J Agric. Res., 2021, Vol. 59(1):13-28
www.jar.com.pk
Agriculture Department
Government of Punjab

BIOTECHNOLOGY
REVIEW PAPER

DNA FINGERPRINTING OF CROPS AND ITS APPLICATIONS IN THE FIELD OF PLANT BREEDING

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Article received on:
28/12/2020
Accepted for publication:
05/01/2021

ABSTRACT

Fingerprinting of crops is gaining importance in plant breeding due to its applications for variety protection, dispute resolution, and forensic science studies. Before the development of genomic and proteomic technologies, varieties were differentiated based on morphological markers using distinctiveness, uniformity, and stability (DUS). However, in the mid-20th century, protein-based markers were discovered and were used for fingerprinting and genetic diversity analysis of crops. Now in the genomics era, deoxyribonucleic acid (DNA) markers are frequently used for fingerprinting of crops. The journey of crop fingerprinting using DNA markers started from RFLPs, which were non-PCR based markers, and progress through to polymerase chain reaction (PCR) based markers randomly emplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPS), simple sequence repeat markers (SSRs), inter simple sequence repeats (ISSRs), single nucleotide polymorphism (SNPs), diversity array technology (DArT). The latest technological advancements in the field of genomics and bioinformatics have shifted focus towards whole genome sequencing of crops rather than using different marker systems. The future of crop fingerprinting is linked with the development of cost-effective whole genome sequencing techniques. Such technologies would allow differentiation of highly similar varieties, mutants, some clones and vegetatively propagated crops. In this review a brief overview of different marker systems is given for their effectiveness in DNA fingerprinting of crops and their putative applications, with recommendations for future developments.

KEYWORDS: DNA markers; genetic diversity; genotyping; Isozymes; PCR-based markers; Pakistan

INTRODUCTION

Variety identification, classification, and sustainability are important during breeding programs, seed production, trade, and product inspection. The examination of genetic variation and relatedness is a critical aspect of the conservation and maintenance of biodiversity and food security (Nybom *et al.,* 2014). Traditionally, species and varieties were identified using distinctness, uniformity, and stability (DUS) approach, based on morphological descriptors (Tiwari *et al.,* 2013). These morphological descriptors were less informative, time-consuming, and unreliable due to multigene nature. Moreover, environmental fluctuations, poor sampling techniques and unknown genetic control make morphological characters less effective for variety identification (Korir *et al.,* 2013).

There is dramatic advancement in molecular biology techniques to develop robust and reliable methodologies to characterize, differentiate, purify, study and understand the genetic variability among the cultivars to facilitate breeding programs (Korir *et al.,* 2013). DNA markers provide reliable and cost-effective approach to differentiate plant genotypes and elucidate the genetic diversity, variability, among varieties and species in time-efficient manner. Molecular methods, especially DNA fingerprinting, are the most promising tools for the identification of plant genotypes (Nybom *et al.,* 2014).

DNA fingerprinting term was coined by Paul Hebert in 2003 as a supplement to traditional morphological based taxonomy and is a widely accepted method for the characterization of genetic differences and relatedness (Hebert *et al.,* 2003). DNA fingerprinting utilizes DNA markers for assigning of breeding lines to various heterotic groups and for variety identification (Jamil *et al.,* 2020b). Moreover, it is least affected by environmental interactions and spatial-temporal changes in gene expression. Some DNA marker techniques are non-PCR based, i.e. Restriction Fragment Length Polymorphisms (RFLPs), but the most commonly used now are PCR based, like Random amplification polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), Single Nucleotide Polymorphisms (SNPs), Diversity Arrays Technology (DArT) and Genotyping by Sequencing

(GBS) (Nadeem *et al.,* 2018).

This review provides an insight on different approaches used for DNA fingerprinting, elucidating their pros and cons. Moreover, it describes the basic methodology of DNA fingerprinting and a critical evaluation of the different techniques, focusing on their reproducibility, sensitivity, cost, and discriminatory power. A detailed analysis on application of DNA fingerprinting for genotyping, genetic diversity assessment and food crop protection is also provided. Further, latest developments in the field of DNA fingerprinting and its various future prospects are discussed.

Crop fingerprinting using different markers system

Earlier morphological markers were replaced by protein-based markers and now DNA based markers are frequently used for fingerprinting purpose as described in (Fig. 1).

Morphological markers: The earliest approach for identification and differentiation of cultivars was morphological markers based. A specific characteristic found in a genotype was labeled as "fingerprint" for its unique identification. Morphological markers such as fruit shape, color, and size, pubescence of leaves, and the number of flowers per spike were most frequently used for variety identification (Walsh and Hoot, 2001). Many crops i.e. bougainvillea (Leonardi and Romano, 2003), sugarcane (Selvi *et al.,* 2003), napier grass (Bhandari *et al.,* 2006), grapevine (Royo *et al.,* 1997), wheat (Ojaghi and Akhundova, 2010), sugarcane and peas (Tar'an *et al.,* 2005) were fingerprinted using morphological descriptors. However, with the advent of latest technologies, morphological approach was considered as a non-reliable source of cultivar identification and genotypic assessment due to following reasons. Different varieties in a species are, although, genetically different but look similar on a morphological basis. Morphological characteristics might arise as a result of genotype to environment interplay. Morphological traits that are controlled by recessive genes and can express only under homozygous form. These characters are quantitative and hence, their calculation and genetic mapping is a complicated job (Bhandari *et al.,* 2006).

Proteins markers (Isozymes): After 1960, Isozymes were employed for fingerprinting because of fast speed, reliability, and relative independence of environmental conditions (Nybom *et al.,* 2014). Isozyme analysis was comprised of samples collection, enzyme extraction, gel electrophoresis, gel staining, photographing and fingerprints searching (Sumarani *et al.,* 2004). Isozymes were used for fingerprinting and characterization of napier grass (Bhandari *et al.,* 2006), grapevine (Royo *et al.,* 1997), paper flower (Hammad, 2009), cassava (Sumarani *et al.,* 2004) and garlic (Ipek *et al.,* 2003). However, Isozymes assay faced some issues i.e. degradation of proteins during samples collection. Protein extraction itself is a tedious and strenuous job (Nybom *et al.,* 2014). Electrophoresis, sometimes, cannot detect polymorphism due to improper gel preparation, unsuitable type and amount of electrophoretic and grinding buffer, grinding, and staining protocols, physiological and ontogenetic conditions of tissues. Variation in sampling time and type of tissue greatly affect the results of isozyme analysis (Johnson *et al.,* 2010).

DNA markers: DNA makers are considered a powerful method of storing allele frequencies for specific genes. These are further divided into four generations. First and second-generation molecular markers include RFLPs, RAPDs, AFLPs, SSR whereas, third and fourth generation marker system includes SNPs, KASper, DArT assays and GBS (Paux *et al.,* 2012). DNA markers are categorized into two classes i.e. PCR based markers and Non-PCR based markers. The detail of studies conducted for DNA fingerprinting of crops and fruits using Non-PCR and PCR based markers is listed in Table 1.

Non-PCR based markers

Restriction fragment length polymorphisms (RFLPs): RFLPs were the earliest used DNA based markers in eukaryotic species. These are created due to mutations, inversions, deletions and translocation events. RFLPs are hybridization-based polymorphism approach which depends on genomic DNA cleavage by restriction enzymes followed by hybridization to DNA labelled probes to detect DNA fragments of equal size differing in one base pair. These are co-dominant which means that both the alleles in heterozygote samples will be detected hence these are useful for detection of the recessive traits (Ben-Ari and Lavi, 2012; Uddin and Cheng, 2015). RFLPs were used for taxonomic studies and understanding of species relationships in various crops i.e. lentil, oats, tomatoes, peanuts and *Brassica napus* (Wang *et al.,* 2011a,b). However, genotyping using RFLPs is a complicated, time consuming and expensive methodology. RFLPs need high quantity and quality of DNA and use radioactive isotopes. DNA probes for RFLPs are not available for many plant species. Due to single-locus nature, it is difficult to detect more than one base pair difference. Oligo-nucleotide probes are sensitive to minute changes in temperature during hybridization and it is a complicated process (Ben-Ari and Lavi, 2012).

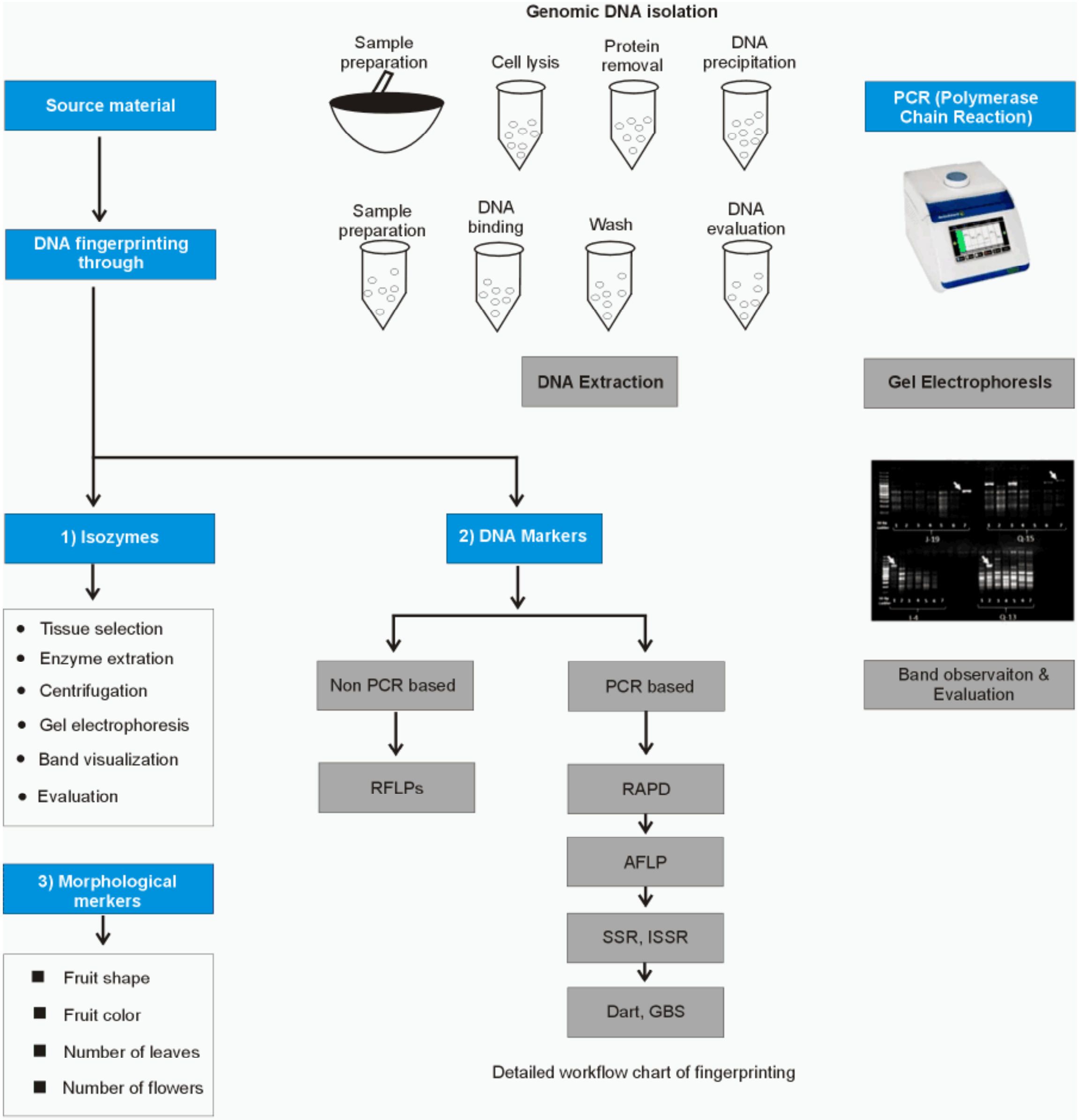

Fig. 1. Detailed strategy for fingerprinting of crops using morphological markers,isozymes and DNA markers

PCR based markers: Reliance on hybridization-based approaches for DNA fingerprinting was minimized with the advent of PCR-based techniques, as later have certain advantages. PCR-based approaches are categorized into Single-locus and Multi-locus methods (Nybom *et al.,* 2014) as described below.

Randomly amplified polymorphic DNA (RAPD): PD developed by William and colleagues (Williams *et al.,* 1990) were used frequently for fingerprinting studies (Gomes *et al.,* 2008; Nybom *et al.,* 2014). These require small sample size, provide rapid results in relatively less time, are less expensive and need no prior information of the genomic sequence. The technique uses primers with arbitrary sequences to generate PCR fragments from genomic DNA which after electrophoretic analysis, result in multi-locus banding patterns which are visualized through UV-trans illuminator. Differences in the size range of PCR products are analysed for genotype characterization and fingerprinting (Selvakumari *et al.,* 2017).

In past, RAPD was used to estimate genetic diversity, molecular characterization and fingerprinting of economically important food crops e.g. corn, wheat, rice, sorghum, barley, rye, and oats (Salem *et al.,* 2007; Iqbal *et al.,* 2019). Similarly, RAPD was used for the identification and characterization of many medicinal plants e.g. eggplant, ginger, mint, holy basil and cinnamon (Selvakumari *et al.,* 2017). But there are

Table 1. Historical overview of ifferent markers system used for DNA fingerprinting of some important crops

Crops	Marker System	References
Cereals & grasses		
Wheat	Morphological markers, RAPD, AFLP, SSR, ISSR, SNPs, DArT	(Gupta *et al.,* 2003; Terzi *et al.,* 2005; Salem *et al.,* 2007; Ojaghi and Akhundova, 2010; Talebi and Fayyaz, 2012; Etminan *et al.,* 2016; El-Esawi *et al.,* 2018; Hao *et al.,* 2020)
Rice	RFLP, RAPD, AFLP, ISSR, DArT	(Ge *et al.,* 2001; Jaccoud *et al.,* 2001; Salem *et al.,* 2007; Chandel *et al.,* 2010; Singh and Sengar, 2015; Suvi *et al.,* 2020; Yan *et al.,* 2020)
Maize	RAPD, SSR, ISSR, SNPs, GBS	(Salem *et al.,* 2007; Elshire *et al.,* 2011; Lenka *et al.,* 2015; Song *et al.,* 2017; Ghonaim *et al.,* 2020; Jamil *et al.,* 2020b)
Sorghum	RAPD, AFLP	(Perumal *et al.,* 2007; Salem *et al.,* 2007)
Rye	RAPD	(Salem *et al.,* 2007)
Sugarcane	Morphological markers, SSR, RAPD and SNP	(Selvi *et al.,* 2003; Pang *et al.,* 2019; Wang *et al.,* 2020)
Oats	RFLPs, SNPs	(Moser and Lee, 1994; Terzi *et al.,* 2005)
Barley	RAPD, SSR, SNPs, DArT, GBS	(Thiel *et al.,* 2003; Wenzl *et al.,* 2004; Terzi *et al.,* 2005; Salem *et al.,* 2007; Elshire *et al.,* 2011; Singh and Sengar, 2015)
Napier grass	Morphological markers, isozymes markers	(Bhandari *et al.,* 2006)
Lemongrass	ISSR	(Adhikari *et al.,* 2015)
Oilseeds		
Brassica	RFLP, SNPs	(Wang *et al.,* 2011a; El-Esawi, 2017)
Olive	ISSR, SNPs, SCAR, DArT	(Bautista *et al.,* 2003; Reale *et al.,* 2006; Atienza *et al.,* 2013; Saddoud Debbabi *et al.,* 2020)
Soybean	AFLP, SSR, SNPs	(Akkaya *et al.,* 1992; Faqir *et al.,* 2017)
Cotton	AFLP, ISSR, SNPs	(Murtaza, 2006; Li et al., 2016; Nadeem *et al.,* 2018)
Sunflower	SSR	(Pashley *et al.,* 2006)
Peanuts	RFLP, DArT	(Olukolu *et al.,* 2012; Qin *et al.,* 2012)
Vegetables		
Tomato	RFLPs, ISSR	(Miller and Tanksley, 1990; Liu *et al.,* 2007)
Ginger	RAPD, SNPs	(Salem *et al.,* 2007; Ismail *et al.,* 2016)
Eggplant, Mint, Cinnamon	RAPD	(Selvakumari *et al.,* 2017)
Potato	RAPD, SSR, RFLP, SNP, and GBS	(McGregor *et al.,* 2000; Simko, 2004)
Pulses		
Chickpea	ISSR, SNPs	(Gautam *et al.,* 2016; Deokar *et al.,* 2019)
Lentil	RFLP, ISSR	(Havey and Muehlbauer, 1989; Yigezu *et al.,* 2019)
Pea	Morphological markers, DArT	(Yang *et al.,* 2011)
Fruits		
Mango	AFLP, ISSR, SNPs	(Yamanaka *et al.,* 2006), (Ariffin *et al.,* 2015), (Sherman *et al.,* 2015)
Datepalm	SNPs	(Faqir *et al.,* 2017)
Sugarbeet	SNPs	(Schneider *et al.,* 2007)
Pineapple	DArT	(Kilian *et al.,* 2014)

some limitations i.e. non-specific primers of random sequences are used which may cause an imperfect hybridization between target DNA and primer. It is impossible to distinguish whether the amplified fragment is from the heterozygous or homozygous locus as nearly all RAPDs markers are dominant. RAPD are sensitive to slight changes in reaction, therefore; strictly controlled and optimized working conditions are required (Weising *et al.,* 2005). The usefulness of RAPDs as systematic characters is limited because of difficulties in assessing character homologies and poor reproducibility (Bagley *et al.,* 2001; Salem *et al.,* 2007; Sherman *et al.,* 2015).

Amplified fragment length polymorphisms (AFLP): AFLP marker system combines RFLP with PCR resulting in a higher reproducible banding pattern. These detect restriction fragments in the genome; in that respect, these are similar to RFLP. However, PCR amplification is used instead of using southern hybridization for the detection of genomic restriction fragments and represents only the presence and absence of restriction fragments instead of length differences. The dominant nature of AFLP facilitates simultaneous analysis of two genetic loci per experiment (Meena *et al.,* 2017). AFLPs were used in mango, sorghum, wheat and sweet potato as an effective tool to observe genetic diversity, tagging of important agronomic traits, fibre-quality traits and fingerprinting studies in cotton, sweet-potato, Bt rice and soybean (Zargar *et al.,* 2017).

AFLPs are bi-allelic dominant markers and are not able to distinguish between homozygous and heterozygous individuals. These need a high quantity and quality of

DNA. Further, there is a possibility of non-homology of migrating fragments of different loci. AFLPs bands are not always independent (e.g. in case of insertion between two restriction sites the amplified DNA fragment results in increased band size. This will be interpreted as the loss of a small band and at the same time as the gain of a larger band. Hence, this technology also has some limitations in the DNA fingerprinting of crops (Al-Samarai and Al-Kazaz, 2015).

Simple sequence repeat markers (SSRs): SSRs are tandem repeats of short nucleotide sequences (1-6 bp) distributed all over the genome (Kelkar *et al.,* 2010). These are frequently used marker for population genetic, functional genomics, association mapping, DNA fingerprinting, diversity analysis, comparative mapping and gene tagging studies because of excellent reproducibility, higher polymorphism level, and high mutation rates. SSR markers are co-dominant thus; provide information either the targeted locus is heterozygous or homozygous. Being ubiquitous in all eukaryotic genomes, they are used universally in population genetic analysis (Guichoux *et al.,* 2011). SSR based DNA fingerprinting technique is easy to proceed involving a pair of flanking markers/primers, PCR products are separated by Gel electrophoresis and banding patterns are monitored by radiography, fluorography or staining with silver nitrate or ethidium bromide (Nybom *et al.,* 2014).

SSRs are used for fingerprinting of many crops i.e. *Helianthus*, barley, soybean, wheat, date palm, rice and maize (Wang *et al.,* 2011a; Jamil *et al.,* 2020a; Jamil *et al.,* 2020b). Main issues concerning SSR based fingerprinting approaches is the generation of SSR markers from genomic DNA because much effort is required to isolate nuclear microsatellites from plants so expressed sequence tags; EST-Database of different species has been used to develop SSR markers known as EST-SSR (Squirrell *et al.,* 2003; Nybom *et al.,* 2014). EST databases are inexpensive sources and rapid approach to develop SSR markers (Gupta *et al.,* 2003). Other inherent issues include redundancy and null alleles that result in no bands (Varshney *et al.,* 2005). Moreover, database entries for the development of flanking primers are still limited. In many cases, molecular cloning is required to retrieve the sequence data for the development of flanking markers (Weising *et al.,* 2005).

Inter simple sequence repeats (ISSRs): ISSRs are PCR-based multi-locus molecular markers which are widely used in DNA fingerprinting since 1994. This technique uses specific 16-20 base pair long microsatellite sequences as primers in polymerase chain reaction (PCR) to produce multi locus markers by amplifying inter-specific SSR sequences of different sizes. ISSR is an efficient and simple method which combines several advantages of AFLPs and SSRs to universality of RAPD. Further, Sequence of ISSR primers are generally larger than RAPD primers which allow higher annealing temperature, as a result more reproducible bands are formed than RAPD (Meena *et al.,* 2017). This technique has a great advantage because no prior information of genome is required for the use of ISSR markers.

ISSRs have been widely used as an efficient tool for assessment of genetic diversity, linkage studies and QTL mapping in many crops i.e. coconut, lentil, maize, chickpea, wheat, tomato, wild olives, *Bt* cotton and rice. There are some downsides of ISSR markers as well i.e. these have low reproducibility as compared to other markers and are generally not transferable and are dominant. But these markers take advantage of high polymorphism and therefore, mostly used in genome mapping, gene tagging, phylogeny, linkage studies, genetic diversity and evolutionary biology studies (Reddy *et al.,* 2002).

Single nucleotide polymorphism (SNPs)*:* SNPs are an efficient and popular DNA fingerprinting approach which was firstly proposed by Lander in 1996 (Al-Samarai and Al-Kazaz, 2015). These describe a sequence polymorphism in the genome which occurs as a result of change or mutation in a single nucleotide (A, T, C, or G) at the specific locus and include single base transversion, transitions, deletions, and insertions. Transitions are most common than other types of SNP mutations. SNPs are genetically stable, abundant and genotyping chips are amenable to automation allowing high throughput analysis. However, SNPs can also be mined from the genome database just like SSRs. Bi-allelic nature makes SNPs a strong tool for data management by creating a large database of marker information because there are two alleles per locus. Recently, the SNP concept has arisen for the need for high-density genetic markers for multi-factorial disease studies and QTL based mapping (Zhang *et al.,* 2020). The principle of SNPs is based on the hybridization of DNA fragments with SNP chips (DNA probe arrays of high density) and then, SNP allele is named according to results of hybridization (Yang *et al.,* 2013).

SNPs markers are widely used as an important tool for linkage mapping, QTL analysis, DNA fingerprinting and genetic diversity in sugar beet, grapevines, chickpea, mango, olive, cotton, brassica, datepalm, common bean and soybean (Faqir *et al.,* 2017; Raatz *et al.,* 2019; Fu *et al.,* 2020) due to high levels of polymorphism. Further SNPs are used as a powerful tool for species identification and fingerprinting in different cereals (e.g. durum and bread wheat, oats, rice, maize and barley. There are some limitations to the SNPs marker system as well. As compared to microsatellites, SNP lack of information per locus and are less useful on per locus basis (Wang *et al.,* 2017). Further, SNP marker development needs complete knowledge of organism genome.

Diversity array technology (DArT): DArT is one of the recently developed multi-locus molecular marker technique that generates genome-wide fingerprints (Jaccoud *et al.,* 2001). DArT is a hybridization-based microarray technique that is capable of measuring thousands of DNA fragments to hybridization arrays with high levels of multiplexing (Wenzl *et al.,* 2008). This is a non-exclusive technology that has great potential for mapping studies and genetic diversity in many ‘’orphan’’ crops of Third World countries. Further, this technique has the advantage of a high level of polymorphism and no prior information of whole-genome is required (Ribaut and Ragot, 2019).

Detailed methodology of DArT technology is as follows: Specific restriction enzymes are used to digest genomic DNA followed by restriction fragments ligation to adapters. PCR is used to reduce genomic complexity by using specific primers with a complementary sequence to selective overhangs and adapters. Specific fragments are cloned from representation sites and then vector-specific primers are used to clone and purified fragments are arrayed on the solid support (microarray) that results in a discovery array. Labeled genomic representations, which are prepared from an individual genome, are hybridized to discovery array. DArT markers are capable of showing variable intensities of hybridization signals for different individuals and these polymorphic clones are assembled in genotyping array for routine genotyping of species (Kumar *et al.,* 2020).

DArT was mostly used for genetic linkage map construction, QTL analysis and genetic diversity in many crops i.e. rice, cassava, orphan crops, pigeon pea, chickpea, groundnut, olive, wild potato, wheat, cotton and barely. DArT markers are less commonly used due to requirement of advanced software (DArTdb, DArTsoft) for analysis, heavy investment, up-to-date laboratory facilities, and skilled manpower. Further DArT markers are dominant and are of variable intensity, which limit their use in some genomic applications (Kesawat and Kumar, 2009).

Genotyping by sequencing (GBS): With the advances in next-generation sequencing (NGS) a new approach GBS, was introduced to exploit genome-wide genetic diversity in different plant species (Chung *et al.,* 2017). GBS offers robust genome-wide scanning and multiplexed sequencing platforms using NGS technologies. GBS is based on genome reduction with restriction enzymes (Peterson *et al.,* 2014). The main steps in GBS protocol are sample preparation, selection of restriction endonuclease and adapters, NGS library assembly, sequencing, SNP calling (polymorphism detection in comparison to reference sequence) (Nielsen *et al.,* 2011) and diversity analysis. Sample preparation involves DNA extraction, quantification, restriction digest, and ligation with suitable adapters and PCR amplification with adapter-specific-primers. The library is assembled by pooling samples and size selection followed by adjustment of concentration and preparation of sample sheet. Different bioinformatics tools are used for the next two steps, sequencing and SNP calling e.g. Fastx-collapse, Minia, Bowtie 2, SAM tools, BCF (Elshire *et al.,* 2011; Peterson *et al.,* 2014). GBS combines marker discovery and genotyping thus resulting in rapid, robust, high-throughput, and cost-effective, making it the most favorable technique for crop fingerprinting. It provides direct genotyping of plants by bypassing the time-consuming processes of genome sequencing and markers discovery (Fu *et al.,* 2014). GBS requires a small amount of DNA sample (100-200 ng). It can provide high SNP coverage in gene-rich regions of the genome in a highly cost-effective manner. Fractionated genomic DNA via restriction digestion reduces representation through size selection or the specific combinations of restriction enzymes for further targeting specific genomic regions of interest (Chung *et al.,* 2017).

The flexibility and low cost of GBS make this an excellent tool for many applications and to address research questions in plant genetics and breeding (Zhu *et al.,* 2019). Crop fingerprinting with GBS is not very common yet, but with the key improvements to increase the potential and reduce uncertainties. GBS can be the most attractive technique for crop fingerprinting as it is a simple, inexpensive, specific, highly reproducible, and rapid approach due to the simultaneous detection of SNPs. Use of GBS is highlighted in several studies including the patterning of genetic structure and domestication of artichoke and cardoon (Pavan *et al.,* 2018), Genomic Selection in wheat breeding (Gutierrez-Gonzalez *et al.,* 2019), to assess the diversity in maize and barley (Guo *et al.,* 2020). GBS was not applied widely for genotypic analysis of plants as its application is still facing many challenges. The large amounts of missing data due to use of low coverage sequencing techniques (Fµ and Peterson, 2012) and uneven genome coverage because of restriction endonuclease and fragment size selection (Beissinger *et al.,* 2013). A great variety of potential exists for use of GBS for crop fingerprinting and genomics studies which needs to be explored.

Other systems: Besides some above mentioned PCR-based markers that have been used frequently in crop fingerprinting, some other markers are useful enough but are not being used widely such as Sequence Characterized Amplified Regions (SCAR), PCR based co-dominant marker, represents single locus and identify by amplification of genomic DNA (Paran and Michelmore, 1993). Twenty-two olive cultivars were identified using ten SCAR markers (Bautista *et al.,* 2003). Fingerprinting of Turkish cultivars of sweet cherry was conducted by using SCAR markers (Turkec *et al.,* 2006). Cleaved Amplified Polymorphic Sequences (CAPS) are also PCR based co-dominant markers generated by the digestion of amplicons with a panel of restriction endonucleases. CAPS have been used for mapping of genes in Arabidopsis (Konieczny and Ausubel, 1993). Similarly Expressed Sequence Tags (ESTs) are PCR based markers system which are co-dominant in nature and help in identification of heterozygote as well. ESTs are useful in identification of gene transcript and instrumental in gene identification and gene sequence identification (Nadeem *et al.,* 2018).

Target Region Amplified Polymorphism (TRAP), a PCR-based marker, uses two primers, one from target EST and the other one is the arbitrary primer. TRAP is known for the efficient and rapid genotyping of crop germplasm (Hu and Vick, 2003). Sequence Related Amplified Polymorphism (SRAP) uses two primers for the amplification of genomic DNA. Both the primers are labelled with [γ33P]-ATP (Li and Quiros, 2001). A comparison of different markers systems is summarized in Table 2 for a better understanding of their effectivity for DNA fingerprinting.

Applications of DNA fingerprinting

DNA fingerprinting has a wide variety of applications in plants sciences i.e. genotyping, genetic diversity assessment and food crop protection as discussed below.

Genotyping: DNA fingerprints developed through PCR or non-PCR based markers are used to identify individual cultivars. These are more reliable than traditional morphological and molecular characterization (Iqbal *et al.,* 2019). DNA fingerprinting is necessary for protection of varieties under Plant Breeders Rights Rules. It is a powerful tool for the identification of closely related species & varieties and to estimate genetic relatedness and genetic diversity assessment (Selvakumari *et al.,* 2017; Jamil *et al.,* 2020a). DNA fingerprinting helps in varietal purity assessment hence will help in controlling the marketing of the false and impure seed. Another useful application of DNA fingerprinting is for hybridity testing of the plants. Co-dominant nature of the SSR markers helps its use in hybridity testing and hence will be useful in regulating the marketing of the hybrid seeds. DNA markers also possess a reliable source for confirmation of pedigree parentage of the new crop varieties and will in their registration under Plant Breeders Rights Rules for protection of plant breeders rights (Jamil *et al.,* 2020b).

Plants that are vegetatively propagated share the same fingerprints, regardless of rare mutation rate because their mode of reproduction is asexual while sexually propagated cultivars exhibit non-uniform patterns of fingerprints. For genotyping of asexually propagated cultivars, SSR markers are preferred because they are highly reproducible. Reliability and accuracy of SSR markers is around 99.8% but some loci were observed sensitive to chimera mutations (Ahmad *et al.,* 2019; Ismail *et al.,* 2019; Jamil *et al.,* 2020b; Nasim *et al.,* 2020).

Recently great attention is being paid on the use of SNPs markers for the identification of vegetatively propagated species. SNP chips are used for high throughput genotyping and then information is used for the detection of several QTLs in the genome (Fujii *et al.,* 2013). Several high and low throughput SNP marker arrays have been developed in many crops. Genotyping of seed propagated plants using marker aided selection is difficult due to the high level of outcrossing especially during the seed cycle when foreign pollen fertilizes the egg and creates new genetic variations. A very advance and efficient technology are required therefor; DArT technology is used for discrimination among seed propagated cultivars (Kopecký *et al.,* 2011).

AFLP and SSR are reliable for the identification of inbreeding cultivars. Spontaneous somatic mutations or “ports’’ which deviate minutely from original cultivar but govern economically important traits like leaf and flower and fruit color are difficult to detect by DNA fingerprints since markers cover only small portion within the genome (Franks *et al.,* 2002). It was observed that plants regenerated from different layers i.e. L_1 and L_2 have different SSR loci with variable phenotypic expressions. SSR analysis is used for genotyping of somatic mutations and to identify chimeras clones (Meng *et al.,* 2018) whereas AFLPs are used for fingerprinting of in-vitro propagated crops because these mutations are heritable, very desirable and can have very long regeneration cycles (Kumar *et al.,* 2019).

Table 2. Comparison of widely used markers systems for DNA fingerprinting.

Features	Isozymes	RFLPs	RAPDs	AFLPs	SSRs	SNPs	ISSRs
Genomic abundance	Low	High	Very high	Very high	Medium	Very high	Medium
Type of polymorphism	Amino acid changes	*Single base change	*Single base change	*Single base change	Repeat length variations	*Single base changes	*Single base change
Level of polymorphism	Low	Medium	medium	medium	High	Very high	High
Amount of DNA required	-	High	Low	Medium	Low	Low	Low
DNA quality	-	High	Moderate to high	High	High	High	Moderate to high
Types of primers or probes	Specific Enzymes	Low cDNA clones or low copy region	Usually 10bp random nucleotide sequence	Specific sequences	Specific repeats of DNA sequences	Single nucleotide changes; insertions or deletions	Specific repeat sequences
PCR-based	No	No	Yes	Yes	Yes	Yes	Yes
Inheritance	Co-dominant	Co-dominant	Dominant	Dominant	Co-dominant	Co-dominant	Dominant
Genome coverage	Medium	low copy of coding regions	Whole genome	Whole genome	Whole genome	whole genome	Whole-genome
Detection of alleles	———	Yes	No	No	Yes	Yes	No
Reproducibility	Medium	High	Unreliable	Medium	High	Very high	Medium to high
Ease of use	Initially difficult	Intense labor	Easy	Initially difficult than easy	Easy	Easy	Easy
Amenable to automation	Low	Low	Moderate	Moderate	High	High	High
Radioactive detection	No	Yes	No	Yes/No	Yes		No
Developmental costs	Medium	High	Medium	Low	High in start	High	Medium

*insertion, deletions, and inversion

Genetic diversity assessment: Another fruitful use of DNA fingerprinting is to study genetic relatedness among genotypes/species. Parentage analysis is mostly done for this purpose which is an efficient way to find gene flow. SSR markers are mostly used but it is also found that other multi-locus markers can also be used with great confidence such as AFLPs when dominant alleles are present in between frequency of 0.1 to 0.4 (Barluenga *et al.,* 2011). Genetic relatedness also provides valuable information about the domestication process as data is taken from different ecological zones (Raj *et al.,* 2019). Since accurate measurement of genetic relatedness is not possible by nuclear DNA generated markers as they are only capable of generating phenetic analysis rather than phylogenetic analysis while SSR and AFLPs give satisfying results. HiDRAS project showed the best approach of utilizing phylogenetic analysis among genetically similar apple cultivars in which specific chromosomal regions were studied using a large set of SSR markers which cover the whole genome of cultivars. In this way, genetic relatedness was estimated among apple cultivars (Heo *et al.,* 2019). DNA fingerprinting and genetic profiling of the breeding material helps plant breeders in assigning of inbred lines/ purelines to various heterotic groups and help in deciding of the crossing plan to exploit maximum heterosis. These studies are helpful in breeding of cross pollinated crop for identification of diverse parents for exploitation of maximum heterosis (Silva *et al.,* 2020).

Food/crop protection: Modern fingerprinting techniques have applications in varietal protection and germplasm characterization. The International Association of Breeders has conducted model research in maize and tomato to highlight the capability of fingerprinting techniques to resolve variety protection issues. International Union for the protection of new varieties of plants (UPOV) is deliberately working on the development and use of fingerprinting techniques in DUS testing (Archak, 2000; He *et al.,* 2020). Crop Fingerprinting can be utilized in forensic botany. SSR and RAPD techniques have been used to analyse DNA fragments recovered from suspects' belongings and crime sites to resolve disputes (Virtanen *et al.,* 2007). Fingerprinting is considered a matchless tool to detect adulteration in plant-based food items and drugs. For example, mixing of expensive basmati cultivars with non-basmati cultivars is an important issue. To resolve this issue, several markers have been proposed for adulteration tests (Archak *et al.,* 2007; Ganopoulos *et al.,* 2011). DNA analysis of different food items, juices, and drugs has been conducted to identify plant variety from which it was originated (Nybom *et al.,* 2014).

The molecular fingerprinting of a plant variety is of the utmost importance for protecting plant breeders' rights (Kwon *et al.,* 2005; Jamil *et al.,* 2020b). Since the violation of Plant Breeder's Rights is a major issue in developing countries like Pakistan, a variety of reliable DNA markers can be used to protect Plant Breeder's Rights (Kumar *et al.,* 2001; Jamali *et al.,* 2019). Crop fingerprinting is gaining importance as a tool in plant breeding. The efficiency of a breeding program is dependent on the diversity of parental lines. With the aid of finger-printing techniques, diversity can be easily detected (Ijaz, 2011). Modern fingerprinting techniques give a genetic description of analyzed plant variety that is key for a plant breeder. Microsatellite markers have been used for genome mapping of different plant varieties, microsatellites linkage maps are available for wheat, maize, soybean, rice, avocado, lettuce, chickpea for genome mapping. Moreover, fingerprinting techniques are used for marker-assisted selection which is helpful for plant breeder as it reduces the number of generations required for screening of different traits (Chukwu *et al.,* 2019).

Agricultural Biotechnology Research Institute, AARI Faisalabad (ABRI) has launched a DNA fingerprinting project funded by Punjab Agricultural Research Board. Under this project ABRI has developed the DNA fingerprinting of seven crop varieties i.e. olive, maize, cotton, date palm, wheat, tomato and potato cultivated in Pakistan using SSR markers. The ABRI is helping the local breeders for protection of PBRs rights under this facility (Iqbal *et al.,* 2019; Jamil *et al.,* 2020a; Jamil *et al.,* 2020b).

CONCLUSION AND FUTURE PROSPECTS

Dating back in 19th-century varieties were distinguished based on morphological characters. However, over time advancement in technology brought us to DNA based markers. Now in the 21th century DNA fingerprinting move once step ahead with the advent of next-generation sequencing technologies and genotyping is done by sequencing. SSR markers are more frequently used markers because of excellent reproducibility, higher polymorphism level, and high mutation rates.

Advancements in genomics and sequencing alongside the development of advanced bioinformatics tools are providing fast means of identification of novel genomic regions for DNA fingerprinting of crops. The focus should be shifted towards improvements and innovations of sequencing approaches and to develop cost-effective genotyping-by-sequencing methodologies. Despite all these developments choice of markers systems for population genetics, genetic mapping, genetic relatedness, and plant genotype identification studies will depend on the resources, time, and capital of the organization concerned.

DNA fingerprinting has a lot to offer in the future particular variety protection under Plant Breeders Rights Rules, disputes resolution; forensic activities related to plant sciences, and will help broaden the genetic base of approved varieties of different crops (Wang *et al.,* 2019). A global DNA fingerprinting database of all crops should be established in the future and DNA fingerprints of all-important crop varieties should be deposited there. It will also help in dispute resolution of different trademarks i.e. Basmati Rice is claimed both Pakistan and India as their trademark (Chandola, 2006). The development of a global database of crop varieties will prevent such issues in the future.

For low income countries that lack funding for development of high throughput systems, SSR markers are still a reliable system for DNA fingerprinting of crop varieties (Jamil *et al.,* 2020a). It is proposed that all nations should develop Plant Breeders Rights and should protect them through DNA fingerprinting of new plant varieties which evolve in their jurisdictions. However there is a need to develop cost effective high throughput DNA fingerprinting systems for efficient DNA fingerprinting and variety protection in middle and low income countries (Rohland and Reich, 2012).

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