, Patiala.

The matter presented in this thesis has not been submitted in any other University or Institute for the award of degree.

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Abstract

Biological diversity is the variation among all species of plants and animals, their genetic material and the ecosystems in which they occur. Genetic diversity is the amount of genetic variability among individuals of a variety, or population of a species i.e. any variation in the nucleotides, genes, chromosomes, or whole genome of organisms. Molecular methods have become an essential part of most studies on genetic diversity analyses and distribution and in the analyses of breeding system. Molecular methods are most useful for estimating gene flow, genetic drift and degree of out breeding. RAPD was the first PCR-based molecular marker to be employed in genetic variation analyses. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine. They exhibit incredible range of diversity in shape, size and colour of their flowers. *Aerides multiflorum* is a tropical epiphytic orchid species with attractive flowers arranged in racemose inflorescence, ranks among the important Indian ornamental orchids. Comparative population studies using PCR based markers RAPD and ISSR were performed to assess the genetic diversity of the wild orchid. Among 60 primers tested 20 RAPD and 20 ISSR primers were selected for analysis. In total, 60 RAPD and 181 ISSR fragments were generated. Out of which 13 of RAPD and 71 of ISSR were polymorphic. High level of polymorphism was recorded in ISSR (39.22%) than RAPD (21.66%).

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LIST OF ABBREVIATIONS

%	Percent
Bp	Base pair
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide Triphosphates
EDTA	Ethylene DiamineTetraacetic Acid
G	Gram
ISSR	Inter-simple sequence repeat
Kb	Kilobase
Mg	Milligram
mM	milli Molar
nmol	Nano mole
ng	Nano gram
PCR	Polymerase Chain Reaction
PCA	Principal Component Analysis
RAPD	Random Amplification of Polymorphic DNA
TE	Tris-EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic mean
ul	Microlitre
hr/s	Hour/second
w/v	Weight/volume
v/v	Volume/volume
V	Volt

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INTRODUCTION Chapter 1

Genetic Diversity

Biological diversity is defined as the variation present in all species of plants and animals, their genetic material and the ecosystems in which they occur. Diversity can occur at three levels: genetic diversity (variation in genes and genotypes), species diversity (species richness) and ecosystem diversity (communities of species and their environment) (Suneson, 1960).

Genetic diversity is the amount of genetic variability among individuals of a variety, or population of a species i.e. any variation in the nucleotides, genes, chromosomes, or whole genome of organisms (Brown, 1983). It results from the genetic differences between individuals and may be manifest of differences in DNA sequence, in biochemical characteristics (e.g. in protein structure or isoenzyme properties), in physiological properties (e.g. abiotic stress resistance or growth rate) or in morphological characters such as flower colour or plant form (Porth and Yousry, 2014). Four components of genetic diversity can be usefully distinguished: the number of different forms (alleles) ultimately found in different populations, their distribution, and the effect they have on performance and the overall distinctness between different populations. The variation that underpins genetic diversity arises from mutation and recombination. Selection, genetic drift and gene flow act on the alleles present in different populations to cause variation in the diversity in them. The selection can be natural or it can be artificial, as is the case with much of the variation present in crop species (Suneson, 1960; Frankel, 1977).

Analysis of genetic diversity can be applied to study evolutionary ecology of populations. Genetic studies can identify alleles that might affect the ability of the organism to survive in its existing habitat, or might enable it to survive in more diverse habitats. This is the basis of natural selection due to selective advantage conferred by alleles on the host organism (Karp and Edward, 1995). The presence of unique genetic characteristics distinguishes members of a given population from

those of any other population. Large populations will usually have a greater diversity of alleles compared to small populations. This diversity of alleles indicates a greater potential for the evolution of new combinations of genes and, subsequently, a greater capacity for evolutionary adaptation to different environmental conditions. In small populations, the individuals are likely to be genetically, anatomically, and physiologically more homogeneous than in larger populations decreasing their capability to adapt to different environmental stresses (Wang *et al*., 2013).

Biological diversity enables social and economic systems to flourish in ways that allow the poorest to meet their food and nutritional needs and retain the cultural diversity of countries throughout the world (Shiva, 1994). Plant genetic resources are among the most essential of the world's natural resources and during the last 2–3 decades, major advances have been made in conserving bioresources (Frankel and Bennet, 1970). Genetic diversity data provides information necessary to evaluate the extent to which a collection contains significant gaps in terms of the range of variation found in a species or significant redundancies, that is accessions with very similar characteristics (Caetano-Anolles, 1993).

Steps for species conservation include both *in situ* and *ex situ* measures. *In situ* conservation, where species are conserved in their natural habitats, is considered the most appropriate way of conserving biodiversity. Various associated problems such as poor enforcement and regulation of law, modification of habitat and migration or absence of the pollinators (Swarts, 2007), make it necessary to focus on *ex situ* conservation measures. *Ex situ* conservation is the preservation of components of biological diversity outside their natural habitats. Its measures can be complementary to *in situ* methods. These measures have a valuable role to play in recovery programmes for endangered species. In this context, *ex situ* conservation is very important aspect of orchid conservation which can include both seed banks and *in vitro* culture plant tissue collections (Ellstrand and Elam, 1993).

Molecular methods have become an essential part of most studies on genetic diversity analysis and distribution and in the analysis of breeding system, bottlenecks and other key features affecting genetic diversity patterns. The molecular techniques commonly employed for the studies are RFLPs, RAPDs, AFLPs or SSRs (Bornet and Branchard, 2001). It is important, however, to understand that different markers have different properties and will reflect different aspects of genetic diversity (Karp and Edwards, 1995). Genetic diversity assessment methods vary in

- the way they resolve genetic differences,
- the type of data these markers generate,
- the taxonomic levels at which they can be most appropriately applied.

Molecular methods are most useful for estimating gene flow, genetic drift and degree of out breeding (Doulis *et al*., 2000).

The assessment of genetic diversity within and between populations is performed at the molecular level by DNA analysis, which measure levels of variation directly (Bansal *et al*., 2014). Genetic diversity may also be gauged using morphological, and biochemical characterization and evaluation.

Molecular analysis comprise a large variety of DNA based molecular markers, which can be employed for such analysis. Different markers have different genetic qualities like, they can amplify anonymous or characterized loci and can contain expressed or non-expressed sequences (Padmalatha and Prasad, 2006). A molecular marker can be defined as a genomic locus, detected through probe or specific starters (primer) which, in virtue of its presence, distinguishes unequivocally the chromosomic trait which it represents as well as the flanking regions at the 3' and 5' extremity (Barcaccia *et al.,* 2000). Molecular markers may or may not correlate with phenotypic expression of a genomic trait. They offer numerous advantages over conventional, phenotype-based alternatives as they are stable and detectable in all tissues regardless of growth,

differentiation, development, or defense status of the cell. Additionally, they are not confounded by environmental, pleiotropic and epistatic effects (Lioi and Piergiovanni, 2013).

An ideal molecular marker should possesses the following features: (1) should be polymorphic and evenly distributed throughout the genome; (2) provide adequate resolution of genetic differences; (3) generate multiple, independent and reliable markers; (4) simple, quick and inexpensive; (5) need small amounts of tissue and DNA samples; (6) link to distinct phenotypes; and, (7) require no prior information about the genome of an organism (Mukherjee *et al.,* 2013).

RAPD (Random amplification of polymorphic DNA)

RAPDs were the first PCR-based molecular markers used for genetic analyses (Welsh and McClelland, 1990). RAPD markers are generated through the random amplification of genomic DNA using short primers (decamers). The use of short primers is necessary to increase the probability that, they are able to find homologous sequences suitable for annealing. DNA polymorphisms are then produced by "rearrangements or deletions at or between oligonucleotide primer binding sites in the genome" (Williams *et al.,* 1990). As this approach requires no prior knowledge of the genome, it can be employed across species using universal primers. The major drawback of this method is that the profiling is dependent on reaction conditions which can vary between laboratories; even a difference of a degree in temperature is sufficient to produce different patterns. Additionally, as several discrete loci are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001). Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) and DNA amplification fingerprinting (DAF) are independently developed methodologies, which are variants of RAPD. For AP-PCR (Welsh and McClelland, 1990), a single primer, 10–15 nucleotides long, is used and involves amplification for initial two PCR cycles at low stringency. Thereafter, the remaining cycles are carried out at higher stringency by increasing the annealing temperatures. The RAPD has been successfully used for

studying the genetic diversity among wild populations (Bansal *et al.*, 2014) and clonal fedility of micropropagated plants (Aggarwal *et al.*, 2010).

ISSR (Inter simple sequence repeat)

ISSRs are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp) (Senthil *et al.*, 2009). ISSRs are randomly distributed throughout the genome. Because of the multilocus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Zietkiewicz *et al.*, 1994).

Orchids

Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine. They exhibit incredible range of diversity in shape, size and colour of their flowers. They are important aesthetically, medicinally and also regarded as ecological indicators (Joshi *et al.*, 2009). Several orchid species are cultivated for various economic uses especially in floriculture. Orchids are grown primarily as ornamentals and are valued as cut flowers because of their exotic beauty and their long lasting blooming period (Hew *et al.*, 1997). Though orchids are grown primarily as ornamentals, many are also used as herbal medicines and food. They also possess huge cultural values in different cultures and tribes of different parts of the world (Khasim and Rao 1999; Kasulo *et al.*, 2009). Still large population of orchid is confined to their natural habitat only. Number of orchids is continuously decreasing in many parts of world due to their high demand and population pressure. Habitat destruction and indiscriminate collection further add to decreasing number (Kishore *et al.*, 2008).

Aerides multiflorum Roxb.

Aerides multiflorum Roxb. (Figure1), tropical epiphyte, is a member of family Orchidaceae. *Aerides* flowers are fragrant, colourful and long-lived with leathery and drought-resistant leaves. These plants do not tolerate any kind of disturbance or damage of their root systems in cultivation and do not have pseudobulbs. Species in genus *"Aerides"* range from small to large monopodial epiphytes, having single stem and addition of leaves to apex per year, except for *Aerides krabiensis*, which is a lithophyte. *Aerides multiflorum* is a free-flowering species native to Southeast Asia, Bangladesh, Coromendal Coast. Its flowers are pink, purple and white in colour, produced on long branching spikes in great profusion. It is widely cultivated as an ornamental flower, used as flavouring agent and is also in various medicinal preparations. Leaves are eight inches long, dark green, and are covered with small spots. They can be kept in hanging baskets, teak containers or net pots, which allow their roots to extend into the air. The genus is reported to show much of morphological and molecular diversity (Parab *et al.,* 2008). Therefore, the present study was taken up to study the genetic diversity in the wild populations from Kangra Valley (H.P).

Figure1: *Aerides multiflorum* Roxb.

REVIEW OF LITERATURE Chapter 2

Detection and analysis of genetic variation helps to understand the molecular basis of various biological phenomenon in plants. Since the entire plant kingdom cannot be covered under sequencing projects, molecular markers and their correlation to phenotypes provide with requisite landmarks for elucidation of genetic variation. Markers can be classified into three types: morphological trait based markers, protein based (biochemical) markers and DNA based (molecular) markers. Traditionally, diversity within and between populations was determined by assessing differences in morphology. Its advantages are being readily available and non requirement of sophisticated equipment. However these attributes are subject to change due to environmental factors and vary at different time points. Biochemical markers also have similar limitation of being influenced by environment. Genetic or DNA based marker techniques such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) are routinely being used in ecological, evolutionary, taxonomical, phylogenic and genetic studies of plant sciences (Bansal *et al.*, 2014). These techniques are well established and their advantages as well as limitations have been realized (Agarwal *et al.*, 2008).

Parab *et al.*, (2008) studied genetic variation among the population of *Aerides maculosum*, one of the most important orchid valued for its beautiful flowers, using RAPD and ISSR markers. Among 35 tested markers, 13 RAPD and 6 ISSR markers were studied for analysis. Total of 101 RAPD fragments were generated from 13 RAPD primers 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%). Nei's average genetic identity value for different population ranged from 0.465 to 0.762 with the average of 0.629. Six ISSR primers produced 40 ISSR fragments, with an average of 6.66 bands per primer. Percentage polymorphism across all samples varied from 50 to 100 (average 75%).

Thus RAPD was recorded with high level of polymorphism 90.45% compared to ISSR (72.85%). This study provides important insights for genetic variation in *Aerides maculosum* and facilitates conservation and management of this species.

Pinheiro *et al.,* (2012) studied genetic diversity on Brazilian orchid *Cattleya labiata*, a threatened species due to habitat destruction, using RAPD and ISSR markers. In total 130 accessions were studied, 117 belonging to *Cattleya labiata* and 13 from 10 other species in the same genus. Data generated from 12 ISSR and 12 RAPD primers was used to determine genetic variability via a model-based Bayesian analyses (Structure) and molecular variance analysis. In addition, Shannon index, genetic diversity and Jaccard coefficients were also estimated. The marker data indicated that *Cattleya labiata* has a high level of polymorphism. A total of 272 fragments (151 ISSR and 121 RAPD) all polymorphic, were generated using 24 primers. Jaccard coefficients ranged from 0.14 to 0.82, with average similarity of 0.45. The unweighted pair group method with arithmetic mean dendrogram did not group the samples by origin, which was also confirmed by Bayesian analysis, demonstrating the complex genetic structure of *Cattleya labiata*. Other *Cattleya* species showed no relationship with any *Cattleya labiata* sample. This genetic characterization of *Cattleya* from Northeast Brazil contributes to knowledge of the genetic structure of the species and can be used to define strategies for conservation and breeding programmes.

Parab and Krishnan (2008) studied genetic diversity in wild monopodial epiphytic orchid *Rhynchostlylis*. Among 35 tested primers used, 13 RAPD and 7 ISSR primers produced good number of amplified markers. 13 RAPD primers generated 74 fragments, of which 57 bands were polymorphic for all the populations. The percentage polymorphism across all the samples varied from 33.3 to 100%. Nei's average genetic identity value ranged from 0.405 to 0.932 with the average of 0.709 for different populations. Seven ISSR primers tested generated 30 markers. Out of 30, 16 were polymorphic with mean of 3.2 bands per primer. Percentage polymorphism across samples varies from 40 to 80%. The genetic similarity coefficients varied from 0.733 to 0.933

with average of 0.844. In total 74 RAPD and 30 ISSR fragments were generated. High level of polymorphism was recorded in RAPD 76.13% than ISSR 62.6%. Mean number of amplified RAPD primers was 5.69 bands per primer, more than that of ISSR primers which was 4.28. On the basis of results they suggested that RAPD markers were superior to ISSR markers in their capacity to generate more polymorphic bands.

Wang *et al.,* (2013) studied genetic diversity on rare terrestrial orchid *Calanthe tsoongiana* endemic to China. ISSR markers were employed to access the genetic diversity and differentiation of six populations of *Calanthe tsoongiana*. Based on 124 discernible fragments yielded by 11 selected primers, high genetic diversity (H = 0.3978) was revealed at the species level, however, genetic diversity at the population level was relatively low (H = 0.1240). High-level genetic differentiation (G_{st} = 0.55) among populations was detected based on analysis of molecular variance (AMOVA), indicating potential limited gene flow. Mean within population diversity of *Calanthe tsoongiana* (H = 0.1826) was lower than the average value of plant genetic diversity based on ISSR (H = 0.22). No significant relationship was observed between genetic and geographic distances among the sampled populations. These results suggested that restricted gene flow might be due to habitat fragmentation and reduced population size as a result of human activities.

Tripathi *et al.,* (2013) conducted molecular characterization studies on one of the most important medicinal plant *Coleus forskohlii*. Due to continuous collection of its roots from wild sources, this plant is listed in endangered species. This has necessitated its conservation and sustainable management. Morphological and molecular characterization helps to understand the improvements for optimal yields through breeding. 18 *Coleus forskohlii* genotypes were collected from different places of central India and assessed through RAPD and ISSR markers. 11 RAPD and 10 ISSR primers produced 101 and 80 fragments, respectively. Percentage polymorphism with RAPD was 61.39% and with ISSR was 68.75%.

Mukherjee *et al.,* (2013) used RAPD and ISSR markers to study genetic diversity and phylogenetic analysis of economically important species of *Allium*. They included three varities including eight cultivars of *Allium cepa* L., five cultivars of *Allium sativum* L., four cultivars of *Allium porrum* L., and two other species of *Allium* (*Allium tuberosum*Rottl. and *Allium stracheyi* Baker). For better understanding of the phylogenetic relationships among the *Allium* species, two species related to *Allium* from the family Alliaceae, viz. *Agapanthus africanus* L. and *Nothoscordum fragrans* Kunth., were also included. ISSR showed more polymorphism within *Allium cepa* and *Allium sativum*, while RAPD showed more polymorphism within *Allium porrum*. Total number of polymorphic bands ranged from 365 in RAPD to 189 in ISSR. The number of polymorphic bands per assay unit was higher in RAPD (30.41) as compared to ISSR (17.18). These authors studied the interspecific and intraspecific relationships amongst these species.

Kishore *et al.,* (2008) studied genetic relations in the hybrids of *Aerides vandarum* and *Vanda stangeana* (Orchidaceae) at the protocorn stage. *Aerides vandarum* and *Vanda stangeana* are two rare and endangered vandaceous orchids with immense floricultural traits. The intergeneric hybrids were synthesized by performing reciprocal crosses between them. Determination of hybridity was made as early as the immature seeds or embryo germinated in vitro, using RAPD markers. Out of 15 arbitrarily chosen decamer RAPD primers, two were found to be useful in amplification of polymorphic bands specific to the parental species and their presence in reciprocal crosses. However, a decisive profile that can identify the reciprocal crosses could not be provided by RAPD.

Guasmi *et al.,* (2012) studied genetic diversity among South Tunisian Barley using ISSR and RAPD primers. RAPD and ISSR markers were assayed to determine the genetic diversity of 80 barley specimens from South Tunisia. The ISSR primers showed variation in the percentage of polymorphism, band informativeness (Ib), and resolving power (Rp). The percentage of polymorphism is 66.67%, the average Ib ranged from 0.24 to 0.39, while Rp ranged from 0.74 to

1.16. In RAPD analysis, three primers yielded a total of 17 scorable bands, which are all polymorphic. The three polymorphic primers exhibited variation with regard to average band informativeness (AvIb) and resolving power (Rp). RAPD and ISSR marker systems were found to be useful for the genetic diversity among the barley specimens. The two dendrograms obtained through these markers show different clustering of 80 barely specimens, but it was noted that some clusters were similar in some cases. A poor correlation (= 0 . 1 2) was found between both sets of genetic similarity data, suggesting that both sets of markers revealed unrelated estimates of genetic relationships. Therefore, the ISSR and RAPD molecular markers show two genetic grouping of studied barely specimens.

Giancarla *et al.,* (2012) studied genetic diversity among barley cultivars using RAPD and ISSR markers. The success of a breeding program depends on the genetic variability available in the germplasm of the crop. The objective of this study was to assess molecular variation among different Romanian and foreign barley cultivars, and to determine the level of genetic similarity among them. The results demonstrated that RAPD analyses are useful for evaluation of genetic diversity between different barley cultivars, considering the fact that the average polymorphic rate was 91.17 %, and 7.5 polymorphic bands per primer. Total polymorphism generated by a certain primer (PIC), lies between 0.147 and 0.438. The discrimination index (PI), value is 0.787 for the primer E6 and 2.626 for P1 primer, which had the highest capacity to generate polymorphic bands to barley cultivars studied. Among the tested ISSR primers, only five primers amplified polymorphic loci with an average number of 9.2 bands per primer and the mean percentage of ISSR polymorphism was 89.13%. RAPD and ISSR analysis attested the existence of a high genetic variability among studied cultivars, which can be efficiently exploited in the breeding programs of barley.

Bulpitt, (2005) described that Chinese were the first to cultivate and describe orchids. These plants first received recognition in the herbal writings of China and Japan 3,000 to 4,000 years ago,

along with their medicinal significance recognition. Medicinal orchids belong mainly to genera: *Anoctochilus, Bletilla, Calanthe, Coelogyne, Cymbidium, Cypipedium, Dendrobium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nevilia*and *Thunia* (Arif *et al.,* 2010). Recently, more species belonging to different genera have been reported to have medicinal properties and in future more will be added in the list. Many medicinal orchids are reported to contain alkaloids and posses antimicrobial activity. Recent works have reported isolation of anthocyanins, stilebnoids and triterpenoids and various phytochemicals (Orchinol, hircinol, cypripedin, jibantine, nidemin and loroglossin) from orchids (Hou and Lou, 2011).

MATERIALS AND METHODS Chapter 3

Collection and characterization of the *Aerides multiflorum* from different locations of Kangra, Himachal Pradesh.

Plant Material

A total of 12 different samples of *Aerides multiflorum* were collected from different locations in Kangra, Himachal Pradesh (Table1). These were multiplied by vegetative propagation and were maintained in the nursery at Thapar University Campus, Patiala (30 35' N, 76 36' E).

Table1: Accessions of *Aerides multiflorum* collected from various locations around Kangra, H.P.

Serial No.	Sample Code	Place of Collection
1	TA1	Takipur, Kangra (Old Mango Tree)
2	KA1	KangraKhad
3	KA2	KangraKhad
4	KCA1	Kangra City
5	KCA2	Kangra City
6	KCA3	Kangra City
7	ARAM1a	Airport Road
8	ARAM1b	Airport Road
9	KOA1a	Kohli
10	KOA1b	Kohli
11	MA1a	Matour
12	MA1b	Matour

Chemicals, glassware and plastic ware

All routinetly used chemicals (AR Grade) were purchased from HiMedia Laboratories, Mumbai, India. Growth regulators, antibiotics and other fine chemicals were procured from Sigma Chemical Co. (St Louis, MO, USA). Taq DNA Polymerase was procured from Larova (Teltow, Germany). Plasticware such as sterile disposable filter sterilization units were purchased from Tarsons Products Pvt. Ltd. (Kolkata, India). Glassware such as conical flask, measuring cylinders etc were procured from Borosil Glass Works Ltd. (Mumbai, India). Glass culture bottles of 300ml capacity were procured from Kasablanka Corporation (Mumbai, India).

Molecular Characterization

PCR-based molecular markers are widely used in many plant species for identification, phylogenetic relation among the population and genetic linkage mapping (Williams *et al.,* 1990). Both RAPD and ISSR markers have proved to be a reliable, easy to generate, inexpensive and versatile set of markers that rely on repeatable amplification of DNA sequence using single primer.

Isolation of genomic DNA

Genomic DNA was isolated from actively growing shoots using the modified CTAB method (Doyle and Doyle, 1990). 2.0 g fresh tissue of each sample was washed with distilled water, dried and grounded in liquid nitrogen to fine powder, followed by immediate transfer to 50 ml centrifuge tube. To the samples pre-warmed CTAB extraction buffer (10.0 ml) was added to make slurry and incubated at 60 ^{0}C for 1 hr in water bath. Equal volume of chloroform and isoamylalcohol (24:1 v/v) was added to the above slurry and mixed for about 3 mins, followed by centrifugation at 5000 rpm for 10 mins. Aqueous phase was removed with the help of wide-bore pipette and transferred to clean tube. Chloroform extraction step was repeated again in case of coloured extracts. DNA was precipitated with 0.66 volume of cold isopropanol followed by incubation for 1 hr at -20 ^{0}C. After centrifugation (10,000 X g for 15 mins) the supernatant was discarded and the pellet was dissolved in 1 ml TE buffer and transferred to microfuge tube. To the

above solution 2 µl of pre heated RNase solution (10 mg/ml stock) was added and incubated at 37 ^{0}C for 1 hr. To the samples equal volume of phenol and chloroform was added (1:1v/v) followed by gentle shaking and centrifuged (10000 X g for 10 mins). Aqueous layer was retained. To this aqueous solution 0.3 volume of 3M sodium acetate (Appendix I) and 0.6 volume of chilled isopropanol was added and incubated for 1 hr at -20 ^{0}C. Following incubation, samples were centrifuged (10000 X g for 10 mins). The pellet was retained, dried and dissolved in TE Buffer and stored at -20 ^{0}C.

Electrophoresis of DNA on agarose gel

Quality of DNA was checked on 0.8% agarose gel (w/v). Gel was prepared by adding 0.32 g of agarose (Life Technologies India Pvt. Ltd.) in 40 ml of 0.5X TAE (Tris-Acetate-EDTA) buffer. The agarose was melted in microwave oven until dissolved completely. The molten agarose was cooled and 0.1 µl of ethidium bromide (10 mg/ ml) was added and poured into casting tray inserted with combs and allowed to solidify at room temperature. 5.0 µl of DNA sample were mixed with 0.2 volume of 6X gel loading buffer and loaded into well. The gel was electrophoresed on horizontal electrophoresis apparatus (Amersham Bioscience, U.S.A) in TAE running buffer at 50 V for one hr and visualized on a U.V. transilluminator (Vilber Loumart, France).

Quantification of DNA

The concentration of extracted DNA in suspension was estimated by spectrophotometric measurement using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at $A_{260.}$ The quality of DNA was also evaluated by taking the ratio of absorbance at 260 nm and 280 nm. Ideally, the A_{260}/ A_{280} ratio should be 1.8-2.0, if it is less than 1.8 indicate the contamination of proteins, while ratios greater than 2.0 indicate the contamination of RNA.

PCR based markers (RAPD and ISSR)

PCR amplification was performed in 20 µl volume using 40 RAPD decamer primers (Table2) for the random amplified polymorphic DNA analysis and 20 ISSR (16-20 nucleotide) primers (Table3). The reaction mixture consisted of 40 ng of genomic DNA, 1.0 U Taq DNA polymerase

(Larova, Teltow, Germany), 100 µM dNTPs mixture, 2.0 µl reaction buffer (10X), and 10 nmol primer, Mill-Q water (Millipore India, Banglore, India) was added to make up the final volume to 20ul. Amplifications were performed in thermal cycler model Gene Amp 9700 (Applied Biosystem, San Francisco, USA). Amplification conditions were initial denaturation at 94 ^{0}C for 5 mins; 41 cycles of : denaturation at 94 ^{0}C for 60 sec, annealing at 36 ^{0}C (55 ^{0}C in case of ISSR) for 90 sec and extension at 72 ^{0}C for 90 sec; with final extension at 72 ^{0}C for 5 mins. The amplified products were separated on a 1.2 % (w/v) agarose gel at 50 volts on vertical midi gel electrophoresis system (Life technologies, USA).

Agarose gel electrophoresis

Amplified products were separated in 1.2 % agarose gel containing ethidium bromide using 0.5X TAE buffer. A constant voltage of 55V was provided for 4-5 hrs. DNA fragments were visualized under UV light. The patterns were photographed using Geldoc system (BioRad) and stored as digital pictures. The reproducibility of the amplification was confirmed by reporting each experiment three times.

Phylogenetic analysis

The size of the amplicons was determined from gel photographs by comparing with molecular weight markers. Each band of amplified DNA fragment was transformed in to discrete variables or binary characters matrix, '1' (to mark presence) and '0' (to mark absence). The binary data matrices were used to estimate the level of polymorphism by dividing the number of polymorphic bands (not present in all samples) by the total number of scored bands. Amplified fragments in the size range 250-3000 bp, were included in the analysis. Data was subjected to analysis by Jaccard's coefficient to generate matrix and the values were used to construct dendrograms of UPGMA using Multivariate Statistical Package 3.2.1 (MVSP; Kovach Computing Services, Anglesay, Wales).

Principal component analysis

A scatter plot of these accessions was also drawn by PCA using the RAPD and ISSR data (SPSS 16) to reveal pattern of relatedness within matrix coordinates in two dimensions for each accession.

Table2: Sequences of RAPD primers used in the study

PRIMER NO. (RAPD)	PRIMER SEQUENCE(5'-3')
RAPD1	CAGGCCCTTC
RAPD2	TGCCGAGCTG
RAPD3	AGTCAGCCAC
RAPD4	AATCGGGCTG
RAPD5	AGGGGTCTTG
RAPD6	GGTCCCTGACC
RAPD7	GAAACGGGTG
RAPD8	GTGACGTAGG
RAPD9	GGGTAACGCC
RAPD10	GTGATCGCAG
RAPD11	CAATCGCCGT
RAPD12	TCGGCGATAG
RAPD13	CAGCACCCAC
RAPD14	TCTGTGCTGG
RAPD15	TTCCGAACCC
RAPD16	AGCCAGCGAA
RAPD17	GACCGCTTGT
RAPD18	AGGTGACCGT
RAPD19	CAAACGTCGG
RAPD20	GTTGCGATCC

Table3: Sequences of ISSR primers used in the study

PRIMER NO. (ISSR)	PRIMER SEQUENCE (5'-3')
ISSR1	$(GA)_8CG$
ISSR2	$(GA)_8TC$
ISSR3	$(AC)_8GC\ GC$
ISSR4	$(AC)_{10}$
ISSR5	$(CA)_8GC$
ISSR6	$(GC)_8T$
ISSR7	$(GC)_8A$
ISSR8	$(CT)_8G$
ISSR9	$(GT)_8TC$
ISSR10	$(AT)_8C$
ISSR11	$(AT)_8G$
ISSR12	$(AT)_8GC$
ISSR13	$(AT)_8$
ISSR14	$(GA)_8TG$
ISSR15	$(GA)_8C$
ISSR16	$(GA)_8CT$
ISSR17	$(GA)_8CA$
ISSR18	$(GA)_8CC$
ISSR19	$(GA)_8T$
ISSR20	$(CT)_8T$

RESULTS Chapter 4

The plants of *Aerides multiflorum* were collected (Table 4) from different locations around Kangra valley. These were grown in the pots and were used to study the genetic variability using PCR based molecular markers namely RAPD and ISSR.

RAPD

Table 4: List of various RAPD primers used, primers code, amplicon size, total number of bands amplified, polymorphic bands along with percentage polymorphism

S. No	Primer code	Sequence of primer	Amplicon size (bp)	No. amplified bands	No. of polymorphic bands	% polymorphism
1	RAPD1	5'CAGGCCC TTC3'	750-2500	3	0	0
2	RAPD2	5'TGCCGAG CTG3'	500-2500	4	2	50
3	RAPD3	5'AGTCAGC CAC3'	500-3000	5	1	20
4	RAPD4	5'AATCGGG CTG3'	1000	1	0	0
5	RAPD5	5'AGGGGTC TTG3'	750-2500	2	1	50
6	RAPD6	5'GGTCCCT GACC3'	1000-2500	2	0	0
7	RAPD7	5'GAAACGG GTG3'	700-3500	11	6	54.54
8	RAPD8	5'GTGACGT AGG3'	2500	1	0	0
9	RAPD9	5'GGGTAAC GCC3'	600-2000	6	2	33.33
10	RAPD10	5'GTGATCG	750-2000	2	0	0

		CAG3’				
11	RAPD11	5’CAATCGC CGT3’	750-1700	2	1	50
12	RAPD12	5’TCGGCGA TAG3’	600-750	2	0	0
13	RAPD13	5’CAGCACC CAC3’	1200	1	0	0
14	RAPD14	5’TCTGTGCT GG3’	500-1000	2	0	0
15	RAPD15	5’TTCCGAA CCC3’	1500	1	0	0
16	RAPD16	5’AGCCAGC GAA3’	250-750	2	0	0
17	RAPD17	5’GACCGCT TGT3’	500-1500	3	0	0
18	RAPD18	5’AGGTGAC CGT3’	500-1000	2	0	0
19	RAPD19	5’CAAACGT CGG3’	750-1500	3	0	0
20	RAPD20	5’GTTGCGA TCC3’	250-1000	5	0	0
				Total no. Of bands=60	Total no. Of polymorphic bands=13	Average polymorphism=21.66

Genetic relationship among the 12 accessions has been carried out using RAPD. In this investigation, 20 random decamer oligonucleotide primers were used for amplification of 12 accessions of *Aerides multiflorum*. A total of 13 polymorphic bands were observed with selected primers RAPD2, RAPD3, RAPD5, RAPD7, RAPD9, RAPD11. The highest number of polymorphic bands i.e. 6 was obtained with primer RAPD7, with 54.54% polymorphism. The average percentage of polymorphism was 21.66 %.

Table5: List of sample codes, their collection place and lane number in gels.

LANE NO.	SAMPLE CODE	COLLECION PLACE
1	1KB Ladder	-
2	Negative Control	-
3	TA1	Takipur, Kangra
4	KA1	Kangra Khad
5	KA2	Kangra Khad
6	KCA1	Kangra City
7	KCA2	Kangra City
8	KCA3	Kangra City
9	ARAM1a	Airport Road
10	ARAM1b	Airport Road
11	KOA1a	Kohli
12	KOA1b	Kohli
13	MA1a	Matour
14	MA1b	Matour

Figure2 shows amplification with RAPD markers using different primers. Gel pictures showed various monomorphic and polymorphic bands. The size of amplicons varied from 250bp to 2500bp. In Figure 2A a sharp monomorphic band can be seen at 2500bp and few polymorphic bands are also present below 2000bp upto 250bp. Figure 2B, 2C, 2D shows good intensity of polymorphic bands. No amplification was seen in negative control. Ladder used was 1kb. Maximum number of bands are obtained between 500bp and 2500bp. This figure shows good number of polymorphic bands.

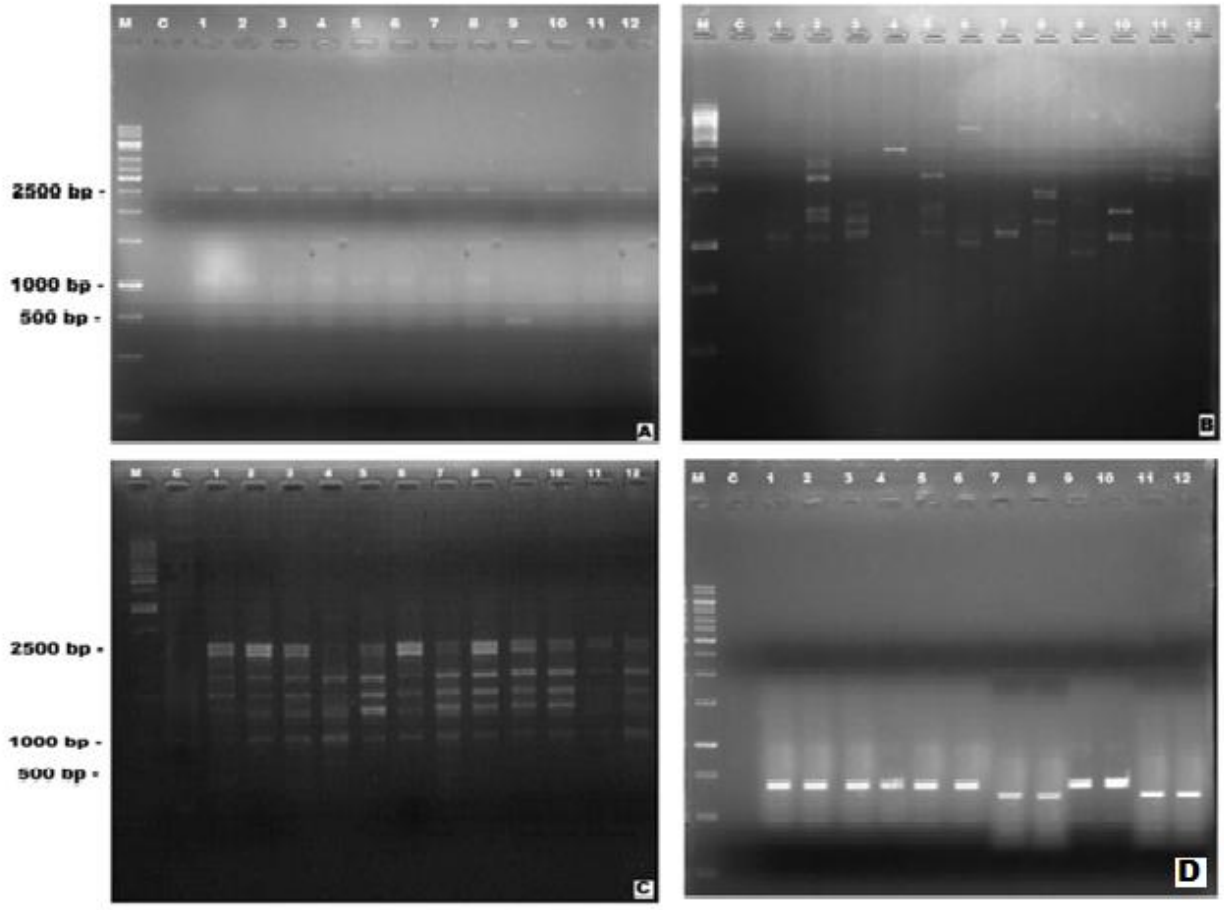

Figure2: RAPD profiles of 12 accessions of *Aerides multiflorum* showing the banding pattern. A) RADP profile using primer RAPD2; B) RAPD profile using primer RAPD4; C) RAPD profile using primer RAPD6; D) RAPD profile using primer RAPD8. Maximum number of markers were obtained between 1000bp and 2500bp. Lane M= 1kb marker, Lane C= Negative control, Lane 1-12= Various accessions as mentioned in Table 5.

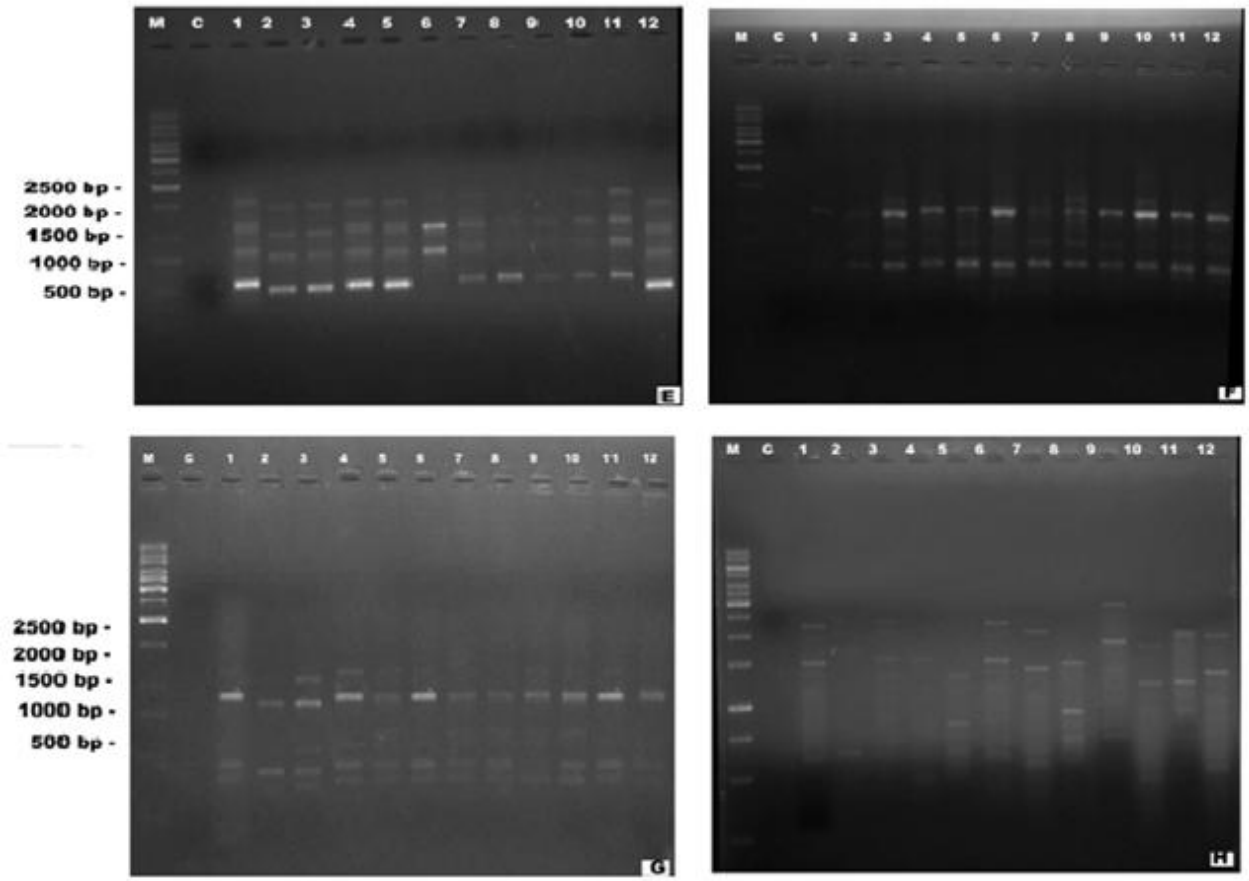

Figure3: RAPD profiles of 12 accessions of *Aerides multiflorum* showing the banding pattern. E) RADP profile using primer RAPD10; F) RAPD profile using primer RAPD12; G) RAPD profile using primer RAPD14; H) RAPD profile using primer RAPD16. Maximum number of markers were obtained between 150bp and 2000bp. Lane M= 1kb Marker, Lane C= Negative control, Lane 1-12= Various accessions as mentioned in Table 5.

Figure 2E, 2F, 2G and 2H shows good number of polymorphic bands.

Cluster analysis

Clustering is the process of organizing objects into groups based on the similarity among accessions. Cluster analysis was performed by using Jaccard's coefficient which is a statistic used to compare similarity and diversity of sample sets.

Table6: Jaccard's Coefficient of 12 accessions of *Aerides multiflorum* using RAPD data

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	1.000											
2	0.791	1.000										
3	0.682	0.711	1.000									
4	0.651	0.644	0.775	1.000								
5	0.717	0.783	0.837	0.727	1.000							
6	0.762	0/750	0.805	0.775	0.837	1.000						
7	0.721	0.711	0.762	0.775	0.795	0.805	1.000					
8	0.609	0.604	0.721	0.690	0.717	0.682	0.721	1.000				
9	0.636	0.667	0.756	0.769	0.711	0.756	0.714	0.674	1.000			
10	0.682	0.711	0.805	0.821	0.795	0.805	0.805	0.682	0.800	1.000		
11	0.644	0.674	0.609	0.614	0.717	0.644	0.805	0.644	0.600	0.682	1.000	
12	0.682	0.750	0.682	0.732	0.717	0.721	0.850	0.682	0.674	0.721	0.850	1.000
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

1:TA1; 2:KA1; 3:KA2; 4:KCA1; 5:KCA2; 6:KCA3; 7:ARAM1a; 8:ARAM1b; 9:KOA1a; 10:KOA1b; 11:MA1a; 12: MA1b

The Jaccard's similarity coefficient of 12 accessions of *Aerides multiflorum* based on RAPD revealed that similarity value among accessions ranged from 0.600 to 0.850 indicating low to moderate level genetic similarity. Maximum similarity value of 0.850 was recorded between MA1a (Matour) and MA1b (Matour).While the lowest similarity value was obtained between MA1a (Matour) and KOA1a (Kohli).

Dendrogram

A dendrogram is a branching diagram that represents relationship of similarities among a group of entities. It consists of clades (branches) and leaves (nodes). Arrangment of clades tell us about the similarity of leaves. The dendrogram was contructed using algorithm UPGMA.

Figure 4 shows that MA1b and ARAM1b are highly similar followed by KA2 and KCA3, KCA1 and KOA1b, atlast by TA1 and KA1. Dendrogram based on RAPD data separated all accessions into two major clusters. The first major cluster was divided into two subclusters, the first sub-cluster consisted only two accessions TA1 (Indore) and KA1 (Katni) while, the second sub-cluster comprised MA1a (Matour), MA1b (Matour), ARAM1a (Airport Road), KOA1a (Kohli), KOA1b (Kohli), KCA1 (Kangra City), and KCA2 (Kangra City), KCA3(Kangra City), KA2 (KangraKhad). The second major cluster contains only ARAM1a.

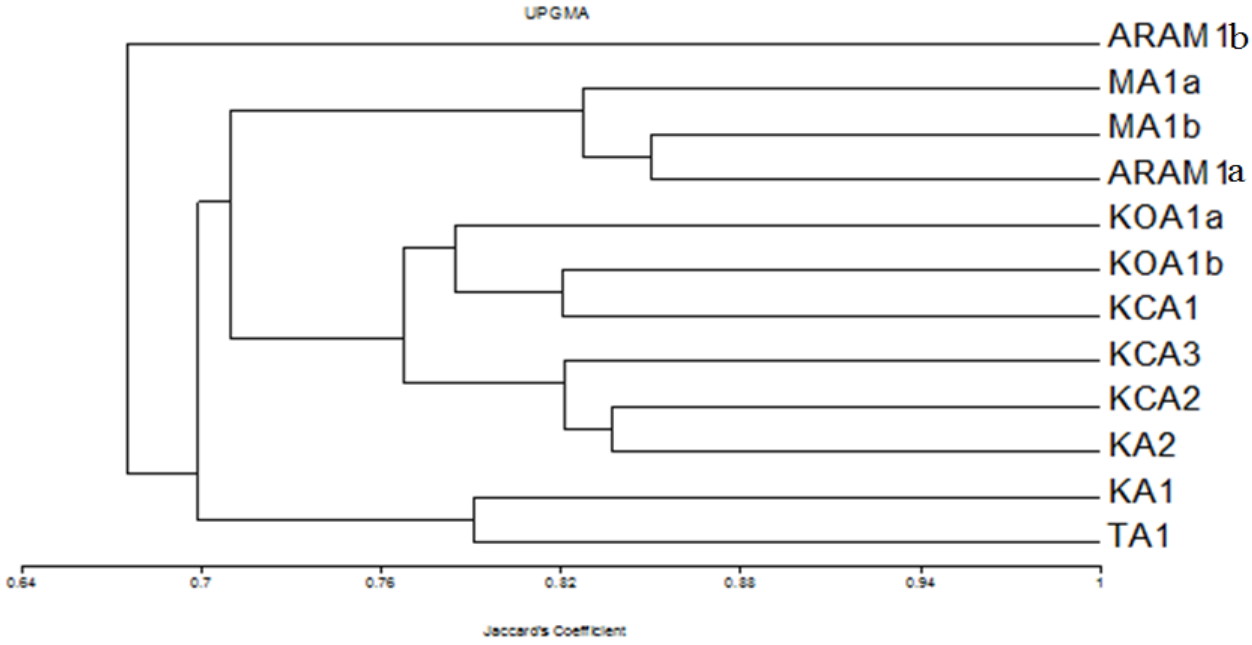

Figure4: Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from RAPD data of 12 accessions of *Aeridus multiflorum*

Principal Component Analysis

It is a mathematical procedure which converts numbers of possibly correlated variables into smaller number of uncorrelated variables called principal component. Below in given Figure5 we can see that all the accessions are positively correlated on component 1, whereas on component 2 both positive and negative correlations are observed. In this two-dimensional PCA scatter plot accession MA1a and MA1b are separated out from all the accessions. Among all accessionsKCA3 and KOA1b are highly correlated.

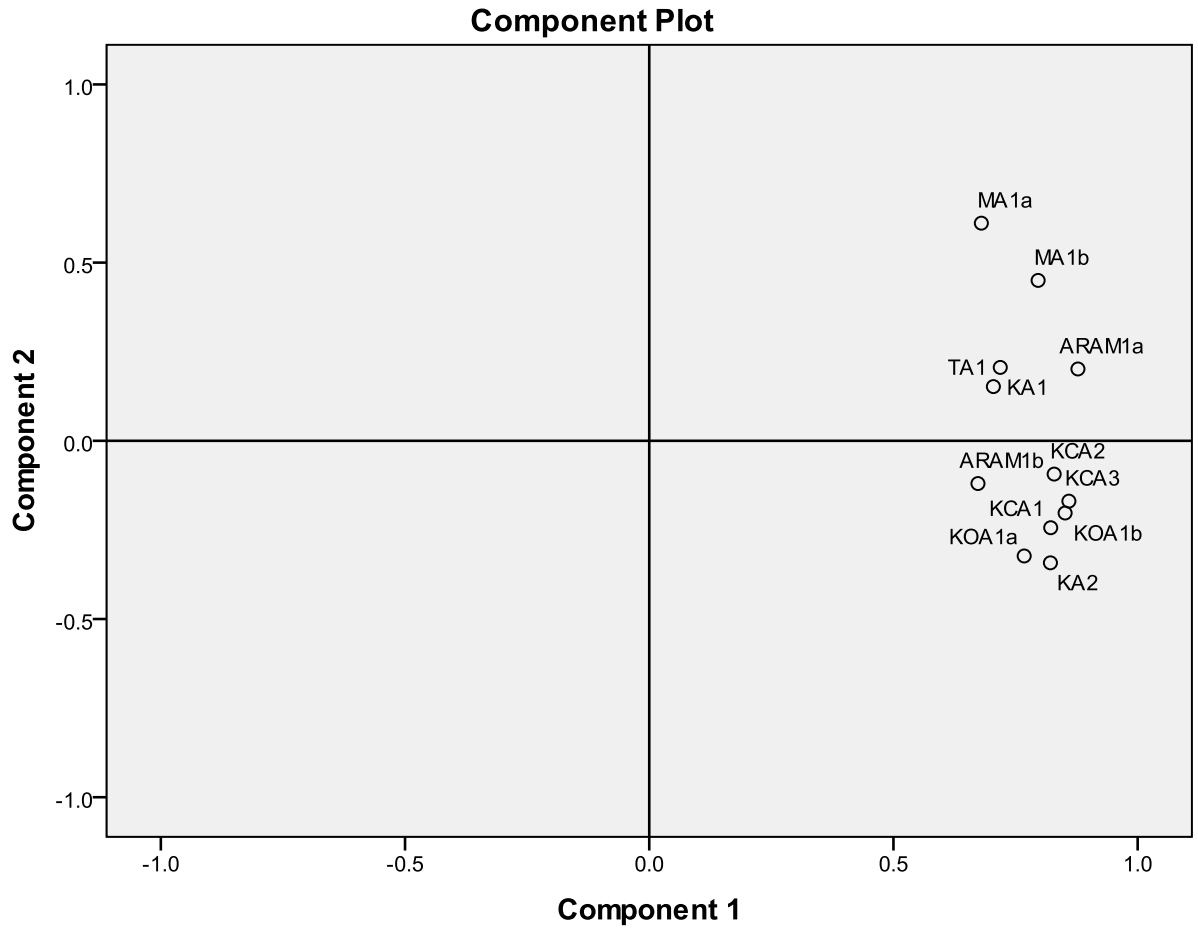

Figure5: Scatter plot of Principle component analysis (PCA) of RAPD data of 12 accessions of *Aerides multiflorum*

ISSR

Table7: List of various ISSR primers used, primers code, amplicon size, total number of bands amplified, polymorphic bands along with percentage polymorphism.

Serial No.	Primer Code	Sequence of Primer	Amplicon Size (bp)	No. Of amplified bands	No. Of polymorphic bands	Percent polymorphism
1	ISSR2	$(GA)_8CG$	250-1500	9	5	55.55
2	ISSR3	$(GA)_8TC$	250-1500	10	4	40
3	ISSR4	$(AC)_8GC$ GC	250-1500	7	3	42.86
4	ISSR5	$(AC)_{10}$	250-1500	9	1	11.11
5	ISSR7	$(CA)_8GC$	250-1500	9	4	44.44
6	ISSR9	$(GC)_8T$	250-2500	14	5	35.71
7	ISSR10	$(GC)_8A$	250-3000	12	6	50
8	ISSR12	$(CT)_8G$	230-2500	9	4	44.44
9	ISSR16	$(GT)_8TC$	250-1500	9	1	11.11
10	ISSR17	$(AT)_8C$	250-1500	9	4	44.44
11	ISSR18	$(AT)_8G$	250-1500	8	3	37.5
12	ISSR19	$(AT)_8GC$	250-3000	7	3	42.86
13	ISSR20	$(AT)_8$	250-2500	8	4	50
14	ISSR21	$(GA)_8TG$	250-1000	4	2	50
15	ISSR22	$(GA)_8C$	250-3000	9	4	44.44
16	ISSR23	$(GA)_8CT$	250-3000	17	7	41.176
17	ISSR24	$(GA)_8CA$	250-1500	7	1	14.286

18	ISSR25	$(GA)_8CC$	250-1500	13	6	46.154
19	ISSR26	$(GA)_8T$	250-1000	2	0	0
20	ISSR27	$(CT)_8T$	200-1500	9	4	44.44
				Total no. Of markers=181	Total no. Of polymorphic markers=71	Average polymorphism =39.22

The oligonucleotide sequences of the primers and the resultant multiple band patterns are summarized in Table 7.

All 20 primers used for amplification produced clear and reproducible bands. A total of 181 fragments in the size range of 250-4000bp were amplified with an average of eight bands per primer. Out of 181 markers, 71 were polymorphic. The analysis showed the percentage of polymorphic bands is 39.22%. The Polymorphic bands suggested that ISSR primers were the most suitable polymorphic markers to detect the genetic diversity of accessions of *Aerides multiflorum* at the molecular level.

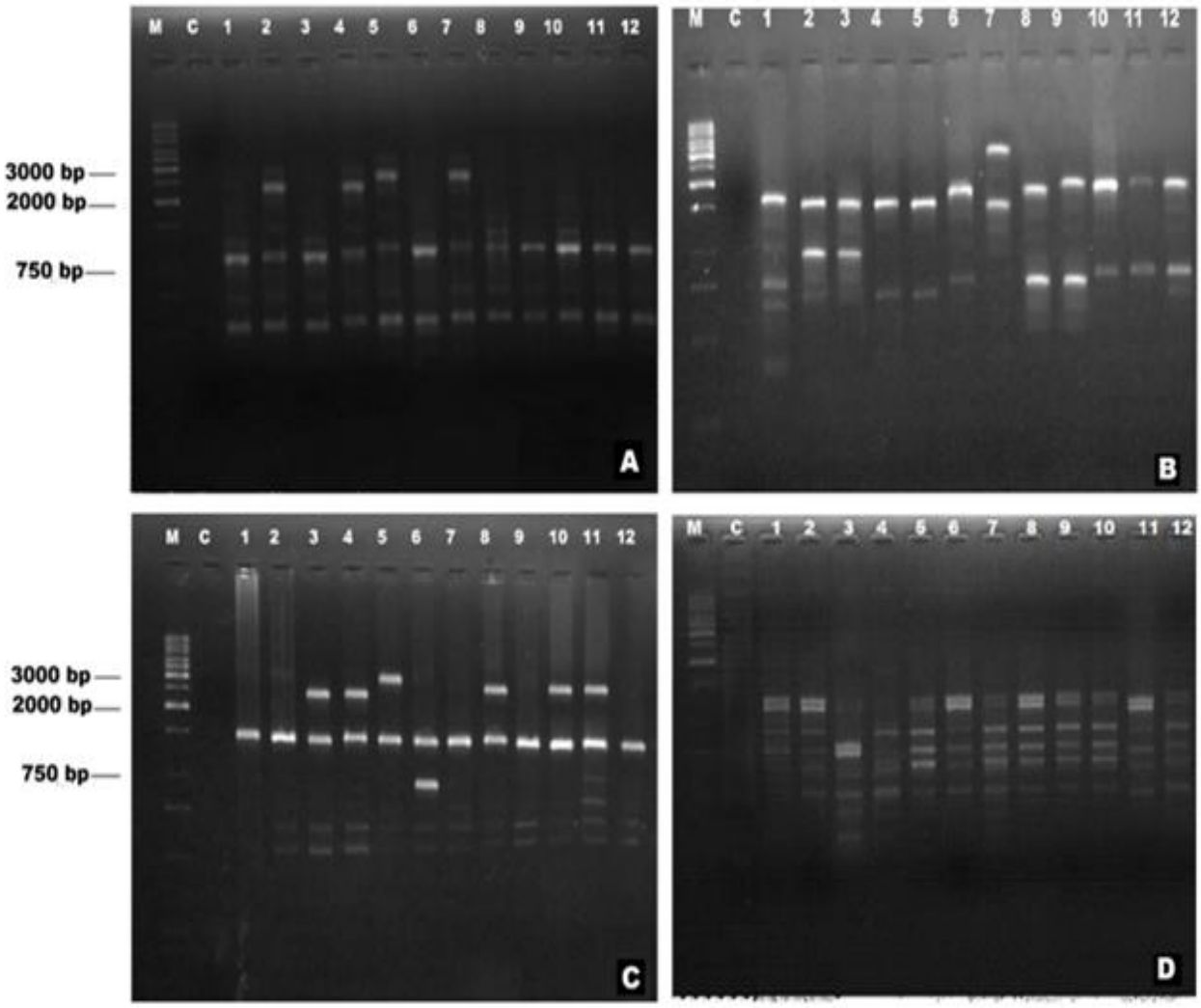

Figure6: ISSR profiles of 12 accessions of *Aerides multiflorum* showing the banding pattern. A) ISSR profile using primer ISSR1; B) ISSR profile using primer ISSR3; C) ISSR profile using primer ISSR5; D) ISSR profile using primer ISSR7. Maximum number of markers were obtained between 250bp and 3000bp. Lane M= 1kb Marker, Lane C= Negative control, Lane 1-12= Various accessions as mentioned in Table 5.

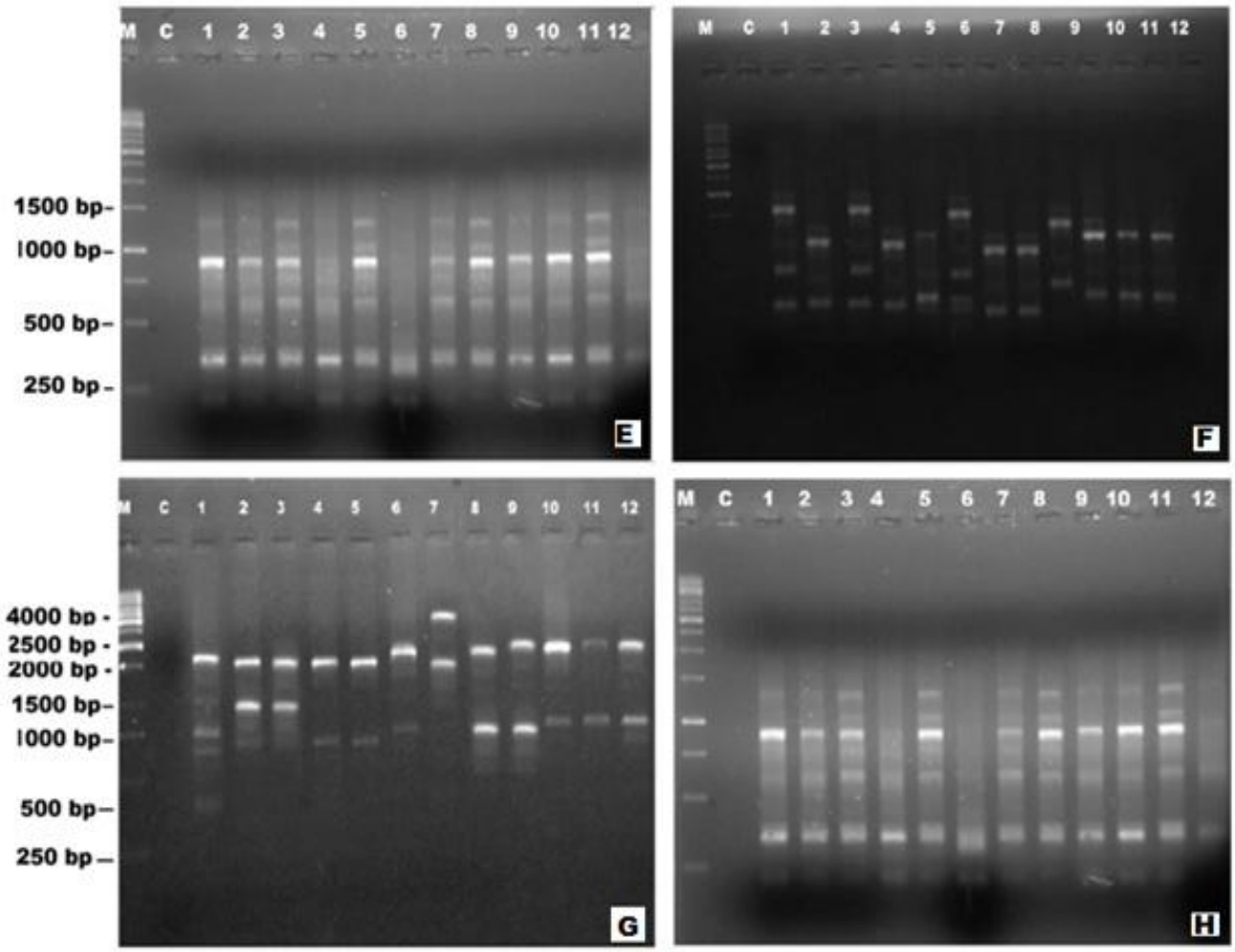

Figure7: ISSR profiles of 12 accessions of *Aerides multiflorum* showing the banding pattern. E) ISSR profile using primer ISSR11; F) ISSR profile using primer ISSR13; G) ISSR profile using primer ISSR15; H) ISSR profile using primer ISSR17. Marker size is upto 4000bp and maximum number of markers were obtained between 250bp and 2000bp. Lane M= 1kb Marker, Lane C= Negative control, Lane 1-12= Various accessions as mentioned in Table 5.

Figure 6A, 6B, 6C, 6D, 6E, 6F, 6G and 6H contains good number of polymorphic bands.

Cluster analysis

Table8: Jaccard's Coefficient among 12 accessions of *Aerides multiflorum* using ISSR data

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	1.000											
2	0.353	1.000										
3	0.388	0.516	1.000									
4	0.275	0.580	0.478	1.000								
5	0.358	0.420	0.515	0.426	1.000							
6	0.330	0.394	0.333	0.430	0.370	1.000						
7	0.415	0.489	0.511	0.398	0.631	0.465	1.000					
8	0.398	0.464	0.515	0.411	0.558	0.457	0.713	1.000				
9	0.376	0.448	0.409	0.372	0.500	0.476	0.635	0.571	1.000			
10	0.402	0.441	0.448	0.355	0.505	0.402	0.613	0.667	0.590	1.000		
11	0.355	0.444	0.495	0.406	0.475	0.394	0.568	0.602	0.529	0.699	1.000	
12	0.250	0.372	0.382	0.358	0.363	0.378	0.430	0.459	0.459	0.526	0.623	1.000
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

1:TA1; 2:KA1; 3:KA2; 4:KCA1; 5:KCA2; 6:KCA3; 7:ARAM1a; 8:ARAM1b;9:KOA1a; 10:KOA1b; 11:MA1a; 12: MA1b

In order to estimate the genetic distances among these accessions, the similarity matrix was computed with the Jaccard method. The obtained matrix showed that Jaccard's coefficient of similarity values ranging from 0.250 to 0.713 revealed a considerable level of genetic diversity among these 12 accessions. The smallest similarity value (0.250) suggested the high divergence between TA1 and MA1b while, the maximum similarity value (0.713) was scored between ARAM1a and ARAM1b indicating that both accessions are most similar.

Dendrogram

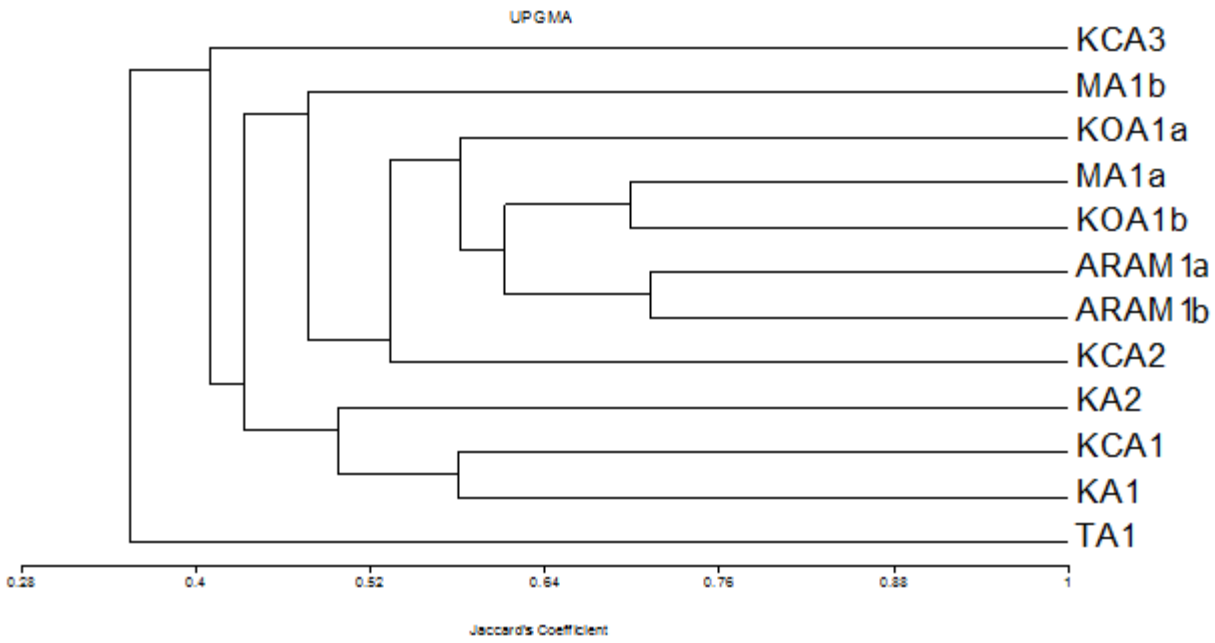

Figure8: Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from ISSR data of 12 accessions of *Aerides multiflorum*

The Dendrogram for 12 *Aerides multiflorum* accessions revealed two main clusters. The first major cluster further divided into two subclusters. The first subcluster contained only one accessions KCA3 (Kangra City) while, the second subcluster comprised ten accessions, namely MA1b (Matour), KOA1a (Kohli), MA1a (Matour), KOA1b (Kohli), ARAM1a (Airport Road), ARAM1b (Airport Road), KCA2 (Kangra City), KA2 (KangraKhad), KCA1(Kangra City), and KA1(KangraKhad).

The second main cluster is TA1. So from the above figure we can analyze that ARAM1a and ARAM1b share most of the similarity, followed by MA1, KOA1b then KA1, KCA1. TA1 is most distantly related from all the remaining samples.

Principal Component Analysis

Figure 9 shows two components. All the accessions are positively correlated on component 1, whereas KCA1, KA1, KA2, KCA3, KCA2 are positively correlated on component 2 and all other accessions are negatively correlated. Accessions KOA1a and ARAM1b are highly correlated. KCA1, KA1, KA2, KCA3, KCA2 accessions are forming scattered cluster while ARAM1a, ARAM1b, KOA1a, KOA1b, MA1a, MA1b are forming cohesive cluster except TA1 which is seperated out from the group.

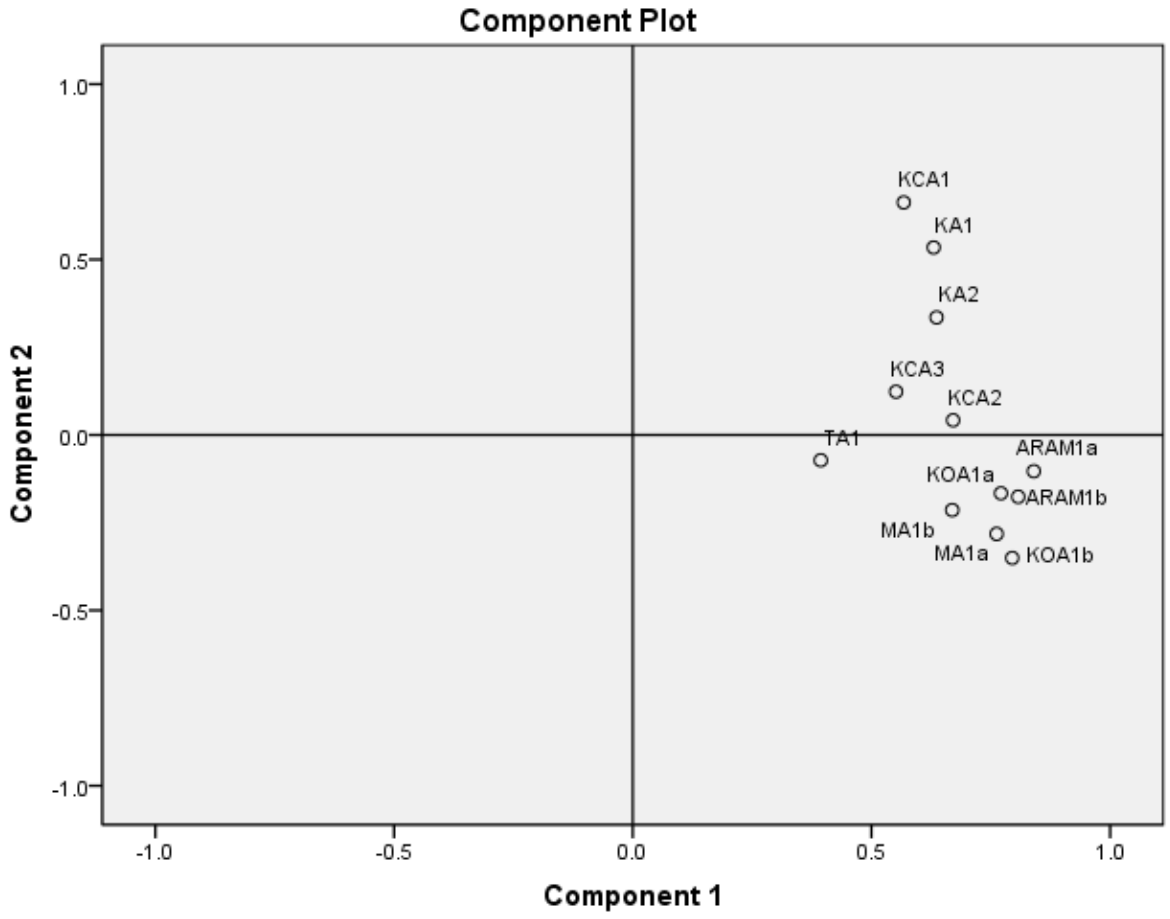

Figure9: Scatter plot of Principle component analysis (PCA) of ISSR data of 12 accessions of *Aerides multiflorum*

RAPD+ISSR

Cluster analysis

Table9: Jaccard's Coefficient among 12 accessions of *Aerides multiflorum* based on combined data of ISSR and RAPD

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	1.000											
2	0.483	1.000										
3	0.476	0.580	1.000									
4	0.386	0.603	0.569	1.000								
5	0.467	0.534	0.614	0.522	1.000							
6	0.458	0.507	0.469	0.540	0.510	1.000						
7	0.511	0.564	0.591	0.516	0.688	0.575	1.000					
8	0.463	0.510	0.579	0.496	0.610	0.529	0.715	1.000				
9	0.460	0.523	0.515	0.496	0.571	0.569	0.664	0.606	1.000			
10	0.489	0.529	0.555	0.492	0.599	0.526	0.678	0.672	0.661	1.000		
11	0.441	0.517	0.531	0.471	0.551	0.472	0.643	0.616	0.553	0.693	1.000	
12	0.386	0.500	0.481	0.484	0.482	0.496	0,571	0.535	0.538	0.597	0.701	1.000
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**

1:TA1; 2:KA1; 3:KA2; 4:KCA1; 5:KCA2; 6:KCA3; 7:ARAM1a; 8:ARAM1b;9:KOA1a; 10:KOA1b; 11:MA1a; 12: MA1b

Cluster analysis was performed from combining data of two markers (RAPD and ISSR) and a dendrogram was generated distinguishing the samples into two clusters. The similarity index ranged from 0.386 to 0.715.

Dendrogram

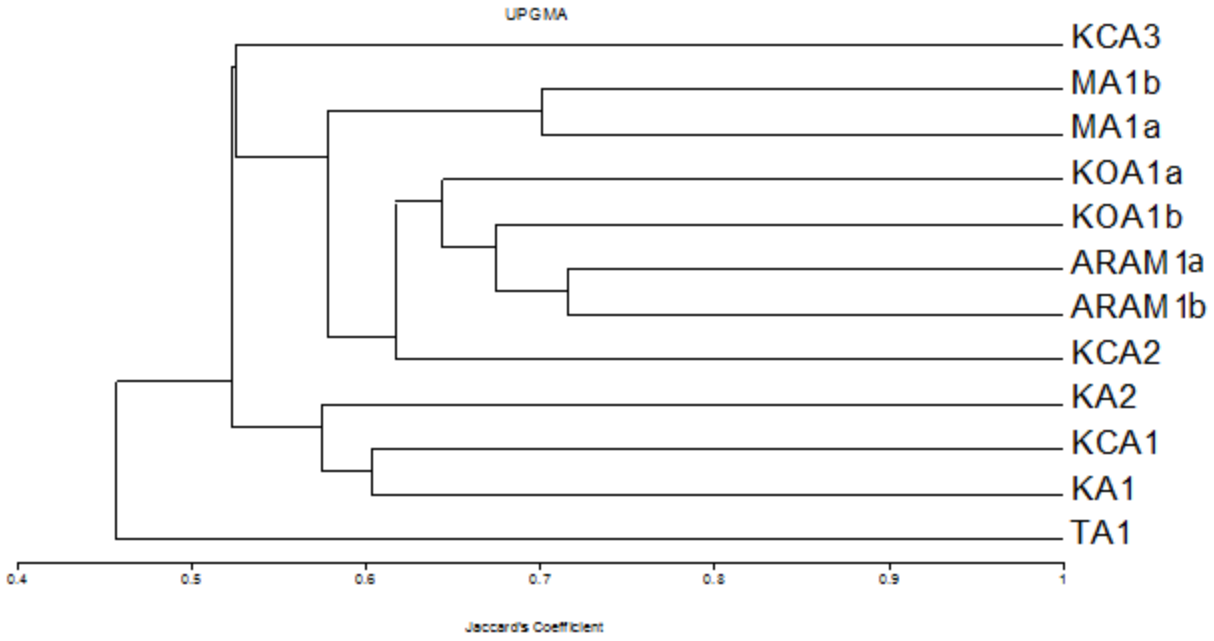

Figure10: Unweighted pair group method with average (UPGMA) cluster based on Jaccard's coefficient calculated from combined RAPD and ISSR data of 12 accessions of *Aerides multiflorum*

The dendrograms based on RAPD, ISSR combined markers showed partially different genetic similarity compared to individual data for RAPD, ISSR analysis. However, clusters of ISSR had more similarity with the combined cluster analysis as compared to that of RAPD. Figure 8 shows that ARAM1a and ARAM1b share highest similarity with each other, followed by KOA1b then KOA1a. These all show a good similarity with MA1a and MA1b. This data also shows that all the sequences are diverged from TA1.

Principal Component Analysis

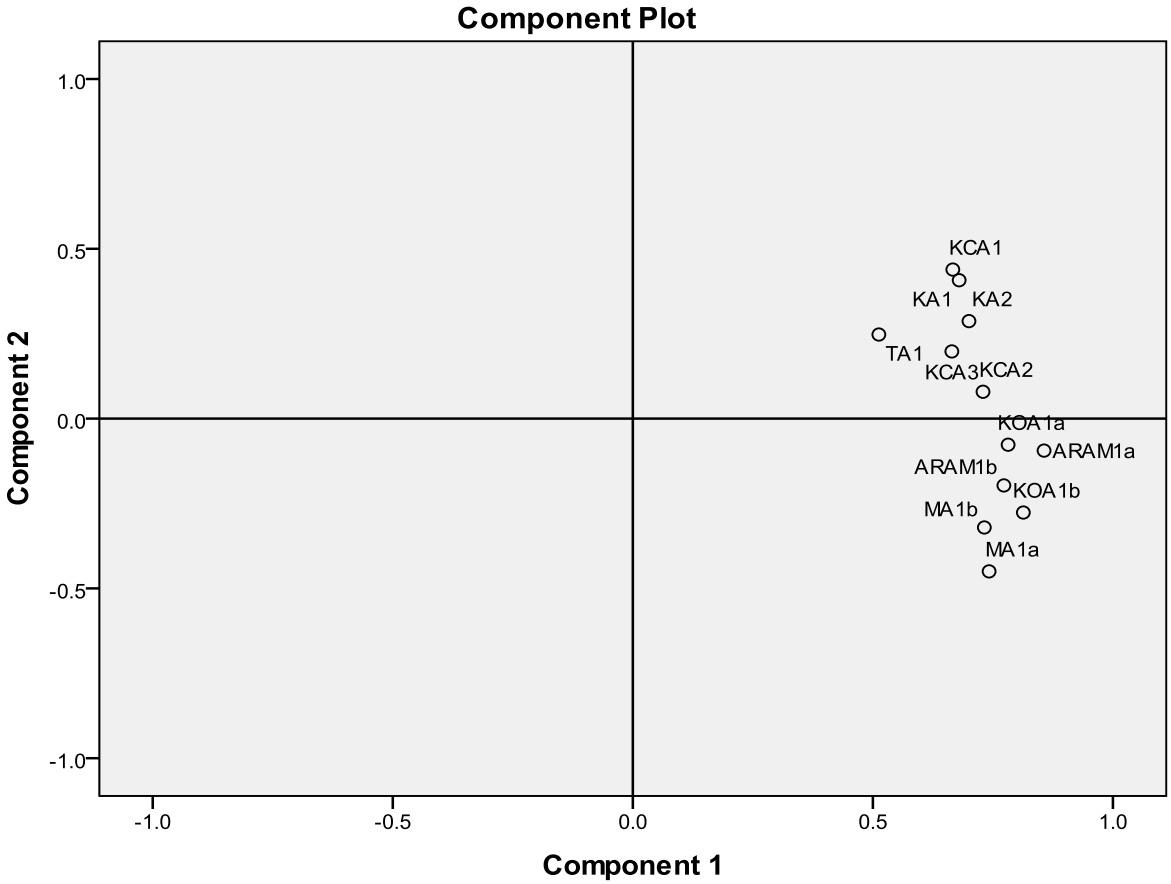

Figure11: Scatter plot of Principle component analysis (PCA) of combined RAPD and ISSR data of 12 accessions of *Aerides multiflorum*

All the accessions are positively correlated on component 1 whereas KCA1, KA1, KA2, TA1, KCA2, KCA3 are positively correlated on component 2 and all other accessions are negatively correlated, which shows that combined results of RAPD and ISSR data are very similar to RAPD and ISSR results individually. A strong correlation can be seen among all the accessions.

DISCUSSION Chapter 5

Aerides multiflorum Roxb., a very beautiful ornamental flower with antibacterial properties is a tropical epiphyte belong to family Orchidaceae. Although its seeds are produced in millions but only very few are able to germinate because seeds are under developed and tiny seeds which cannot enough food for them. Infact seed germination in nature can only be achieved following mycorrhizal association. Therefore, there is a felt need to conserve important ornamental and medicinal orchid species. There are various methods of conservation like *exsitu* which includes seed banks and *in vitro* plant tissue culture and in-situ where plants are conserved in their natural habitats (McCauley, 1995). But the most important one is the genetic conservation. Most of the threatened and critically endangered species are at the verge of extinction. This is due to their very narrow genetic base which makes them homozygous and equally responsive for negative stresses (Mukherjee *et al.,* 2011).

So study of genetic diversity is very important as it can show a clear picture of species that whether they can survive in long run or not. A population with low genetic diversity cannot tolerate negative environmental impacts as most of the population is identical (Frankel, 1977). But population with higher genetic diversity can lead to new genetic makeup of individuals which make them to survive under adverse conditions (Frankham, 1996).

As orchids are limited to certain localizations, so destruction of their habitat can lead to their loss (Khasim and Rao, 1999). So there is an immediate need to study the genetic variation in populations so that required conservation measures can be taken (Ouborg 1999). DNA based molecular markers are used to study genetic diversity, because they can detect the minor sequence differences.

In this study RAPD and ISSR markers were used to study diversity among 12 accessions of *Aerides multiflorum*. RAPD markers are polymorphic, require very low amount of DNA, no

radioactive assays are required, and does not require specific probe libraries (Williams *et al.*, 1990). ISSR markers are co-dominant markers, simple and easy to use, easily detected by PCR, and perfectly suited for map based cloning (Giancarla *et al.,* 2012). Earlier these markers have been effectively used to study the diversity in *Aerides maculosum* (Parab *et al.,* 2008).

Population genetics in varieties of *Aerides multiflorum* were studied using RAPD and ISSR markers. In present study, the applicability of ISSR and RAPD is compared as genetic marker to characterize the population of *Aerides multiflorum*. The results indicate that percentage of ISSR polymorphic bands (39.22%) are higher than RAPD polymorphic bands (21.66%),which is in accordance with work done on *Coleus forskohlii* where percentage polymorphism with RAPD was 61.39% while with ISSR was 68.75% (Tripathi *et al.,* 2013). Total number of fragments obtained in ISSR were 181, out of which polymorphic bands were 71 which is more as compare to polymorphism obtained with RAPD. The utility of PCR based markers in studying population genetics has been well recognized (Mukherjee *et al.,* 2013).

Genetic relationship among the 12 accessions has been carried out using RAPD. In this investigation, 20 random decamer oligonucleotide primers were used for fingerprinting of 12 accessions of *Aerides multiflorum*. These results are in line with earlier reports on other places (Tripathi *et al.,* 2013; Parab and Krishnan, 1996). In case of *Rhynchostylis* only 30 ISSR fragments were generated from seven primers while 74 RAPD fragments were generated from 13 primers (Parab and Krishnan, 1996). Similarly 101 RAPD fragments are generated from 13 RAPD primers, and 94 fragments from six ISSR primers (Parab *et al.,* 2008).

The results of the present study also suggest that ISSR markers were superior to RAPD markers in present study to reveal more polymorphic bands. These results corroborate the earlier findings (Bansal *et al.,* 2014). Dendrograms generated using both RAPD and ISSR markers are different. In RAPD highest genetic diversity can be seen between ARAM1a and KA2, while in ISSR highest genetic diversity is seen between ARAM1a and TA1. When both RAPD and ISSR are combined

same results can be seen like ISSR. Jaccard's coefficient analysis of RAPD shows that MA1a and MA1b shares 80% similarity, while 60% (minimum) between ARAM1b and TA1. In ISSR maximum similarity recorded was70% between KOA1b and MA1a, while minimum was 25% in TA1 and MA1b. Results shown by ISSR Jaccard's coefficient were in accordance with RAPD+ISSR.

RAPD and ISSR markers have been used in many studies for DNA fingerprinting and Phylogenetic analysis (Tripathi *et al.*, 2013). ISSR primers produce more reliable and reproducible bands than RAPD primers. However in our study, we have seen that reproducibility of RAPD and ISSR markers depends on right PCR conditions (Mukherjee et al., 2013). Similar results were found with *Chilean nothofagus* species (Bansal et al., 2014).

From this study, it can be concluded that there is significant variation among 12 accessions of *Aerides multiflorum* collected from different locations of Kangra Valley on the basis of RAPD and ISSR data. As number of polymorphic markers obtained is higher in ISSR so we can say that level of variation recorded with ISSR data is higher as compared to RAPD data.

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