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Advances in biochemical and molecular marker techniques and their applications in genetic studies of orchid: A review

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Abstract
Characterization is most important for correct identification of plants. It helps to understand the genetic diversity, to trace out the phylo-genetic relationship, taxonomical status, registration, plant variety protection, farmer's right etc. The use of biochemical and molecular markers for genetic diversity analysis as a selection tool is a high priority area for new orchid cultivars and the exploitation of species diversity. Biochemical markers, molecular markers and DNA sequence analysis allow a more complete characterization and understanding of the genetic relationships between species and cultivars. Various types of techniques are used to estimate genetic studies such as isozymes, allozymes, phyto-chemical and DNA markers like amplified fragment length polymorphism (AFLP), cleaved amplified polymorphic sequence (CAPS), DNA amplification fingerprinting (DAF), diversity arrays technology (DArT), next generation sequencing technology (NGST), expressed sequence tags (EST), inter-simple sequence repeat (ISSR), microsatellite primed PCR (MPPCR), multiplexed allele-specific diagnostic assay (MASDA), random amplified microsatellite polymorphisms (RAMP), random amplified microsatellites (RAM), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), sequence characterized amplified regions (SCAR), sequence specific amplification polymorphisms (S-SAP), sequence tagged microsatellite site (STMS), sequence tagged site (STS), short tandem repeats (STR), simple sequence length polymorphism (SSLP), simple sequence repeats (SSRs), single nucleotide polymorphism (SNP), single primer amplification reactions (SPAR) and variable number tandem repeat (VNTR). Among these techniques, some techniques have been used for genetic diversity analysis of orchid. Today, new techniques are frequently being developed and no such techniques are ideal yet these fulfill all requirements needed by plant researchers. Each technique has its own advantages and limitations. This review is an attempt to discuss a basic description of different biochemical and molecular techniques that can be utilized for genetic studies in orchids.

Keywords: biochemical, molecular marker, genetic studies, orchid

1. Introduction
Floriculture is a branch of horticulture concerning cultivation of flowering and ornamental plants for gardens and floristry. It includes cut flowers, cut greens, bedding plant, houseplants, flowering garden and potted plants etc. Commercial floriculture has higher potential per unit area than the field crops and is therefore evolving as a lucrative business all over the world (Misra and Sudip, 2016) [92]. Indian floriculture industry stands 2nd in world production (Shilpa and Singh, 2016) [113] and contributes rupees 455 crores which is 0.06 percent of global trade (De and Singh, 2016) [22]. Among the flowering crops, Orchidaceae is one of the largest and most diverse families of flowering plants 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) [64,114]. India accounts for 1141 species of orchids belonging to 166 genera they are mainly distributed in the Himalayan, northeastern and peninsular regions of the country (Bhanwra *et al.* 2006) [11]. 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) [20]. Orchids are well known for their beauty (Griesbach, 2003) [45] and cover an exceptionally wide range of different shapes, forms, sizes and colouration (Dressler, 1993, Thomas and Michael, 2007) [29,126]. It has been documented that 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 Basac, 1998, Lopez and Runlde, 2005; Attri *et al.* 2008) [150,75,5].In addition, the species of this genus are able to produce large crops. *Orchids* varieties on the market are diverse and complex; however, it is difficult to distinguish one species from another, because of their very similar outward appearance before flower development, making identification problematic.

Plant breeders have traditionally used morphological attributes to classify genetic resources of crops. The morphological traits that are controlled by a single locus can be used as genetic markers. They could be affected by environmental conditions, thus, they might not be appropriate for accurate analysis (Goodman and Paterniani, 1969, Gerdes and Tracy, 1994) [44,41]. Higher cost and time required for data collection and lack of knowledge of genetic control of phenotypic traits are other limitations of morphological markers leading germplasm curators towards more reliable and faster methods of characterization.

Now a days, molecular markers are considered to provide a better estimate of genetic diversity as they are unaffected by environmental factors, which affect the phenotype. Before the DNA revolution, Biochemical markers such as isozymes were the first molecular tool to be used for genetic characterization (Tanksley and Orton 1983, Smith, 1986, Soltis and Soltis, 1990) [123,117,119]. Biochemical markers may also be biased since these markers represent a small portion of the genome and generally they exhibit low polymorphism. The assessment is rapid and may be largely automated at the reducing cost. Molecular approaches collectively represent a potential gold mine of important information that can be applied as an efficient tool for effective characterization of germplasm. DNA based markers, for cultivar identification and diversity analysis, are gaining importance since fifteen years. DNA based markers can be used for direct analysis of genome. Moreover, these markers are tissue and age neutral and are not affected by the environment. Power of discrimination between cultivars is very high. It is important to use biological molecular markers as an identification method which may be useful for crop improvement programme. DNA based markers have been used in various ornamental plants for genetic diversity studies including bougainvillea (Chatterjee *et al*. 2007) [15], chrysanthemum (Baliyan *et al*., 2014 a,b,c and Kumar *et al*. 2018) [6,7,8and65] gladiolus (Kumar *et al*. 2016, Chaudhary *et al*. 2018) [66,16], jasminum (Mahmood *et al*. 2013) [86], rose (Baydar *et al*. 2004) [9] and tuberose (Sirohi *et al*. 2017[a and b]) [115,116]. Molecular markers provide a vast number of descriptors that can be used in addition to morphological data where these are unable to distinguish varieties/germplasm. Among them some markers have been developed as reliable tool to obtain more consistent information on the existing genetic diversity (Zhang *et al*. 2014a,b, Fajardo *et al*. 2017 and Tian *et al*. 2018) [159,160,34,127]. In addition, the taxonomy, phylogeny, breeding of *Dendrobium* species and advances of molecular markers used in orchids in the past two decades have been discussed in the earlier (Teixeira da Silva *et al*. 2016) [125]. This review is an attempt to evaluate critically the role of different biochemical and DNA based markers their importance and advantages in the characterization of orchids. These markers can be categorized of four types.

1. Morphological markers

It is also known as morpho-agronomic traits. Morphological markers can visually distinguish qualities like seed structure, flower colour, growth habit and other important agronomic traits. Morphological markers are easy to use, with no requirement for specific instruments. The main disadvantages of morphological markers are: they are limited in number, influenced by the plant growth stages and various environmental factors (Eagles *et al*. 2001) [30]

2. Cytological markers

Markers that are related with variations present in the numbers, banding patterns, size, shape, order and position of chromosomes are known as cytological markers or it refers to the chromosomal banding produced by different stains; for example, G banding. These chromosome landmarks can be used in the differentiation of normal and mutated chromosomes. Such markers can also be used in the identification of linkage groups and in physical mapping (Jiang, 2013) [56].

3. Biochemical markers: Biochemical markers can be categorized in two types

1. Protein markers: Polymorphisms in protein profile as detected by electrophoresis followed by specific staining of a discrete protein sub-class have been used successfully as biochemical markers in plant breeding and genetics. Much of the detectable protein variations identify allelic variability in structural genes encoding the proteins. Alternatively some protein variation may appear due to post translational modification. Two classes of proteins isoenzymes /isozymes and alloenzymes /allozymes, are used as markers. Isozymes are allelic variants of the same enzyme, generally encoded by different loci (Weeden *et al*. 1988, Weeden and Wendel, 1989) [136,137], while allozymes are different proteins encoded by different genes performing the same enzyme function. Isozymes are the most commonly used tool in protein based marker. In 1959 Markert and Moller [87], introduced the term isozyme to define each one of the possible manymultiple forms of an enzyme existing in the same population of an organism. In such analysis, a tissue extract is prepared and proteins of the extract are separated according to their net charge and size by electrophoresis using a polyacrylamide or starch gel. The gel is stained for a particular enzyme by adding a substrate and a dye under appropriate reaction conditions, resulting in band(s) at position where the enzyme polypeptide has migrated showing relative enzyme activity. Depending upon the number of loci, their state of homo /heterozygosity in the individual, and the enzyme molecular configuration, one to several bands were visualised. The positions of the bands are polymorphic and thus informative. Isozymes are generally co-dominant. It should be noted that in most cases the polymorphism of isozyme markers is rather poor within a cultivated species or varieties. As a result, even with the use of isozymes as genetic markers, the full potential of genetic mapping in plant breeding has yet to be realized.

Another type of protein-based genetic marker utilizes two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). Unlike allozymes where single known enzymes are assayed individually, the 2-D PAGE technique simultaneously reveals all enzymes and other proteins present in the sample preparation. The proteins are revealed as spots on gels and marker polymorphisms are detected as presence or absence of spots. Although 2-D PAGE has the potential advantage that many marker loci can be assayed simultaneously on a single gel, assays are more difficult than in allozyme analyses, and the markers are often dominant in their expression.

2. Phytochemical markers: The discovery of novel compounds (phyto-chemicals) from wild plant species is an achievement toward the enhancement of the eradication of the human diseases. With the advancement of modern techniques such as mass spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR) combined with separation techniques facilitated the identification and structural elucidation of molecules. These phyto-chemical analyses are valuable tools for taxonomic differentiation within species or for evaluating the effect of environmental factors (Hawkes, 1992) [51]. Variation in biosynthesis of these metabolites could be a result from both genetic and environmental factors, which play important roles in the development of phenotypic variations in plants.

4. DNA marker: DNA markers are related to variations in DNA fragments generated by restriction endonuclease enzymes are called DNA markers/genetic markers or A gene or DNA sequence having a known location on a chromosome and associated with a particular gene or trait refers to DNA marker. Those characters which can be easily identified are called marker characters. A number of DNA based marker methods have been used for genetic diversity analysis in crops.

AFLP (amplified fragment length polymorphism): AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA (Lynch and Walsh, 1998) [83]. The key feature of AFLP is its capacity for "genome representation. AFLP is a high multiplex PCR-based system (Vos *et al*. 1995) [131] having the potential to generate a large number of polymorphic loci (Powell *et al*. 1996) [99].

Randomly Amplified Polymorphism DNA (RAPD): RAPDs are based on the PCR amplification of random DNA segments with primers of random nucleotide sequences that were inexpensive and easy to use. The primers bind to complementary DNA sequences and where two primers bind to the DNA sample in close enough for successful PCR reaction. The amplified of DNA products can then be visualized by gel electrophoresis (Williams *et al.* 1990) [139] (Gupta and Varshney, 2013) [48]. In RAPDs, Polymorphisms are detected only as the presence or absence of a band of a certain molecular weight, with no information on heterozygosity (Jiang, 2013) [56].

Restriction Fragment Length Polymorphisms (RFLPs): RFLP is the most widely used hybridization-based molecular marker. The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. The polymorphisms detected by RFLPs are as a result of changes in nucleotide sequences in recognition sites of restriction enzymes, or due to mutation events (insertions ordeletions) of several nucleotides leading to obvious shift in fragment size (Tanksley *et al.* 1989) [124]. The main advantages of RFLP markers are co-dominance, high reproducibility, no need of prior sequence information, and high locus-specificity. Most plant breeders would think that RFLP is too time consuming procedure and it requires relatively large amounts of pure DNA, tedious experimental procedure. Additionally, each point mutation has to be analysed individually (Wong 2013, Edwards and Batley, 2009) [140, 31]

Microsatellite or Simple Sequence Repeats (SSRs): Microsatellite also known as Simple Sequence Repeats (SSRs) short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS), are PCR-based markers. They are randomly tandem repeats of short nucleotide motifs (2-6 bp/nucleotides long). Di-, tri- and tetra-nucleotide repeats, e.g. (GT)n, (AAT)n and (GATA)n, are widely distributed throughout the genomes of plants and animals. The copy number of these repeats varies among individuals and is a source of polymorphism in plants. Microsatellite marker firstly developed by (Litt and Luty, 1989) [74], a molecular tool which is a highly reliable marker system. It is an elegant technique that can be used for DNA profiling and diversity analysis because of the following reasons. To development of microsatellite markers, a summary is presented in Fig.2

1. They are co-dominant, which makes them more informative for linkage analysis than dominant marker.
2. They are PCR based therefore, determination of the process for marker generation and analysis are possible.
3. They are usually multiallelic and hyper variable which make identification of polymorphism much easier, even in mapping populations derived from elite plant material within a species.
4. They appear to be randomly and uniformly dispersed throughout eukaryotic genome (Hamada *et al.* 1982) [49].
5. They are accessible to other research laboratories via a published primer sequence (Saghai-Maroof *et al.* 1984) [106].

ISSR (inter-simple sequence repeat): The microsatellite repeats used as primers for ISSRs can be di-nucleotide, tri-nucleotide, tetra-nucleotide or penta-nucleotide. The primers used can be either unanchored (Meyer *et al*. 1993, Gupta *et al*. 1994, Wu *et al*. 1994) [90,47,141] or more usually anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al*. 1994) [164]. It offers many advantages, such as requirement of only low quantities of template DNA, no need for sequence data for primer construction, random distribution throughout the genome, the generation of many informative bands per reaction etc. (Shi *et al*. 2010) [112]. This method uses single primers of 15–20 nucleotides with a 30or 50anchor sequence (Pharmawati *et al*. 2004) [98].The primers are not proprietary and can be synthesized by anyone. The technique is simple, quick, and the use of radioactivity is not essential. ISSR markers usually show high polymorphism (Kojima *et al*. 1998) [62] although the level of polymorphism has been shown to vary with the detection method used.

Retrotransposon-based insertion polymorphism (RBIP)
Retrotransposon-based insertion polymorphism (RBIP) was developed by Flavell *et al*. [1998] [39]. In this technique, the presence or absence of retrotransposon sequences is investigated, which can be used as molecular marker. In this, DNA amplification is achieved through a primer having 3' and 5' end regions flanking the retrotransposons insertion site. Detection of the presence of insertion is achieved through the development of primer from Long terminal repeats (LTRs) retrotransposons.

SCAR (Sequence Characterized Amplified Region): A SCAR marker is a genomic DNA fragment that is identified by PCR amplification using a pair of specific oligo-nucleotide primers (Paran and Michelmore, 1993, McDermott *et al*. 1994) [95, 88]. SCARs are derived by cloning and sequencing

the two ends of RAPD markers that appeared to be diagnostic for specific purposes (e.g., a RAPD band present in disease resistant lines but absent in susceptible lines). SCARs are advantageous over RAPD markers as they detect only a single locus, their amplification is less sensitive to reaction conditions, and they can potentially be converted into co-dominant markers (Paran and Michelmore, 1993) [95]. These markers are more reliable and robust in comparison to the dominant RAPD and ISSR markers (Dhyaneshwar *et al*. 2006, Li, *et al*. 2010, Rajesh *et al*. 2013) [27,69,101].

Single Nucleotide Polymorphisms (SNPs): SNP is a single nucleotide base difference between two DNA sequences or individuals. SNPs provide the simplest and ultimate form of molecular markers as a single nucleotide base is the smallest unit of inheritance, and therefore they can provide a great marker density. The probability to find polymorphisms in a target gene are increases due to high density of SNP markers which provides a huge advantage over previous markers that are at best closely linked to a locus of interest and not within (Ganal *et al*. 2009) [40]. SNP frequencies are in a range of one SNP every (100 - 300) bp in plants. SNPs may present within coding sequences of genes, non-coding regions of genes or in the intergenic regions between genes at different frequencies in different chromosome regions (Edwards and Batley 2009, Edwards *et al*. 2007) [31,32].

Expressed Sequence Tagged Polymorphisms (ESTPs) ESTPs are PCR-based genetic markers that are derived from expressed sequenced tags (ESTs). Expressed sequenced tags are partial cDNA sequences that have been obtained by automated DNA sequencing methods The EST databases contain hundreds of thousands of entries from a variety of organisms, The ESTs are routinely compared to DNA sequence databases to determine their biochemical function. It is also a goal of most genome projects to place the ESTs onto genetic linkage maps. Expressed sequenced tags can be genetically mapped by a variety of methods, all of which rely on detecting polymorphism for the ESTs, hence the name ESTPs for the genetic marker.

CAPS (Cleaved Amplified Polymorphic Sequence): CAPS is a combination of the PCR and RFLP, and it was originally named PCR-RFLP (Maeda *et al*. 1990) [85]. The technique involves amplification of a target DNA through PCR, followed by digesting with restriction enzymes (Konieczny and Ausubel 1993; Jarvis *et al*. 1994; Michaels and Amasino, 1998) [63,55,91]. Compared to RFLPs, CAPS analysis does not include the laborious and technically demanding steps of Southern blot hybridization and radioactive detection procedures

DArT (Diversity Arrays Technology): DArT is a microarray hybridization-based technique that enables the simultaneous typing of several hundred polymorphic loci spread over the genome (Jaccoud *et al*. 2001, Wenzl *et al*. 2004) [54,138]. The efficacy of DArT markers in the analysis of genetic diversity, population structure, association mapping and construction of linkage maps has been demonstrated for a variety of species, especially for plants (http://www.diversityarrays.com/dart-resources-papers) [52]. Contrary to other existing SNP genotyping platforms, DArT platforms do not rely on previous sequence information. With the development of next generation sequencing (NGS), DArT technology faced a new development by combining the complexity reduction of the DArT method with NGS. This new technology named DArTseq™ represents a new implementation of sequencing of complexity reduced representations (Altshuler *et al*. 2000) [2] and more recent applications of this concept on the next generation sequencing platforms (Elshire *et al*. 2011) [33]. DArTseq™ is rapidly gaining popularity as a preferred method of genotyping by sequencing (Kilian *et al.* 2012, Courtois *et al*. 2013, Cruz *et al*. 2013, Raman *et al*. 2014) [60,19,21,102]

Next Generation Sequencing Technology (NGST)
Next generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionized genomic research. Different types of molecular markers have been developed and extensively used during the last three decades for identifying the germplasm, pedigree analysis, linkage between genes and markers, discovering quantitative trait loci (QTLs), pyramiding desired genes and performing marker assisted foreground and background selections for introgression of desired traits (Varshney and Tuberosa, 2007) [129]. Another application of NGS is in parental genotyping of mapping populations or of wild relatives, which can accelerate the development of molecular markers, e.g. simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) markers. These markers can be used to construct genetic maps, to identify QTLs and to monitor alien genome introgression in the case of wide crosses. These QTL-associated markers for a trait of interest can then be used in selecting progenies carrying favorable alleles via marker-assisted selection (MAS) (Varshney *et al.* 2009) [128] (Fig. 3). However, these markers are based mostly on electrophoresis separation of DNA fragments, which limits detection of genetic polymorphism. In large plant breeding populations, genotyping may take up several months depending on marker system, adding more cost to genotyping. The next generation sequencing technology would thus demand more efficient technologies to develop low cost, high-throughput genotyping for screening large populations within a smaller time frame. The advent of next generation sequencing (NGS) technologies and powerful computational pipelines has reduced the cost of whole genome sequencing by many folds allowing discovery, sequencing and genotyping of thousands of markers in a single step (Stapley *et al*. 2010) [121]. NGS has emerged as a powerful tool to detect numerous DNA sequence polymorphism based markers within a short timeframe growing as a powerful tool for next generation plant breeding. NGS technologies have been used to screen germplasm collections, multiparallel resequencing studies, genome-wide association studies in crop plants, genome-wide marker discovery, sequence informed conservation and utilization of PGR (Kilian and Graner, 2012) [61].

Use of Molecular Markers as Marker-Assisted Selection (MAS): A method that uses molecular markers associated with the traits of interest to select plants at the seedling stage, thus speeding up the process of conventional plant breeding and reducing the cost involved in maintaining fields. Marker Assisted Selection (MAS) facilitates improvement of traits that cannot easily be selected using conventional breeding methods.

Advantages of DNA markers:
a. They are highly polymorphic.
b. They have simple inheritance (often co-dominant).

c. They abundantly occur throughout the genome.
d. They are easy and fast to detect.
e. They exhibit minimum pleiotropic effect.
f. Their detection is not dependent on the developmental stage of the organism.

Importance of DNA markers:

1. DNA markers are useful in the assessment of genetic diversity in germplasm, cultivars identification and advanced breeding material.
2. DNA markers can be used for genetic mapping, QTL mapping, QTL mapping populations, selection of markers for QTL mapping and constructing genetic linkage maps.
3. DNA markers are useful in identification of new useful alleles (Different DNA sequences at a locus) in the germplasm and wild species of crop plants.
4. DNA markers are used in the marker assisted or marker aided selection. Mass Assisted Selection (MAS) has several advantages over straight selection.
5. DNA markers are useful in the study of crop evolution
6. DNA markers are usefull in investigation of heterosis
7. DNA markers are usefull for identification of haploid/diploid plants and cultivars genotyping
8. Molecular markers are usefull in backcrossing for a gene of interest

The use of molecular markers for genetic diversity analysis and as a selection tool is a high priority for new efforts in the development of ornamental cultivars and the exploitation of species diversity. Molecular marker and DNA sequence analysis of extant and new floricultural germplasm collections should allow a more complete characterization and understanding of the genetic relationships between species and cultivars (Dore *et al*. 2001, Meerow, 2005) [28,89]. The comparison of different molecular markers with their characteristics and results profiling generated by molecular markers are presented in (Table.1 and Fig.1).

Table 1: Comparison of important characteristics of the most commonly used molecular markers (Nadeem *et al*. 2018) [93].

Characteristics	RFLP	RAPD	AFLP	ISSR	SSR	SNP	DArT	Retrotransposons
Co-dominant/Dominant	Co-dominant	Dominant	Dominant	Dominant	Co-dominant	Co-dominant	Dominant	Dominant
Reproducibility	High	High	Intermediate	Medium–High	High	High	High	High
Polymorphism level	Medium	very high	High	High	High	High	High	High
Required DNA quality	High	High	High	Low	Low	High	High	High
Required DNA quantity	High	Medium	Low	Low	Low	Low	Low	Low
Marker index	Low	High	Medium	Medium	Medium	High	High	High
Genome abundance	High	Very high	Very high	Medium	Medium	Very high	Very high	High
Cost	High	Less	High	High	High	Variable	Cheapest	Cheapest
Sequencing	Yes	No	No	No	Yes	Yes	Yes	No
Status	Past	Past	Past	Present	Present	Present	Present	Present
PCR requirement	No	Yes	Yes	Yes	Yes	Yes	No	Yes
Visualization	Radioactive	Agarose gel	Agarose gel	Agarose gel	Agarose gel	SNP-VISTA	Microarray	Agarose gel
Required DNA (ng)	10000	20	500–1000	50	50	50	50–100	25–50

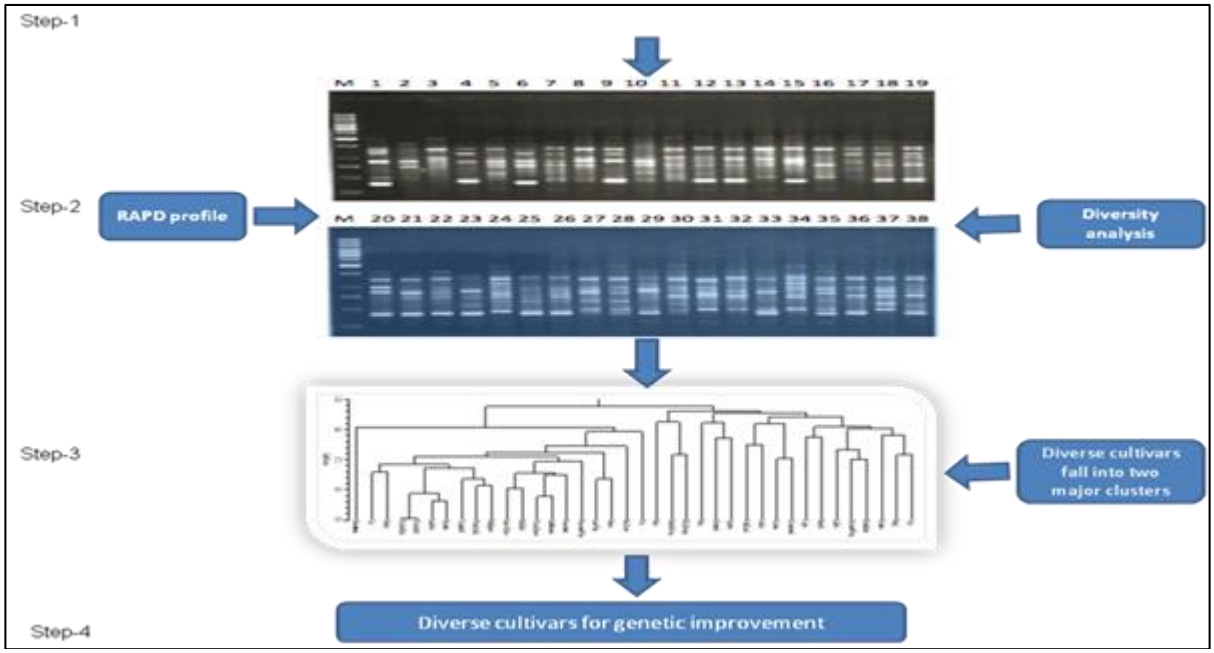

Fig 1: DNA profiling and diversity analysis of chrysanthemum by RAPD markers (Kumar *et al*. 2017) [67]

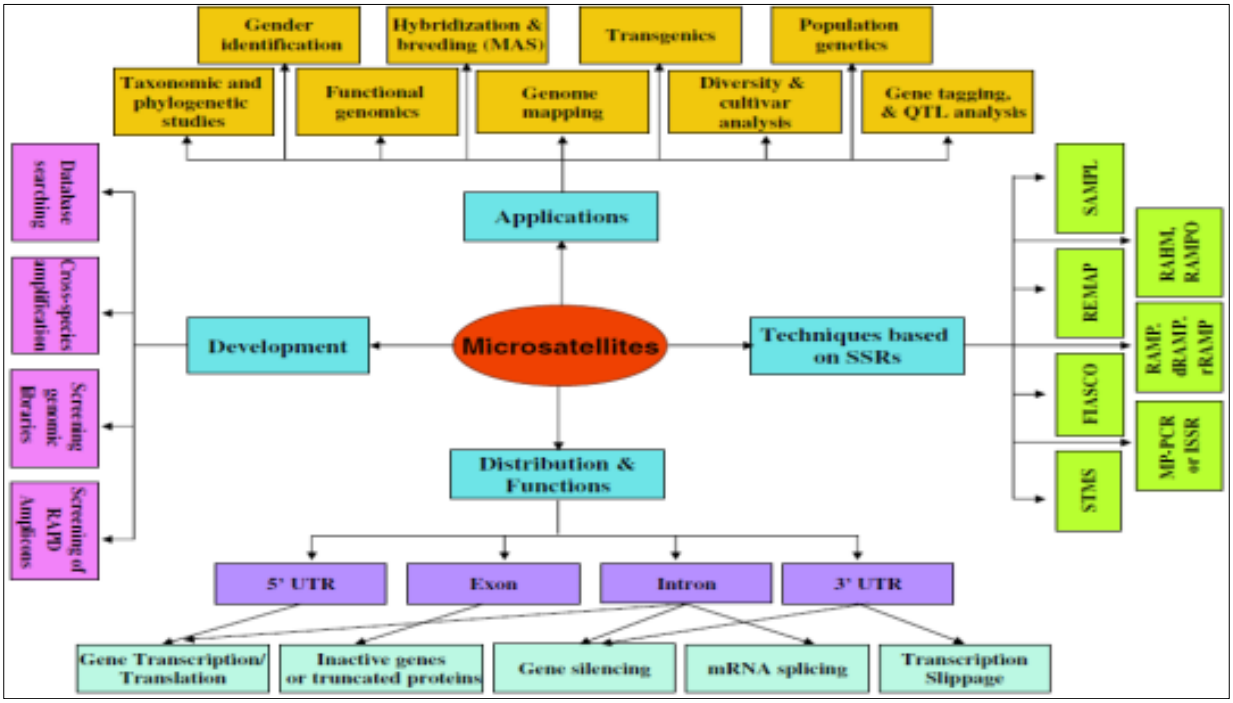

Fig 2: Microsatellites: A summary of development, distribution, functions and applications (Kalia *et al*. 2011) [58].

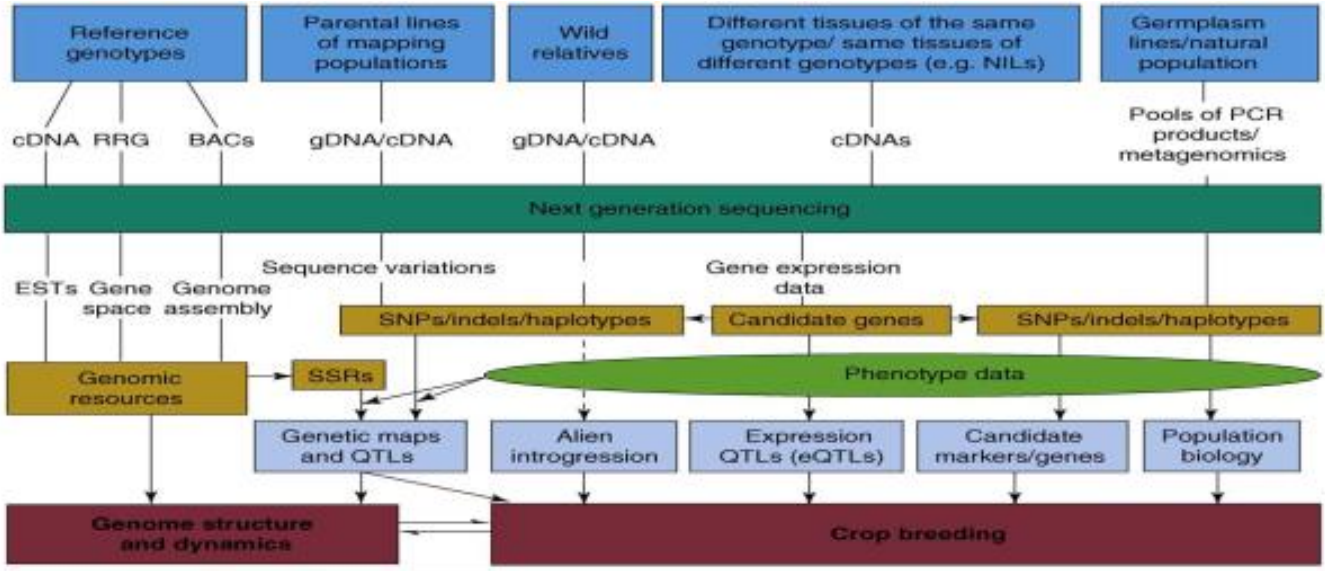

Fig 3: Overview of Next Generation Sequencing (NGS) applications in crop genetics and breeding (Varshney *et al*. 2009) [128].

Application of biochemical and molecular markers in diversity analysis in orchids

Smith *et al.* (2002) [118] observed very low genetic diversity for terrestrial woodland orchid *Tipularia discolor* in eastern United States based on ISSR markers. The findings revealed that polymorphism varied from 0.00 to 18.2% and gene diversity ranging from 0.00 to 0.069. Ahmad *et al.* (2003) [1] revealed variations among the local genotypes with immense potential for future improvement in Seabuckthorn on the basis of SDS-PAGE. The results indicated that Electro-phoretic pattern of the protein fractions are directly related to the genetic background and can be used to certify the genetic makeup. Molecular systematic research using the internal transcribed spacer (ITS) region of the 18–26S nuclear ribosomal DNA repeat was carried out by Clements (2003) [18] who reported that molecular results in combined with morphological data, provide a strong basis for a reassessment of the phylogeny of part of the study. Cheng *et al.* (2004) [17] collected three Dendrobium species and differentiated by the sequences of partial rDNA (ribosomal DNA) sections which contain the ITS 1, 5.8S, and ITS 2 regions. The Dendrobium species included in this research were *Dendrobium tosaense*, *Dendrobium officinale*, and *Dendrobium moniliforme*. The length of PCR products was 632 nt for *D. tosaense*, 627 nt for *D. officinale*, and 62 nt for *D. moniliform* respectively. The similarities of the rDNA region between the species pairs showed in the range from 91% to 95%. The method could efficiently used to obtain the variation in different species. Peng *et al.* (2004) [96] characterized *Dendrobium species* by RAPD markers. Ten decanucleotide primers (10 bp) were selected from 70 primers, and 99 amplification bands were obtained. Yu *et al.* (2004) [154] carried out DNA fingerprinting of five species by AFLP primer. The combinations were screened from 64 primer combinations. Among the species, four species of Dendrobium were clustered into one big group, whose relationships were clearly distinguished. Cozzolino and Widmer (2005) [20] discussed how deception affects orchid mating systems, the evolution of reproductive

isolation, speciation processes and neutral genetic divergence among species. The results suggested that pollination by deceit is one of the keys to orchid floral and species diversity. A better understanding of its evolutionary consequences could help evolutionary biologists to unravel the reasons for the evolutionary success of orchids. However, Ding *et al*. (2005) [23] evaluated genetic differences and extensive genetic diversity among the wild populations. The findings indicated that RAPD markers are informative and useful tool for assessing genetic diversity, evaluation and authentication of wild populations of *D. officinale*. Goh *et al.* (2005) [42] demonstrated that RAPD markers are useful tool for identifying *Phalaenopsis* orchids up to the specific and/or sub generic levels. Li *et al*. (2005) [73] screened of multiple species-specific probes from genomic DNA for closely related *Dendrobium* species. The identification was done based on DNA array hybridization. Fourteen species-specific probes were screened from five closely related *Dendrobium* species, *D. aurantiacum* Kerr, *D. officinale.* Shen *et al.* (2005) [111] used 7 ISSR primers out of 10 primers amplified polymorphic bands. The amplification patterns of two primers, namely UBC-807 and UBC-864 had higher polymorphism and amplified band ratio. Each of them could distinguish all 9 examined species. Shen *et al*. (2006) [109] authenticated the 8 populations of *D. officinale* by using 10 primers selected from 76 ISSR primers. A total of 127 DNA fragments were amplified, of which 115 were polymorphic. To enhance the efficiency of authentication, ISSR fingerprinting codes were constructed using 6 polymorphic bands for authenticating 8 *D. officinale* populations. Wang *et al*. (2006) [133] used 10 RAPD primers and amplified a total of 188 DNA bands out of which 180 were polymorphic and 08 bands were monomorphic. The average level of polymorphism was 95.74%. Cluster analysis with UPGMA method showed that 13 genotypes could be classified into three types at a genetic distance of 0.63. Xu *et al*. (2006) [145] studied on diversity in DNA sequences among various species. The results showed that ITS divergence ranging varied from 3.2% to 37.9% in ITS1 and 5.0% to 26.6% in ITS2. Variation within species was very low, ranging in sequence divergence from 0% to 3.0% in ITS1 and from 0% to 4.0% in ITS2. These species could be easily distinguished based on the divergent ITS regions. Yue *et al.* (2006) [157] identified 14 polymorphic SSR markers when screened against 42 popular commercial Dendrobium hybrids. The average number of allele was 12.0 ± 1.9 and average observed heterozyosity was 0.70. All 42 hybrids tested, except for two tissue culture mutants, were uniquely identified with the markers used. The results also exhibited that sibling hybrids were closely clustered. The findings also revealed that hybrids were also closer (unquantified) to parents. Wu *et al.* (2006) [142] reported higher genetic variation in natural and cultivated populations of *Gastrodia elata* by using ISSR. Li and Ge (2006) [70] investigated the level of genetic diversity in the orchid species (*Changnienia amoena)* endemic using RAPD technique. Based on the results obtained from the study, 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. However, another study exhibited low level of diversity at species and population level between glaciated and un-glaciated sites of *Cypripedium reginae* reported by Kennedy and Walker, 2007 [59] based on RAPD markers. No difference was observed when RAPD markers used for genetic diversity in wild and cultivated group of *Vanilla planifolia* Schluter *et al.* (2007) [107]. The microsatellite markers developed by Gu *et al*. (2007) [46] who isolated 12 microsatellite loci which derived from two microsatellite-enriched libraries and characterized. These loci were polymorphic and displayed 3–12 alleles per locus. The observed and expected heterozygosities ranged from 0.150 to 0.624 and from 0.162 to 0.605, respectively. Ren *et al*. (2007) [103] tested the genomic DNA of 10 Dendrobium germplasms with 17 arbitrary 10-mer primers RAPD primers which were screened from 100 primers. A total of 200 polymorphic RAPD bands were obtained from the study. The average number of polymorphic bands per primer was 11.8. The RAPD based genetic similarity ranged from 0.356 to 0.676. UPGMA cluster analysis based on RAPD molecular markers data divided 10 species into four groups. Wang *et al*. (2007) [134] detected a total of 346 fragments out of 342 were polymorphic, with 98.8% polymorphism in 13 species. All the 13 species were clustered into three groups on a coefficient level of 0.54. The outcome corresponded to the classification based on a morphological classification. Ying *et al*. (2007) [153] analyzed 109 Dendrobium species based on rDNA ITS sequences of the 164 samples and quoted from Gen Bank, allele-specific diagnostic primers QH-JB1 and QH-JB2 were designed. A DNA fragment of about 300 bp was amplified from *D. thyrsiflorum* with annealing temperature at 63.5^0 C and annealing time of 1 min; no other DNA fragments were amplified from the remaining *Dendrobium species*. Zhang *et al*. (2007) [161] amplified a total of 142 bands out of 118 bands were polymorphic (83.1% of all amplified loci). Clustering results revealed that the dendrogram constructed with RAPD molecular makers was identical with the classic taxonomic system. Boonsrangsom *et al*. (2008) [12] constructed two genomic libraries from DNA digested with either MseI or TaqI. A total of 195 positive clones were sequenced. 62.8% and 88.8% of which, respectively contained SSR motifs. 73 clones were chosen for primer design. Eight primer pairs could amplify the products giving the expected sizes and detect genetic polymorphism in the population with the allele numbers ranging from 4 to 7 (average = 5.25 alleles/locus), Ho = 0.0612–1.0000 (average = 0.7398), He = 0.0788–0.7323 (average = 0.5871) and effective number of allele (ne) of 1.0855–3.7355 (average = 2.7850). All Dendrobium samples could be identified but they were not clearly separated into distinct clusters with NTSYS-pc software. Ding *et al*. (2008) [24] analyzed a total of 84 individuals from 9 wild populations of *D. officinale* by using SRAP. A high level of genetic diversity was detected (PPB = 88.07%, He = 0.2880) at the species level. However, the genetic diversity at the population level was lower (PPB = 51.68%, He = 0.1878). Based on analysis of molecular variation, there was moderate variation between pairs of populations with UST values ranging from 0.1327 to 0.4151 and on average 27.05% of the genetic variation occurred among populations. Two main clusters were shown in UPGMA using TFPGA, which is consistent with the result of PCA using NTSYS-pc software. Fan *et al.* (2008) [35] studied the genetic diversity in Dendrobium by using SRAP markers. Forty primer pairs were selected from 88 amplified products that gave 1782 polymorphic bands with an average of 44.55 polymorphic bands per primer pair. Cluster analysis using UPGMA based on the data of SRAP amplified bands by 40 primer pairs showed that 9 samples could be distinguished into two main groups. Li *et al*. (2008) [71] assessed the genetic diversity and population structure in 12 populations by employing AFLP. A high level of genetic diversity was detected (He = 0.269) with POPGENE. As

revealed by AMOVA analysis, there was moderate variation between pairs of populations with UST values ranging from 0.047 to 0.578 and on average 26.97% of the genetic variation occurred among populations. Three main clusters were shown in UPGMA dendrogram using TFPGA, which is consistent with the PCO result using NTSYS. Qiu *et al.* (2008) [100] used RAPD for studying the genetic diversity of Dendrobium. A total of 70 RAPD primers were screened out of 103 random primers were applied to random amplification. A total of 520 DNA bands were detected out of which 471 DNA bands were polymorphic; average polymorphism was 94.42%. The result of cluster analysis by UPGMA showed that 9 genotypes could be classified into two types in genetic distance of 0.44. Ren *et al*. (2008) [104] tested the genomic DNA of 9 Dendrobium with using 18 SRAP primer pairs which were screened from 30 primer pairs. A total of 285 polymorphic SRAP bands were generated with an average of 15.8 polymorphic bands per primer pair. SRAP-based genetic similarity ranged from 0.179 to 0.636, suggesting that the materials used in the experiment possessed broad genetic variation. UPGMA cluster analysis based on SRAP data divided 9 accessions into four groups. Xiao *et al*. (2008) [143] characterized *Dendrobium species* and its adulterant species by RAPD. The genetic distance of the Dendrobium species varied from 0.0762 to 0.68421 with an average 0.4438, and the average genetic distance between *Dendrobium species* and *Pholidota chinensis* was 0.71734. Verma *et al.* (2009) [130] analyzed the inter relationship amongst the cultivated, wild and hybrid of *Vanilla* using ISSR and RAPD analysis. The findings revealed considerable amount of genetic diversity by both analysis. While, Ding *et al*. (2009) [25] used ISSR and RAPD markers for diversity in *D. officinale* populations. Both molecular markers revealed a high percentage (>89%) of polymorphic bands and ISSR markers detected more diversity than RAPD markers in nine natural populations. AMOVA revealed that 78.84% (ISSR) and 78.88% (RAPD) of variability was partitioned among individuals and within populations. This genetic structure was probably due to severe genetic drift resulting from habitat fragmentation and human overexploitation since the 1950s. There was a lack of significant association between genetic and geographic distances ($r = 0.276$; $p > 0.05$) in the *D. officinale* populations. Fan *et al*. (2009) [36] developed and designed ten microsatellite loci (out of 15 primer pairs) which were used in diversity analysis in *D. fimbriatum*. These microsatellite loci were used to screen 25 individuals from across the species with their geographical range. Among the loci, 10 loci were polymorphic with 2–19 alleles; three loci were monomorphic, while the rest produced no amplification fragments. Jin *et al*. (2009) [57] used 15 RAPD primers from 100 random primers to perform random amplification in orchid. A total of 152 reproducible bands were detected, 129 of which were polymorphic, and the percentage of polymorphic bands was 84.19%. These results of cluster analysis by UPGMA showed that 11 genotypes could be classified into 4 types in genetic distance of 0.6. Luo *et al*. (2009) [82] used 10-bp random RAPD primers for detection of genetic variation. *D. 'Burana Green*' and *D. 'Pink Thamond*'. Among the varieties, 2 varieties had no genetic variation; the genetic variation of *D. 'Big Panda'* was 3.3%, that of *D. 'Thailand White*' was 23.3%, and that of *D. 'Red Sonia*' in vitro was 10.9%. Ma and Yin (2009) [84] obtained a total of 117 unambiguous and repetitive bands from 114 individuals of sampled populations by using 12 primers selected. The results showed that *D. fimbriatum* exhibited a relatively low genetic diversity at the population level. Shao *et al.* (2009) [108] suggested that the psbA-trnH region of cpDNA can be used as a candidate marker for authentication of *Dendrobium species* of Fengdous The lengths of sequences varied from 721 to 767 bp. Genetic distances varied from 0.0013 to 0.0183 among 15 species while the average genetic distance was 0.0148. There were no population differences in the psbA-trnH region of various species of Fengdous. Wang *et al*. (2009) [132] used 17 ISSR primers to assess the genetic diversity and phylogenetic relationships among 31 *Dendrobium species*. A total of 2368 bands generated from 278 loci with 100% polymorphism at the genus level. All species were unequivocally distinguished based on ISSR fingerprinting. Species-specific ISSR markers were also identified in 9 of 31 tested Dendrobium species. UPGMA grouped the 31 *Dendrobium species* into six clusters, indicating the polyphyletic nature of the genus with several well-supported lineages. ISSR was very effective in determining genetic diversity in Dendrobium at the species level. Cluster analysis based on ISSR profiles clearly identified *Dendrobium species* and provided a molecular diagnostic tool for authentication. Yao *et al*. (2009) [152] reported that the psbA-trnH intergenic spacer region could be used as a barcode to distinguish various Dendrobium species and to differentiate Dendrobium species from other adulterating species. Among the species of study, 17 Dendrobium species exhibited 0.3-0.9% with an average of 1.2% percentages of variation. In contrast, the intra-specific variation among the Dendrobium species studied ranged from 0% to 0.1%. The sequence difference between the psbA-trnH sequences of 17 Dendrobium species and one Bulbophyllum odoratissimum ranged from 2.0% to 3.1%, with an average of 2.5%. Yuan *et al.* (2009a) [155] observed that the ITS sequences could not completely match the classification based on morphological characters with *D. moulmeinense* diverging from the other 35 Dendrobium species. *D. moulmeinense* is not a well-known species from Yunnan province that had been included in the section Dendrobium based on morphological characteristics. Yuan *et al*. (2009b) [156] selected 14 primers with good polymorphism by screening from 55 ISSR primers to conduct ISSR analysis. The data of DNA fragments were used to construct a dendrogram by UPGMA. Clustering results revealed that the dendrogram constructed with ISSR molecular makers was identical with the morphological characteristics. Zha *et al.* (2009) [158] used capillary electrophoretic fingerprints of 69 samples from 10 different areas showed 15 characteristic peaks which could be applied to identify this species. PCA and UPGMA analyses were used to cluster accessions with distinct fingerprints. Zheng *et al*. (2009) [163] screened eight pairs of primer combinations from 64 pairs of primer combinations and generated 1102 amplified loci, 778 of which were polymorphic loci which accounted for 70.6% in the total amplified loci. DNA fingerprinting of 30 introduced varieties or hybrids were set up with AFLP and provided a reliable and convenient technical means for identification, evaluation, protection and innovation of varieties. Asahina *et al*. (2010) [4] conducted phylogenetic analysis by using sequences of two plastid genes, the maturase-coding gene (matK) and the large subunit of ribulose 1,5-bisphosphate carboxylase-coding gene (rbcL), as DNA barcodes for species identification of Dendrobium plants. A total of five medicinal Dendrobium species used in the study i.e., *Dendrobium fimbriatum*, *D. moniliforme*, *D. nobile*, *D. pulchellum*, and *D. tosaense*. The phylogenetic trees constructed from matK data successfully distinguished each species from each other. On the other

hand, rbcL, as a single-locus barcode, offered less species discriminating power than matK, possibly due to its being present with little variation. When results using matK sequences of *D. officinale* that was deposited in the DNA database were combined, *D. officinale* and *D. tosaense* showed a close genetic relationship, which brought us closer to resolving the question of their taxonomic identity. Identification of the plant source as well as the uniformity of the chemical components is critical for the quality control of herbal medicines and it is important that the processed materials be validated. The results suggested that the methods could be applied to the analysis of processed Dendrobium plants and be a promising tool for the identification of botanical origins of crude drugs. A DNA microarray for detecting processed medicinal *Dendrobium* species (*Herba dendrobii*) was constructed by incorporating the ITS1-5.8s-ITS2 sequences of *Dendrobium* species on a glass slide. The established microarray could detect the presence of *D. nobile* in a Chinese medicinal formulation containing nine herbal components (Hao *et al*., 2010) [50]. Huang *et al.* (2010) [53] reported that ITS sequence could be used as a potential barcode for identifying *Dendrobium species* by inter- and intraspecific divergences. Neither rbcL nor matK and trnH-psbA could be used to identify Dendrobium on its own. No single sequence could completely identify different types of *Dendrobium species*, and the combination of different sequences as a DNA barcode for Dendrobium was needed. Xie *et al.* (2010) [144] screened a total of 60 SSR primer pairs out of 15 primer pairs with stable and repeatable polymorphism were selected for genetic polymorphism of 48 *D. officinale* samples. The observed and expected heterozygosities ranged from 0.60 to 0.85 and 0.49 to 0.85, respectively. The PIC of each SSR locus varied from 0.437 to 0.829 with an average of 0.702. 15 primer pairs were used in Dendrobium cross-species amplification and totally 13 primer pairs had transferability in *D. officinale*-related species. The results suggested that SSR is a feasible technique for identification of tissue cultured *D. officinale* plantlets. Xue *et al*. (2010) [149] constructed a genetic map of two Dendrobium species with a double pseudo-testcross strategy using RAPD and SRAP markers. F_1 mapping population of 90 individuals was developed from a cross between both species. A total of 307 markers obtained, including 209 RAPD and 98 SRAP, which were used for genetic LG analysis. The *D. officinale* linkage map consisted of 11 major linkage groups and 3 doublets, covering 629.4 cM using a total of 62 markers with an average locus distance of 11.2 cM between two adjacent markers. The *D. hercoglossum* linkage map had 112 markers mapped on 15 major and 4 minor linkage groups, spanning a total length of 1304.6 cM with an average distance of 11.6 cM between two adjacent markers. The maps constructed in this study covered 92.7% and 82.7% of the *D. hercoglossum* and *D. officinale* genomes, respectively. Cai *et al*. (2011) [14] used 17 SRAP primer combinations which generated a total of 231 clear amplification bands encompassing 187 (80.95%) polymorphic bands in 7 populations of *D. loddigesii*. A high level of genetic diversity was detected (PPB = 80.52%, H = 0.2743, I = 0.4113) at the species level. There was a moderate genetic differentiation (Gst = 0.304) among populations. Two main clusters were detected by cluster analysis using UPGMA. Ding *et al*. (2011) [26] used SNP markers for discriminated *D. officinale* and its adulterants and suggested that SNP could be used as sites and allele-specific PCR primers. There were apparent genetic differences between all the samples tested. Shen *et al*. (2011) [110] used 16 RAMP informative primers to evaluate the genetic similarity in dendrobium. A total of 123 bands were amplified and 86 (69.92%) were polymorphic. Genetic similarity coefficients ranged from 0.250 to 0.813. A UPGMA dendrogram illustrated 9 populations clustered into 3 groups, and the cluster pattern showed a correlation with the locations of the *D. officinale* populations. RAMP effectively evaluated the genetic diversity of wild populations of *D. officinale*. Takamiya *et al*. (2011) [122] identified a total of 21 Dendrobii Herba (medicinal orchid) plant by ITS of nrDNA sequences. An ITS1- 5.8S-ITS2 sequence database of 196 Dendrobium species was used. 13 Dendrobium species were likely to have been used as plant sources of Dendrobii Herba, while unidentified species allied to *D. denudans, D. eriiflorum, D. gregulus, or D. hemimelanoglossum* were also used, i.e., falsification was also detected. *D. catenatum* was synonymous with *D. officinale*, one of the most important sources of Dendrobii Herba). Xu *et al.* (2011) [148] tested nine pairs of polymorphic chloroplast microsatellite primers across a total of 55 individuals from four natural populations (12–15 individuals per population). Allele numbers varied from 2 to 4 per locus, while the number of haplotypes ranged from 4 to 6 per population. Transferability of the 9 polymorphic chloroplast microsatellite primers was checked on an additional set of 51 Dendrobium individuals (belonging to 17 different species). The results showed that three markers could be transfered to all the species tested, while the remaining 6 markers successfully cross-amplified in most species tested. All 9 pairs of polymorphic chloroplast microsatellite primers were polymorphic in *D. moniliforme*, while 7 were polymorphic in *D. loddigesii*. Yang *et al*. (2011) [151] screened a total of 14 ISSR primers for the ISSR-PCR reaction. Gel analysis produced a total of 179 polymorphic loci. The level of polymorphism was 94.71%. Jaccard's similarity index showed the similarity index among *D. candidum, D. officinale* 0.264–0.536, 0.351–0.561 among *D. devonianum* and 0.115–0.371 among different species. Antony *et al*. (2012) [3] used only 6 RAPD primers (from an initial 20 screened) to conduct the genetic stability of cryo-preserved PLBs versus control PLBs. Cai *et al*. (2012) [13] applied SSR markers to study the genetic variation in *Dendrobium loddigesii* Rolfe. The gel analysis exhibited a total of 98 alleles with an average of 8.2 alleles per locus in *Dendrobium loddigesii Rolfe*, an endangered orchid. The expected heterozygosity of each SSR locus varied from 0.454 to 0.857 with an average of 0.690. PIC of each SSR locus ranged from 0.358 to 0.838 with an average of 0.637. The main variation component existed within populations (82.02%) rather than among populations (17.98%). Li *et al*. (2012) [72] constructed a ITS1-5.8S-ITS2 sequence database of the 43 Dendrobium samples to study the relation between morphological and molecular analysis. A total of 35 Dendrobium known samples were divided into 5 clusters, and most of the samples (24 out of 35) were clustered together. The results displayed that the sections of most species divided by ITS were the same with the morphological classification. However, there were several species re-grouped into different sections by ITS. Authentication of the 8 unknown Dendrobium processed dry samples were also identified by rDNA ITS. Lu *et al*. (2012b) [79] isolated and developed a total of 13 novel microsatellite makers from expressed sequence tag sequences of endangered Chinese endemic herb *Dendrobium officinale* and examined them for 38 individuals from Jinhua, Zhejiang, Chian. These loci displayed the mean number of 4.31alleles/locus, and with expected heterozygosity

(He) and observed heterozygosity (Ho) per locus ranged from 0.4438 to 0.6842 and from 0 to 0.4211, respectively. Lu *et al*. (2012a) [80] carried out a study with F_1 generation derived from the controlled inter-specific hybridization between *D. moniliforme* and *D. officinale* to create genetic linkage maps of parental Dendrobium using a double pseudo-testcross strategy. A total of 422 markers, including 66 EST-SSR, 126 SRAP, 74 ISSR, and 156 RAPD markers, showed expected Mendelian segregation ratio and also used to construct a genetic map. The map of *D. moniliforme* was 1127.9 cM in total with 165 marker loci distributed in 17 linkage groups. The *D. officinale* map consisted of 19 genetic linkage groups with a total length of 1210.9 cM positioned by 169 marker loci. Another study carried out by Lu *et al.* (2012c) [78] to develop an integrated genetic linkage map of an F_1 population derived from an interspecific cross between *D. officinale* and *D. aduncum* (both, 2n = 38). Genetic map was constructed by using expressed sequence tag-simple sequence repeats (EST-SSR) and sequence related amplified polymorphism (SRAP). A total of 349 polymorphic loci, including 261 SRAP loci and 88 EST-SSR loci, were identified for genetic linkage analysis. A total of 157 loci were arranged into 27 major linkage groups, each containing a minimum of four markers, and a further 23 markers were distributed to five triplets and four doublets, the frame map covered with a total distance of 1580.4 cM, with a mean of 11.89 cM between adjacent markers. This primary map of the *D. officinale* and *D. aduncum* hybrid provides a basis for genetic studies and also facilitate future studies of medical traits mapping and marker-assisted selection in Dendrobium species. Pandey and Sharma (2012) [94] used 454 GS-FLX sequencing to isolate microsatellites in two species (*Cypripedium kentucki- ense* and *Pogonia ophioglossoides*), and reported pre-liminary results of the study. From 1/16th plate that was subjected to sequencing, 32,665 reads were gen-erated, from which 15,473 fragments contained at least one SSR. A total of 20,697 selected SSRs representing di-, tri-, and tetra-nucleotides. While 3,674 microsatellites had flanking regions on both sides, useable primer pairs could be designed for 255 SSRs. The mean numbers of reads, SSRs, and SSR-containing reads useful for primer design estimated for other 15 orchid species using Sanger sequencing method were 166, 78 and 31, respectively. The findings revealed that the efficiency of microsatellite isolation in orchids is substantially higher with 454 GS-FLX sequencing technique in comparison to the Sanger sequencing methods. Feng *et al*. (2013) [38] constructed two preliminary genetic linkage by using 90 F_1 progeny individuals derived from an inter-specific cross between *D. nobile* and *D. moniliforme* (both, 2n = 38), using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. A total of 286 RAPD loci and 68 ISSR loci were identified and used for genetic linkage analysis. The resulting frame map of *D. nobile* was 1474 cM in total length with 116 loci distributed in 15 linkage groups; and the *D. moniliforme* linkage map had 117 loci placed in 16 linkage groups spanning 1326.5 cM. Both maps showed 76.91% and 73.59% genome coverage for *D. nobile* and *D. moniliforme*, respectively. Lu *et al*. (2013a) [76] selected six primers with a stable and clear diversity from 100 ISSR primers. 847 DNA fragments were amplified from 24 samples, and 141 DNA fragments on average were amplified from each primer, with 100% polymorphic bands. The 24 samples of Dendrobium plants from different habitats were divided into six groups. Lu *et al*. (2013b) [77] developed a new EST-SSR markers and used to evaluate their potential for cross-species implications in phylogeny study of genus *Dendrobium*. 500 SSR loci identified from a total of 1835 unigene ESTs by sequencing a normalized cDNA library. Of these SSRs, 38.0% (190) were the di-nucleotide repeats, followed by trimer (189), 37.8%, hexamer (81, 16.2%), pentamer (19, 3.8%), and tetramer (21, 4.2%). (AG)n and (AAG)n predominated in dimers and trimers, respectively. Primers for 170 *D. nobile* EST-SSR loci were designed and amplified successfully in *D. nobile*, and 142 of them were successfully transferred to at least one of 31 other *Dendrobium species*, with 42% of average transferability in the genus. SSR markers with high cross-species transferability and high genetic diversity were selected for phylogeny analysis, where the results showed a complex genetic architecture among the different sections as well as wide genetic differentiation within section Dendrobium. These microsatellite markers enrich the current resource of molecular markers, which would facilitate further evolution and genetic diversity studies, germplasm appraisal, genetic mapping, and molecular breeding of *D. nobile*. Feng *et al*. (2014) [37] used sequence-related amplified polymorphism (SRAP) markers for molecular phylogeny analysis and species identification of 31 Chinese *Dendrobium species*. Fourteen SRAP primer pairs were used in the study and produced a total of 727 loci, 97% of which (706) were polymorphic. Average polymorphism information content (PIC) of the SRAP pairs was 0.987, ranging from 0.982 to 0.991, showed that there existed a plenty of genetic diversity at interspecies level of Chinese Dendrobium. The molecular phylogeny analysis based on unweighted pair-group method (UPGMA) exhibited that the 31 Dendrobium species can be grouped into six clusters. In other hand, eighteen species-specific markers were obtained, and can be used in identification ten of 31 tested Dendrobium species. The results indicated the SRAP markers system is informative and would facilitate further application in germplasm appraisal, evolution and genetic diversity studies in the genus Dendrobium. Lu *et al*. (2014) [81]developed microsatellite markers for *D. nobile* by mining the ESTs. Twenty-eight EST-SSRs amplified 2–6 nucleotide repeats with a mean number of 2.82 alleles/ locus. The observed and expected heterozygosities per locus ranged from 0.158 to 0.579 and 0.422 to 0.752, respectively. Peyachoknagul *et al*. (2014) [97] developed the PCR-restriction fragment length polymorphism (PCR-RFLP) approach to identify 25 native Dendrobium species in Thailand. PCR-RFLP of the rDNA-ITS with six restriction enzymes and three chloroplast (cp) DNA regions with five primer–enzyme combinations produced a total of 24 types of DNA patterns. Twenty-three out of the 25 species determined in this study were found to belong to unique classes and were successfully differentiated. Two species, *D. crumenatum* and *D. formosum*, possessing the same DNA pattern, however, results were identified after cutting the chloroplast DNA fragment amplified by psbC-trnS primer with MboI enzyme. They suggested that these markers system is an effective procedure for identifying each Dendrobium species. PCR-RFLP of the rDNA-ITS with TaqI, which is the most informative enzyme, was used for the early detection of 16 Dendrobium species. To identify the remaining Dendrobium species, PCR-RFLP analysis was performed using one more primer-enzyme combination. The study provides a rapid, simple, and reliable identification method for these Dendrobium species. Xu *et al.* (2014) [146] described the study on two new orchid species, *D. wenshanense* and *D. longlingense*, from Yunnan, China, based on morphological

and molecular evidence. Morphological analysis indicated that *D. wenshanense* was similar to *D. wilsonii* and *D. fanjingshanense*, but they differ in floral color, length of column foot and shapes/sizes of sepals and petals. The results also showed that *D. longlingense* is similar to *D. longicornu*, but differs in presence of black hairs on adaxial surface of leaf, position of inflorescences, floral color and shapes of lip and spur. Molecular analysis of combined nuclear and plastid datasets (ITS, matK, rbcL, trnH-psbA, trnL intron) reveals that *D. wenshanense* is sister to *D. wilsonii* and *D. fanjingshanense* and a member of section Dendrobium; *D. longlingense* is sister to *D. longicornu* and a member of section Formosae. Zhang *et al*. (2014b) [160] used 10 RAPD primers to assess the relationship among the germplasm in orchids. A total of 113 bands amplified, among which 106 (93.81%) bands were polymorphic. The RAPD approach was efficient in studying genetic background and relationships among these 3 species. Bhattacharyya *et al*. (2015) [10] examined the genetic relatedness and genetic diversity of 60 *D. nobile* individuals from 6 natural populations of Northeast India by using 13 RAPD primers by AMOVA, UPGMA and STRUCTURE analysis. The value of gene flow was low (Nm = 0.27), the relative genetic diversity of the populations was high, the PIC was 0.74 while RP varied between 6.8 and 13.23. Molecular variations were higher between the populations (62.21%) than within the populations (37.79%) and the number of observed alleles was 1.95 while the number of the alleles contributed to the genetic diversity was 1.54. The 60 individuals' cultivars clustered into 2 main groups (at 64% similarity). Leles *et al*. (215) [68] examined the landscape genetics, estimated genetic diversity, and explored genetic relationships with morphological variability and reproductive strategies in seven natural populations of *Cattleya liliputana* (Orchidaceae). Nuclear microsatellite markers were used for genetic analyses. Spatial Bayesian clustering and population-based analyses revealed significant genetic structuring and high genetic diversity (He = 0.733 ± 0.03). Strong differentiation was found between populations over short spatial scales (FST = 0.138, $p < 0.001$), reflecting the landscape discontinuity and isolation. Monmonier´s maximum difference algorithm, Bayesian analysis on STRUCTURE and principal component analysis identified one major genetic discontinuity between populations. Divergent genetic groups showed phenotypic divergence in flower traits and reproductive strategies. Increased sexual reproductive effort was associated with rock outcrop type and may be a response to adverse conditions for growth and vegetative reproduction. Srikulnath *et al.* (2015) [120] analyzed molecular phylogeny of 27 Dendrobium species based on nrITS and chloroplast MatK. Results indicated that the combined data (matK-rDNA ITS) was highly similar to the result from rDNA ITS. All 27 species were clearly grouped into two clusters with some differences relative to the morphological classification. Xu *et al*. (2015) [147] obtained 11 candidate barcodes on the basis of 1698 accessions of 184 *Dendrobium species* from mainland Asia. Five single barcodes, i.e., ITS, ITS2, matK, rbcL and trnH-psbA, could be easily amplified and sequenced with the established primers. Four barcodes, ITS, ITS2, ITS + matK, and ITS2 + matK, had distinct barcoding gaps. ITS + matK was the optimal barcode based on all evaluation methods. Furthermore, the efficiency of ITS + matK was verified in four other large genera including Ficus, Lysimachia, Paphiopedilum, and Pedicularis. The combination of ITS + matK was recommended as a core DNA barcode for large flowering plant genera. Roy *et al*., (2017) [105] studied the genetic diversity in the four orchid species using NGS based ddRAD sequencing data. The assembled nucleotide sequences (fastq) were deposited in the SRA archive of NCBI Database with accession number (SRP063543 for Dendrobium, SRP065790 for Geodorum, SRP072201 for Cymbidium and SRP072378 for Rhynchostylis). Result for these gene sequences (matK and Ycf2 and psbD) indicated that they were not evolved neutrally, but signifying that selection might have played a role in evolution of these genes in these four groups of orchids. Phylogenetic relationship was analyzed by reconstructing dendrogram based on the matK, psbD and Ycf2 gene sequences using maximum likelihood method in MEGA6 program. Zhao *et al*. (2017) [162] assessed the genetic diversity and population structure of 404 *C. tortisepalum* accessions by 13 nuclear simple sequence repeats (nSSR) markers. A total of 199 alleles were detected that ranged in number per locus from 6 (SSR,9) to 26 (SSR,10), with an average of 15.31 alleles per locus. No significant linkage disequilibrium ($P > 0.05$) was found in the study. A high degree of genetic diversity was observed in this species. Low genetic differentiation among the 28 natural populations was detected, which might have been caused by high gene flow. Analysis of molecular variance revealed 89.25% molecular variation within populations. Fajardo *et al*. (2017) [34] evaluated the genetic diversity and structure of *Cattleya granulosa* by 91 ISSR markers. Genetic variability assessed through molecular variance, diversity indexes, clusters of genotypes through Bayesian analysis, and tests for genetic bottlenecks. From all polymorphic loci, genetic diversity (HE) varied between 0.210 and 0.321 and the Shannon index ranged from 0.323 and 0.472. Significant genetic differentiation between populations (ΦST = 0.391; $P < 0.0001$) resulted in the division of the populations into five groups based on the log-likelihood Bayesian analysis. A significant positive correlation was observed among the populations between geographical and genetic distances ($r = 0.794$; $P = 0.017$), indicating isolation by distance. Patterns of allelic diversity within populations suggest the occurrence of bottlenecks in most *C. granulosa* populations ($n = 8$). Therefore, in order to maintain the genetic diversity of the species, the conservation of spatially distant groups is necessary. Tian *et al.* (2018) [127] assessed the genetic diversity for the entire range of endangered orchids native to China, Korea, and Japan by using ISSR and SCoT markers. A total of 17 populations were investigated to assess the three hypotheses i.e. (1), genetic drift has been a primary evolutionary force;(2) that populations in central and western China harbor higher levels of genetic variation relative to those from eastern China; and (3) that *C. japonicum* in China maintains the highest genetic variation among the three countries. The results revealed low levels of genetic diversity at the species level with substantially high degree of genetic divergence, which can be mainly attributed to random genetic drift. Chinese populations harbor the highest within-population genetic variation, which tends to increase from east to west. The results also exhibited close relationship between Korean populations and central/western Chinese populations. Historical rarity coupled with limited gene flow seems to be important factors for shaping genetic diversity and structure of *C. japonicum*. Gomes *et al*., (2018) [43] verified the loss of total genetic variability and the genetic population structure of all known relict *Cattleya lobata* populations using dominant molecular markers (ISSRs). High genetic variability was detected (He = 0.262; I = 0.463), with

most of the variation occurring within the populations (93%), along with a weak but significant genetic structure (UST = 0.074; P\0.001). Pollination by deception and long-distance seed dispersal may explain the higher variability values than those expected for impacted species with small populations. Historical factors, such as a more continuous distribution in the recent past with inter connected populations and perennial habit with long-lived individuals, can contribute to the maintenance of ancestral genotypes and a highly variable and homogeneous gene pool. These factors can reduce the possible effects of genetic drift, even in the most heavily impacted *C. lobata* populations in the tourist areas of Rio de Janeiro. Wang *et al*. (2018) [135] determined the complete chloroplast genome of Korean *C. goeringii* acc. smg222 by Illumina sequencing. The circular double-stranded DNA of 148,441 bp consisted of two inverted repeat regions of 25,610 bp each, a large single copy region of 83,311 bp, and a small single copy region of 13,910 bp. The genome contained 122 genes, of which 104 were unique and 18 were duplicated within the IRs. The 104 unique genes included 70 protein-coding genes, 30 distinct tRNA genes, and four rRNA genes. Phylogenetic tree analysis revealed that *C. goeringii* acc. smg222 was clustered with Cymbidium kanran, a cymbidium species native to Korea.

Conclusion

The literature covered in this review provides important new insights into the applied of biochemical and molecular technologies in orchid research, including for genotype identification, assessment of genetic diversity, molecular phylogenetics, genetic mapping and conventional breeding. The markers differ in their ability to differentiate among individuals, in the mechanism of detecting polymorphism, in genome coverage, and in the ease of application. Therefore, they can be used for identification of desirable traits in orchids. Traditional breeders of orchid crop have much to gain by applying these tools to their selection programs. We can also expect that large-scale genetic mapping efforts will be applied to the highest value of orchid in the near future.

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