Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 2641-2652
Received: 05-07-2018
Accepted: 07-08-2018

Mukesh Kumar
Department of Horticulture, College of Agriculture, SVPUAT, Meerut, Uttar Pradesh, India

Veena Chaudhary
Department of Chemistry, Meerut College Meerut, Uttar Pradesh, India

Ujjwal Sirohi
Department of Ag. Biotechnology, College of Agriculture, SVPUAT, Meerut, Uttar Pradesh, India

Manoj Kumar Singh
Department of Horticulture, College of Agriculture, SVPUAT, Meerut, Uttar Pradesh, India

Sunil Malik
Department of Horticulture, College of Agriculture, SVPUAT, Meerut, Uttar Pradesh, India

RK Naresh
Department of Agronomy, College of Agriculture, SVPUAT, Meerut, Uttar Pradesh, India

Correspondence
Mukesh Kumar
Department of Horticulture, College of Agriculture, SVPUAT, Meerut, Uttar Pradesh, India

Biochemical and molecular markers for characterization of chrysanthemum germplasm: A review

Mukesh Kumar, Veena Chaudhary, Ujjwal Sirohi, Manoj Kumar Singh, Sunil Malik and RK Naresh

Abstract
The development and utilization of biochemical and molecular markers are becoming widely used in floricultural crops for varying purposes including genetic studies, qualitative as well as quantitative traits, genotype fingerprinting, phylogenetic studies and mapping populations. 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), anchored microsatellite primed PCR (AMP-PCR), anchored simple sequence repeats (ASSR), arbitrarily primed polymerase chain reaction (AP-PCR), cleaved amplified polymorphic sequence (CAPS), DNA amplification fingerprinting (DAF), diversity arrays technology (DArT), expressed sequence tags (EST), inter-simple sequence repeat (ISSR), inverse PCR (IPCR), inverse sequence tagged repeats (ISTR), 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 (SSR), single nucleotide polymorphism (SNP), single primer amplification reactions (SPAR) and variable number tandem repeat (VNTR). Among these techniques, some techniques have been used in characterization of chrysanthemum. 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. In this review, we will discuss a basic description of different biochemical and molecular techniques that can be utilized for diversity analysis in chrysanthemum.

Keywords: Biochemical, characterization, development, PCR, DNA

Introduction
Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is a famous traditional flower in world that possesses great aesthetic value and abundant cultural associations, making it the most economically important horticultural crop worldwide (Anderson, 2006) [5]. It is commonly known by names like "Guldaudi", "Queen of the East" and "Glory of the East" (Randhawa & Mukhopadhyay, 1986) [80]. The number of chrysanthemum cultivars available worldwide is about 20,000 to 30,000 (Anderson, 2006) [5]. However, commonly grown *Chrysanthemum*s are hexaploids with average number of 54 chromosomes (Wolff, 1996) [112]. The modern, large, double and exquisitely flowered cultivars owe their origin to relatively small, single and non attractive types. The evaluation of chrysanthemum remained controversial so far; however, it is generally believed that the species is a hybrid complex derived from chance hybridization that naturally occurred between species of *Chrysanthemum vestitum*, *Chrysanthemum indicum*, *Chrysanthemum lavandulifolium* and *Chrysanthemum zawadskii* (Chen *et al.* 1998; Dai *et al.* 1998; Wang *et al.* 2004) [21, 25, 100]. The species has a strong self-incompatibility system like other members of the Asteraceae (Richards, 1986) [81]. Beside decoration, some species of chrysanthemum are also used the production of essential oil (*C. morifolium*), insecticides (*C. coccineum, C. cinerariifolium*) and culinary items (Bose *et al*., 2002) [13].
Taxonomists have traditionally used morphological attributes to classify genetic resources. 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) [35, 34]. Higher cost and time required for data collection and lack of knowledge of genetic control of phenotypic 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) [95, 90, 91]. 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. Various other ornamental plants have been used for DNA marker based diversity studies including bougainvillea (Chatterjee *et al*. 2007) [17], gladiolus (Kumar *et al*. 2016, Chaudhary *et al*. 2018) [54, 20], jasminum (Mahmood *et al*. 2013) [63], rose (Baydar *et al*. 2004) [10] and tuberose (Sirohi *et al*. 2017[a and b]) [88, 89], 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. The registration of a new cultivar requires Distinct, Uniformity and Stability (DUS) from all other registered cultivars, that different individuals are uniform in respect of the distinguishing characters and that the distinguishing characters are stable over time. DUS testing of new varieties is carried out using a classical taxonomic approach based on the manual measurement of a large number of morphological characters mostly pertaining to flowers. It is the DUS process that underpins the granting of plant breeder rights (PBR) and the protection of breeder's income.

DNA based markers, for cultivar identification and diversity analysis, are gaining importance since last five years. In case of DNA based markers, direct analysis of genome is possible. Moreover, these markers are tissue and age neutral and are not affected by the environment. Power of discrimination between cultivars is very high. These markers can be categorized of two types.

Biochemical markers

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) [106, 107], 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 [66], 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.

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) [40]. 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.

2. 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 listed in alphabetical order as like amplified fragment length polymorphism (AFLP; Vos *et al*. 1995) [99], anchored microsatellite primed PCR (AMP-PCR; Zietkiewicz *et al*. 1994) [121], anchored simple sequence repeats (ASSR; Wang *et al*. 1998) [101], arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990) [108]), DNA amplification fingerprinting (DAF; Caetano-Anolles *et al*. 1991) [15], diversity arrays technology (DArT; Jaccoud *et al*. 2001) [44], expressed sequence tags (EST; Adams *et al*. (1991) [10], inter-simple sequence repeat (ISSR; Zietkiewicz *et al*. 1994) [121], inverse PCR (IPCR; Triglia *et al*. 1988) [96], inverse sequence tagged repeats (ISTR; Rohde, 1996) [82], multiplexed allele-specific diagnostic assay (MASDA; Shuber *et al*. 1997) [87], microsatellite primed PCR (MPPCR; Meyer *et al*. 1993) [70], random amplified microsatellite polymorphisms (RAMP; Wu *et al*. 1994) [117], random amplified microsatellites (RAM; Hantula *et al*. 1996) [39], random amplified polymorphic DNA (RAPD; Williams *et al*. 1990) [110], restriction fragment length polymorphism (RFLP; Botstein *et al*. 1980) [14], sequence characterized amplified regions (SCAR; Paran and Michelmore, 1993) [74], sequence specific amplification polymorphisms (S-SAP; Waugh *et al*. 1997) [105], sequence tagged microsatelite site (STMS; Beckmann and Soller, 1990) [11], sequence tagged site (STS; Olsen *et al*. 1989), simple sequence length polymorphism (SSLP; Dietrich *et al*. 1992) [26], simple sequence repeats (SSR; Akkaya *et al*., 1992)[3], single nucleotide polymorphism (SNP; Jordan and Humphries, 1994) [47], single primer amplification reactions (SPAR; Gupta *et al*. 1994) [36] and variable number tandem repeat (VNTR; Nakamura *et al*. 1987) [73]. Among the DNA markers, some are more attractive for genetic diversity analysis in floricultural crops. These markers are gaining

importance for cultivar identification and diversity analysis since last twenty years. In case of DNA based markers, direct analysis of genome is possible. Moreover, these markers are tissue and age neutral and are not affected by the environment. Power of discrimination between cultivars is very high. Important molecular markers which are frequently used in floricultural crops characterization can be grouped into the following categories

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) [61]. The key feature of AFLP is its capacity for "genome representation. AFLP is a high multiplex PCR-based system (Vos *et al*. 1995) [99] having the potential to generate a large number of polymorphic loci (Powell *et al*. 1996) [76].

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) [110] (Gupta, and Varshney, 2013) [37]. 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) [46].

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) [94]. 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) [116, 30].

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) [58], 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.

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) [38].
5. They are accessible to other research laboratories via a published primer sequence (Saghai-Maroof *et al.* 1984) [83].

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) [70, 36, 117] or more usually anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al*. 1994) [121]. 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) [86]. This method uses single primers of 15–20 nucleotides with a 30or 50anchor sequence (Pharmawati *et al*. 2004) [75]. 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) [52] although the level of polymorphism has been shown to vary with the detection method used.

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) [74, 68]. 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) [74]. These markers are more reliable and robust in comparison to the dominant RAPD and ISSR markers (Dnyaneshwar, *et al*. 2006; Li, *et al*. 2010; Rajesh *et al*. 2013) [27, 57, 78].

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) [33]. 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) [30, 31].

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) [62]. 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) [53, 45, 71]. 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) [44, 109]. 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) [41]. 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) [4] and more recent applications of this concept on the next generation sequencing platforms (Elshire *et al*. 2011) [32]. 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) [50, 23, 24, 79].

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) [98]. 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) [97] (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) [92]. 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)[51],

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 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

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, 69]. The use of DNA markers in chrysanthemum are presented in (Fig.1).

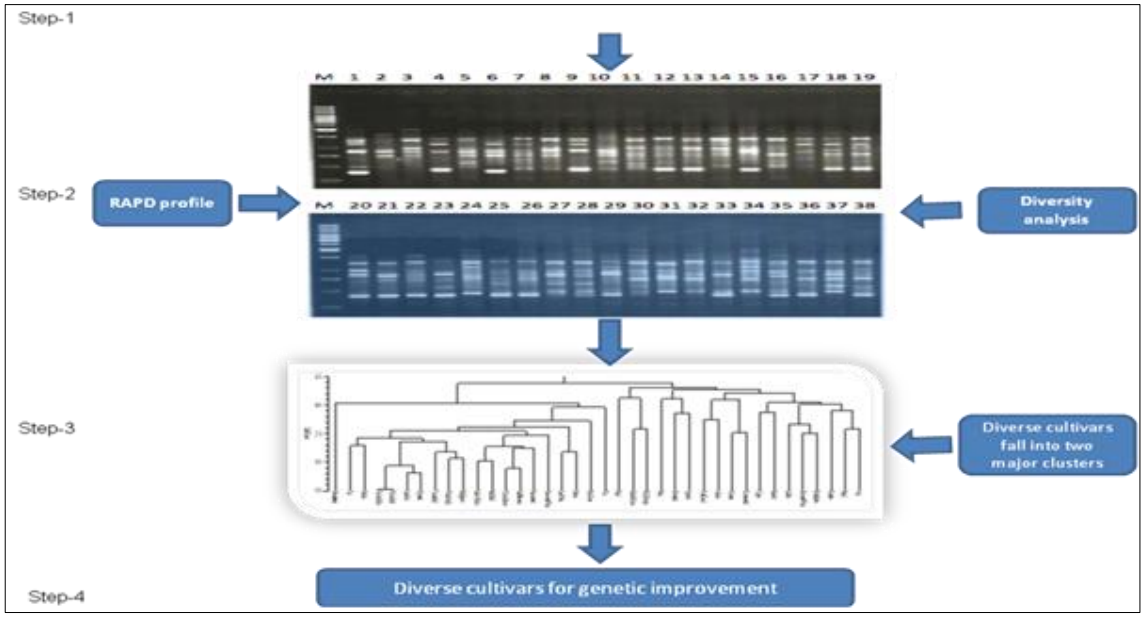

Fig 1: DNA profiling and diversity analysis of chrysanthemum by RAPD markers (Kumar *et al*. 2017) [55]

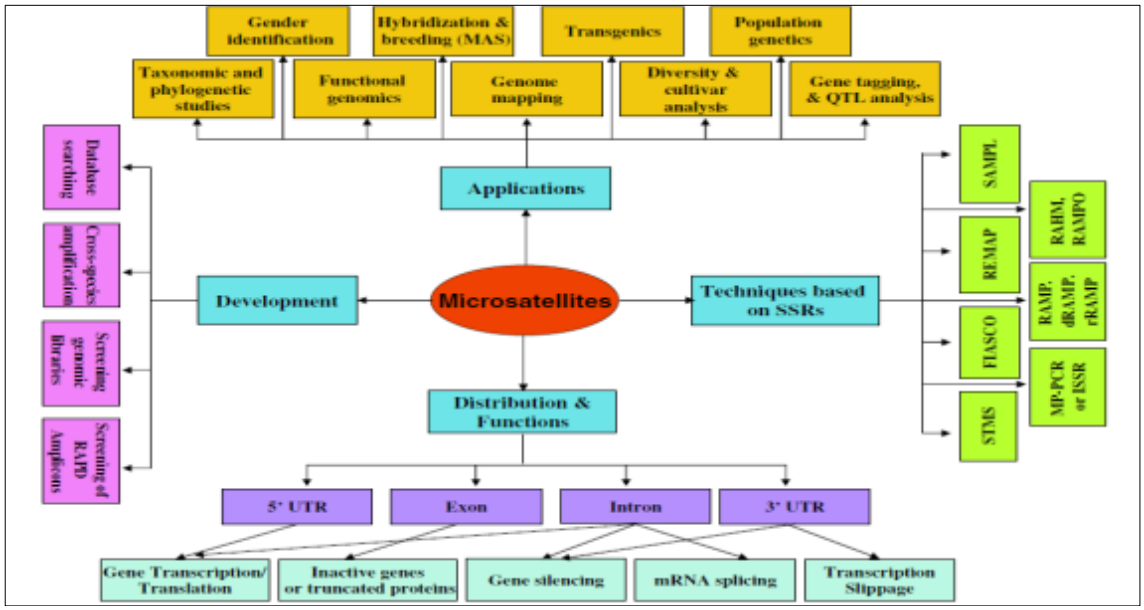

Fig 2: Microsatellites: A summary of development, distribution, functions and applications (Kalia *et al*., 2011) [48].

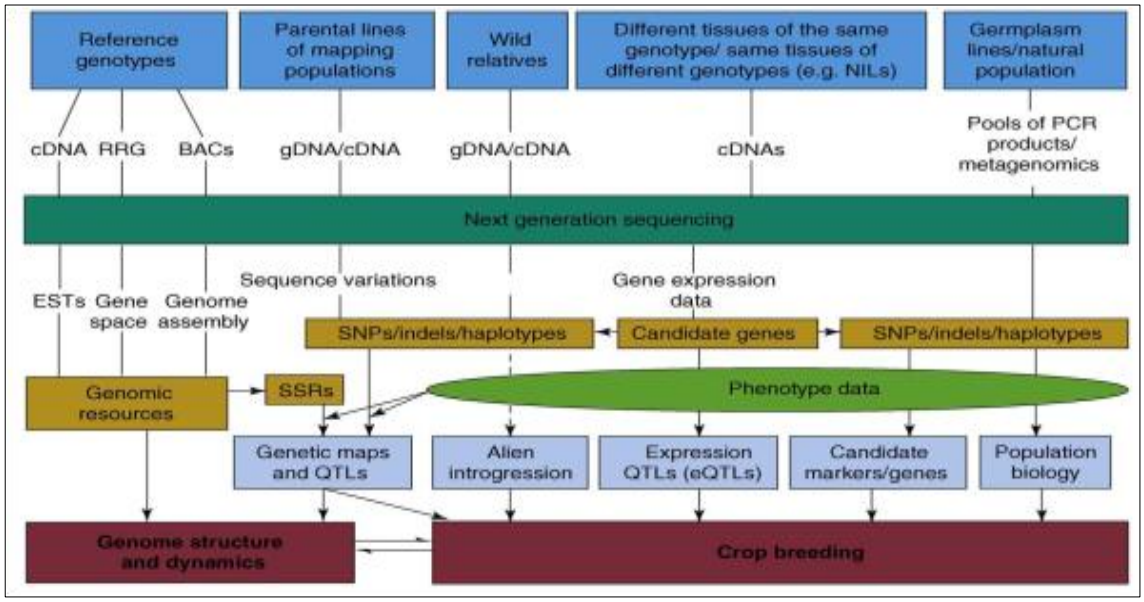

Fig 3: Overview of Next Generation Sequencing (NGS) applications in crop genetics and breeding (Varshney *et al*. 2009) [97].

Applications of biochemical and molecular markers in chrysanthemum

DNA-based molecular markers are becoming increasingly important for characterization and diversity analysis of plant varieties and germplasm, detection of redundancies of gene bank collection and in monitoring genetic changing during maintenance. Among the molecular markers technique, RAPD, AFLP, RFLP ISSR, SSR, CAPS, SCAR, SNP and EST are widely exploited largely due to the fact that results are obtained quickly and are fairly inexpensive to generate. The literatures available on genetic diversity by using molecular markers in chrysanthemum are discussed here. In *Chrysanthemum*, cultivars are identified in flowering trials and breeders' rights are presented by cultivar characteristics including flower, leaf and growth morphology. Dowrick (1953) [29] explained that chrysanthemum (*Denodranthema grandiflora*) is a polyploidy which contained six or seven species and average 54 chromosomes (ranging from 36 to 72) are present. These chromosomes formed regular bivalent at meiosis indicates origination from an allopolyploid (Watanabe, 1977) [104]. Wolff (1991) [114] expected that genetic variability is due to out crossing and many loci are in heterozygous state (Wolf and Jenny Peters-Van Rijn, 1993) [113]. The application of is ozyme technology could largely improve the identification of *Chrysanthemum* cultivars (Roxas *et al*., 1993). However, the level of polymorphism obtained insufficient to distinguish cultivars and the growth conditions may influence the quality and quantity of isozymes (Wolff *et al*. 2005). Technique developed by William *et al.* (1990) [110] *i.e.* RAPD (random amplified polymorphic DNA) is based on amplification of DNA with random primers by using the PCR, in which, information about sequences of DNA and probes is not required in advance. The first study on the identification of chrysanthemum with the application of RAPD markers was carried out by Wolf and Jenny Peters-Van Rijn (1993) [113]. Wolf (1996) [112] reported that for getting the good DNA quantity and optimal fragment pattern in chrysanthemum, polymerase brand, thermal cycler brand, annealing temperature and primer are important factors because each primer has its optimal annealing temperature which is not associated with primer's GC content. Wolf *et al.* (1994) [111] constructed chrysanthemum *Pst I* and *Hind III* genomic libraries and found that 91% of probes of *Pst I* library hybridize to low copy genes and it had only 35% from *Hind III* library. Polymorphic pattern were reflected by *Pst I* (79%) and *Hind* (14%). Wolff *et al*. (1995) [115] managed the RAPD to distinguish between chrysanthemum cultivars, which did not belong to a single group using two or three primers. However, they also suggested that there is no possibility at the DNA level to distinguish mutant family derived vegetatively from one original cultivar. Scott (1996)[84] used DNA amplification fingerprinting (DAF) and only few polymorphic characters uniquely identified which were closely related cultivars within each of the series in chrysanthemum. Malaure *et al*. (1991a, 1991b) [64, 65] showed that plant regeneration from different tissue layers can lead to genetically diverse material, whereas regeneration from leaf tissue or intact florets usually leads to plants identical to the parental plant. Wolf (1996) [112] concluded that it is possible to differentiate cultivars from one family by RAPD, however, a lot of primers should be tested to find primers that differentiate all members of a family from each other. It may eventually lead to locating important genes that are involved in *e.g.* flower pigmentation. Missing or additional RAPD fragments should be analyzed for this purpose. Huang *et al.* (2000) [43] selected 24 RAPD primers out of 45 random primers to detect the molecular marker through RAPD and patterns of molecular markers classified in seven types. There 34.4% to 48.9% of the RAPD markers were found to reveal additivity among parents and off springs in three hybrid combinations. However, 38% to 52.6% of markers were absent in offspring, but 11.6% to 13.1% of unique markers were present in offspring. Moreover, there were no definite rules as to whether markers in offspring were more similar to each other than either was to the offspring. The results clearly indicated that the genetics of chrysanthemum are very complex. However they suggested that RAPDs are a powerful tool to detect different molecular markers in hybrid populations of *Chrysanthemum* cultivars. The characterization analysis carried out by (Martin *et al.* 2002) [67] who revealed a high diversity between the varieties. All of them could be distinguished through the RAPD analysis and the level of similarity was in all the cases less than 80%. The analysis exhibited that most unrelated variety was 'Monnete Blanche', with less than 60% similarity with the others. The highest level of similarity was observed between two varieties i.e. 'Cascade Luminose' and 'Miracle Dautome', which showed almost 80% similarity. Qin *et al*. (2002) [77] analyzed eighteen cultivars of chrysanthemum using random amplified polymorphic DNAs (RAPDs). Three primers out of 80 random primers showed high polymorphism, the polymorphic amplification fragment length ranged from 600 bp to 1300 bp. The 1200 bp fragment generated by primer OPD15 was present only in cv. `Dahongtuogui', while the 1100 bp fragment generated by primer OPA17 was only absent in cv. `Yulingguan'. The cultivars with similar petal type had high similarity coefficient (SC) amongst eighteen cultivars. Zhou and Dai (2002) [120] used AFLP (Amplified Fragment Length Polymorphism) technique to assess the diversity in twelve *Dendranthema* taxons. Digestion with enzymes EcoRI/ MseI and amplification with 5 E+3/M+2 primer combinations resulted 287 AFLP fragments. Among them, 85.7% were polymorphic. Cluster analysis based on UPGMA indicated little genetic differences between two cultivars of *Dendranthema grandiflorum* used in this experiment. This cultivar was found to be relatively close to *D. vestitum, D. indicum and D. lavandulifolium; D. zawadskii* came second and *D.chanetii* is the *most distinct.* They predicted that the relationship between *D. indicum, D. lavandulifolium, D. nankingense* was very close and had frequent gene flow among the species distributing in the same area. Sehrawat *et al.* (2003) [85] studied genetic variation in chrysanthemum by using 60 random decamer primers, out of these, 31 primers amplified genomic DNA. Tested cultivars had 2 to 21 bands for each primer and a total of 257 clear and reproducible bands generated, 239 bands were polymorphic. The amplified DNA fragments normally ranged from 0.55 to 2 Kb. RAPD data of different cultivars were used for cluster analysis which revealed that genetic variation amongst cultivars was high and divided them into two major groups. These groupings were in consistent with their morphological differences and geographical distribution. The first group consisted of Snow Ball, Ajina Purple and Sonar Bangla cultivars, while the second group accounted for Nagpur Red, Haldighati, Cardinal, Puja, Jaya, Suneel, Vasantika, Gauri, Flirt and Baggi. The results indicate that RAPDs are efficient for identification of chrysanthemum cultivars and for determination of genetic relationships. No single RAPD primer was able to distinguish all the cultivars by producing polymorphic bands. However, the cultivars were

distinguishable with the combinations of polymorphic bands generated by various primers. One of the advantages of the RAPDs method is that the entire plant genome is targeted for primer annealing, facilitating development of a higher density map. Polymorphisms can be successfully scored and used for studying genetic variation and diversity. Lema-Ruminska (2004) [56] used RAPD markers for molecular characterization of radiomutants cultivars of chrysanthemum. Analysis of genetic similarity indices revealed low diversity within the radio-mutants. The lady group cultivars, derived from one original cultivar by radio-mutation, could be distinguished from each other by using RAPD markers of only a single primer or sets or three primers. PCR analysis proved the efficiency of the RAPD method for DNA fingerprinting of the original cultivar 'Richmond' and its new radio-mutants. Chatterjee *et al*. (2005) [19] carried out a study to estimate the genetic relationship among the different chrysanthemum cultivars with relation to their morphological, biochemical characteristics and geographical distribution. Genetic distance between four mini chrysanthemum cultivars was studied through RAPD analysis. A total of 40 primers were screened and four were selected and used for all the four genotypes. Similarity among the cultivars was very high showing low genetic diversity. One of these primers could differentiate cultivars from each other, thus estimating the genetic distance and also the species identification. Bhattacharya and Silva (2006) [12] studied molecular systematics and genetic differences between 10 original chrysanthemum cultivars and 11 mutants by using RAPD markers. The similarity among the cultivars and mutants varied from 0.17 to 0.90 by using RAPD analysis. Two distinct groups were found among the cultivars. Two cultivars were present as a separate group showing differences from all other cultivars. Mutants with different flower color could be identified at the molecular level using RAPD techniques. A high genetic distance among the different chrysanthemums showed that there exists a possibility of introgressing new and novel genes from the chrysanthemum gene pool. Chatterjee *et al.* (2006) [18] observed the similarity among the cultivars and mutants varied from 0.17 to 0.90 using RAPD and found two distinct groups. Two cultivars were present as a separate group showing differences from all other cultivars. Mutants with different flower colour could be identified at the molecular level using RAPD technique holding promise to identify unique genes as SCAR markers. A high genetic distance among the different chrysanthemum showed that there exists a possibility of intro-gressing new and novel genes from the chrysanthemum gene pool. Wu *et al*. (2007) [118] screened sixty five chrysanthemum cultivars for molecular diversity. Six pairs of primer combinations generated 244 bands out of which 178 bands were polymorphic. The average percentage of polymorphic bands was 72.95%. They suggested that molecular diversity can be used as stability analysis for plant variety protection in future. Liu *et al*. (2008) [59] used AFLP technique to study the genetic diversity of twenty six chrysanthemum varieties. Seven primer combinations were used to discriminate between genotypes and produced 385 AFLP-bands, 285 of which were AFLP polymorphic bands (74.03%), with an average of 40.7 bands produced per primer pair. The cluster analysis based on AFLP data showed that 26 *Dendranthema* varieties could be divided into 5 groups according to pedigree. Results indicated that it is practical to categorize chrysanthemum varieties with AFLPs. Minano *et al*. (2009) [72] used RAPD analysis for molecular characterization of eight cut flowers and two pot plant cultivars of chrysanthemum. Among the cultivars, three namely ('Refocus', 'Red Reagan', and 'Sheena Select') were established *in vitro* and the occurrence of somaclonal variation studied using the same molecular technique. Likewise, the effect of the culture age was considered in assessing genetic stability. Monthly subcultures were carried out, identifying the origin and history of the shoots, throughout a nine-month proliferation period followed by acclimatization. Molecular markers were obtained in every subculture cycle and from the acclimatized plants. Only one shoot from the 7th subculture of the cultivar 'Refocus' showed a different band pattern. Barakat *et al*. (2010) [9] applied RAPD analysis for the detection of genetic polymorphism among chrysanthemum mutants and their parent. Callus induction and shoot formation percentages were affected by gamma ray doses, whereas, the variation between medium protocols and the variations due to the interaction among medium protocols and doses were statistically insignificant. Five RAPD primers were used to amplify DNA segments from the genomic DNA of chrysanthemum and its 13 soma-clones. The genetic similarity among the fourteen genotypes ranged from 0.43 to 0.95. The chrysanthemum cultivar and its 13 soma-clones were classified into five clusters. Liu and Yang (2010) [60] utilized RAPD markers to analysis of genetic relationship in ten wild species under genus *Dendranthema* and twelve grown species. A total of 50 random primers were screened, 14 primers showed good level of polymorphism were used for genetic diversity analysis in chrysanthemum. The level of polymorphic loci was 96.4% polymorphism. Wang *et al.* (2013) [102] acquired SSR markers by transcriptome sequencing and reported that SSRs are potentially useful for marker-assisted breeding and genetic analysis in the genus chrysanthemum and its related genera. Wang *et al*., (2014) [103] used EST-SSRs to characterize polymorphism among 29 Chrysanthemum and Ajania species accessions of various ploidy levels. Most EST-SSR loci were readily transferable between the species, 29 accessions were separated into three groups in terms of the number of fragments. It was concluded from the study that EST-SSR could provide a potential molecular basis of adaptation during evolution, while whole genome duplication has a major effect on the mutational dynamics of EST-SSR loci, which could also affect gene regulation. Baliyan *et al*. (2014^{a}) [6] studied on genetic diversity in chrysanthemum by using morpho-agronomic traits and ISSR markers. A total of 10 ISSR (inter simple sequence repeat) markers were used for cultivar identification in 24 genotypes of chrysanthemum. Multivariate analysis was performed on field data using Mahalanobis's D2-statistics, Tochers method of clustering and combined analysis of variance. Analysis of variance revealed considerable differences among the genotypes for all the morphological traits studied. Genotypes namely SKC-83, Ratlam Selection, Gaity and Selection 69 were found to be more diverse in both morphological and molecular analysis. Results suggested that both morphological traits and ISSR marker are highly useful for assessing genetic diversity and parental selection studies in chrysanthemum. Baliyan *et al*. (2014^{b}) [7] examined the genetic variation among 24 chrysanthemum cultivars by RAPD markers. A total of 79 fragments was produced with 10 RAPD primers and out of which 64 (81.01%) were found polymorphic and 15 bands (18.99%) were monomorphic. The number of polymorphic fragments varied from 4.0 (OPF13) to 15 (OPF06) with an average of 7.9 bands per primer. The PIC varied from 0.10 to 0.66 with an average. 50, MI varied from

o.36 to 6.99 with an average 2.92 and RP value was noted in the ranged from 5.17 to 14.50 with an average 9.40. UPGMA clustering revealed two major groups i.e. GroupI, containing 11 genotypes and Group II, contained 13 genotypes. Among the 24 genotypes, Poncho, Terri, Rangoli, Sweta, Ravikiran and Nanco are divergent and may be useful for breeding programme. The results suggested that RAPDs are highly useful for assessing the genetic diversity analysis among the chrysanthemum germplasm and parental selection studies in chrysanthemum. Baliyan *et al*. (2014[c]) [8] investigated the genetic diversity among 24 chrysanthemum cultivars by 07 Simple Sequence Repeats (SSRs) markers. A total of 16 bands were produced out of which 15 bands were found polymorphic and 01 band monomorphic. The number of polymorphic fragment varied from 02 (RM1) to 03 (RM433) with an average 2.14 fragment per primer and percent polymorphism varied from 66.75 to 100% with an average of 93.75%. The PIC varied from 0.42 to 0.95 with an average of 0.74. The RP and MI ranged from (0.83 to 0, 57) to (4.0 and 2.76) with an average (2.03 and 0.57) respectively. The UPGMA clustering revealed two major groups and found considerable amount of genetic diversity. Among the 24 cultivars, Ravikiran, Selection 44, Kundan, Terri, Sonton and Poncho were found divergent and may be used for breeding programme. Zhang *et al.*, (2014) [119] identified a broad collection of 480 Chinese traditional chrysanthemum fultivars using the unique DNA fingerprints and molecular identified by 20 simple sequence repeat markers. Five loci, which distinguished all of the selected cultivars, were identified as the core loci to establish unique fingerprints and molecular identities with 19 denary digits for each cultivar. Population structure and Principal component analysis was further performed to verify the classification results. On the basis of the Q-matrices of K = 10, a total of 19 traits were found to be associated with 42 markers. Taken together, these results can serve as starting points for the identification and classification of chrysanthemums based on the polymorphism of microsatellite markers, which is beneficial to promote the marker-assisted breeding. Chong *et al*. (2016) [22] assessed the genetic diversity, evolutionary relationships and the identification of candidate genes in chrysanthemum by SNP. A genome-wide association analysis revealed that seven SNPs lying within six genes were predictive of three important traits (ray floret type, cultivated type and flower shape), but no association with flower color was detected. The study has provided a number of novel insights into evolutionary relationships, the population structure and the genetic basis of some key ornamental traits. Kameswari and Girwani (2017) [49] used RAPD and ISSR markers for genetic relatedness in 37 genotypes of chrysanthemum. A total of 27 RAPDs and 10 ISSRs markers generated 271 and 107 polymorphic bands respectively and accounted 97.4% and 93.86% polymorphism respectively. However, Kumar *et al*. (2017) [55] studied on genetic diversity and population structure in chrysanthemum by using RAPD markers. A total of 70 clear and scorable bands were produced by 10 RAPD primers. Out of 70 bands, 66 were polymorphic and four (4) were mono-morphic bands generated 94.28% polymorphism. Population structure analysis grouped among the genotypes into two sub-population and mixed population was observed among the genotypes. Chang *et al*. (2018) [16] used 10 simple sequence repeat (SSR) markers to identify a collection of 88 chrysanthemum and its related genera accessions. A total of 42 effective alleles across 88 accessions were detected; 3429 bands were obtained by PCR amplification, including 2630 polymorphic bands. The similarity coefficient ranged from 0.53 to 0.88. Cluster analysis based on UPGMA illustrated that the wild species and large-flower cultivars were first divided into two clusters, then the large-flower cultivars formed five distinct groups according to petal type, indicated that petal type can be a classification criteria of chrysanthemum. In the wild species cluster, *C. vestitum* and *C. zawadaskii* grouped with *A. trilobata*, suggested that the *Ajania* genera was closely related to the *Chrysanthemum* genera. 'Hangbaiju', 'Gongju' and 'Chuju' were grouped together, and 'Boju', *O. longilobus* and *C. mongolicum* constituted another branch, showed a correlation with geographic region of origin. Population structure analysis grouped among the genotypes into five subpopulations, which was nearly consistent with the clustering results. Principle component analysis was further performed to verify the classification results. The results showed that these SSR markers are very powerful for studying genetic relationships and will be useful tools in the identification and classification of chrysanthemum.

Conclusion

The literature covered in this review provides important new insights into the applied of biochemical and molecular technologies in chrysanthemum research, including for genotype identification, assessment of genetic diversity, molecular phylogenetics, genetic mapping and conventional breeding. Traditional breeders of chrysanthemum 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 chrysanthemum in the near future.

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