DNA-based marker systems and their utility in entomology

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Morphological differences and similarities have been used to group and classify organisms, such as insects, into major taxonomic groups. However, discerning finer differences among strains, races and biotypes is usually difficult due to the influence of environment. Protein-based marking of individuals was used extensively before DNA-based markers were employed. Variation at the DNA level is remarkable, and the unit change is heritable in a simple manner. At present, many DNA-based marker systems are available to address specific questions, both in basic and applied entomological research, that can circumvent the limitations of conventional approaches to a large extent. DNA-based markers, being neutral to environmental influence and abundant, have helped understand genetics of complex traits in animal and plant systems. The present review primarily aims at familiarizing the DNA-based marker systems along with their utility. The techniques described include restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites/simple sequence repeats (SSRs), expressed sequence tag (EST) based marker system, single nucleotide polymorphisms (SNPs) and other derived marker systems along with their genetic nature and relative comparison.

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1. Introduction

In recent years, DNA-based marker systems have been increasingly employed in diverse areas of biology including phylogenetic studies, evolution, ecology, population genetics, population dynamics and genetics of complex traits in both plant and animal systems. This has been possible because of the rapid advances in molecular biological methods and bench-level protocols for wider application (Morin *et al*. 2004). The utility of molecular markers as additional tools in basic and applied entomology has led to 'molecular entomology', also referred to as 'precision entomol-

ogy' (Ananthakrishnan 2005). Over a long time, significant contributions have been made in the field of insect systematics through morphometric traits, wherein a number of difficulties were encountered due to genotype-environment interactions (Cruickshank 2002). The limitations in using morphological, physiological and cytological markers for assessing genetic diversity and population dynamics have been largely circumvented by the developments in DNA-based markers (Cruickshank 2002). Molecular markers by nature are neutral to the stage of development, physiological status and environmental influences (Black *et al*. 2001, Heckel 2003). Isozymes and other proteins as markers are often expressed codominantly and discriminate homozygous and heterozygous individuals. However, the limited number of proteins and isozymes as markers and requirement of different protocols for each enzyme/protein limit their utility. Unlike morphological and protein-based markers, several DNA-based markers are available to elicit the differences between individuals and populations, or they can be developed for each specific purpose. Although a large number of samples can be analyzed quickly, a number of other factors such as cost, speed and requirement of technical skills are the major concern. DNA-based markers can generate large amount of high quality data compared to several biochemical marker systems, but degree of polymorphism detected and the statistical dependability of the results vary among marker systems (Weising *et al*. 1995).

All the information encoded by the DNA present in the nucleus and mitochondria in an organism is called the genome, which varies from species to species by size and sequence of nucleotides. Studies involving structure, function and evolution of whole genomes is referred to as genomics; for further details, see Heckel (2003). Variation among individuals of a species arises by mutation, segregation and recombination of genes, and rearrangements in the genome, and it is the basis of all morphological changes, such as adaptive variations. Although not all changes in nucleotide sequence lead to morphological changes, there is plenty of DNA level variation available for marking the genome. The term genomic DNA refers to the DNA from a biological sample that is used for routine molecular analysis. Any region of DNA that is recognized is known as a locus or marker; a locus may have alternate forms, contributed by male and female parents. A locus may vary between individuals (in population) in its length and/or sequence of nucleotides. This heterogeneity is detected using different molecular biological techniques and using different markers. The term gene refers to a region of DNA that codes for RNA (mRNA, tRNA or rRNA), regardless of whether the RNA is ultimately modified post-transcriptionally and translated into an enzyme and structural protein or has other functions within the cell as transfer RNA or ribosomal RNA; locus, on the other hand, refers to the physical location of a particular DNA sequence (it can be as small as a base or a stretch of bases) in a genome, whether it codes for RNA or not. Polymorphism is a general term used to describe the difference between individuals either at morphological or at molecular level.

Genetic polymorphism is classically defined as the simultaneous occurrence of a trait in two or more discontinuous variants or genotypes of the same population. Earlier, a number of studies have shown specific amino acid substitutions in enzymes resulting in a new allele (reviewed in Richardson *et al*. 1986). With the help of novel tools and methods, it is now possible to pinpoint such variations at DNA level much more precisely. Single base substitutions, deletions, additions, frame-shifts etc., are detectable both at individual and population level by suitable molecular methods and techniques. Although DNA sequencing is a straightforward approach for identifying variations at a locus, it is expensive and laborious. It is also difficult to setup such facilities due to the requirement of high-end instrumentation and high cost involved. A wide variety of techniques have been developed in the past few decades for visualization of DNA sequence polymorphism (Sambrook *et al*. 1989, Doyle 1996), which are useful in entomological research as well. A number of review papers giving details on elaborate technical aspects and their relative cost analysis are available. The present review is primarily aimed at familiarizing the general entomologists with DNA-based marking methods and techniques that might be useful in specific investigations. A brief account of DNA based marker systems, their utility in entomological re-

search, with examples wherever available is presented to prompt further reading and application. The techniques described include restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites/simple sequence repeats (SSRs), expressed sequence tag (EST) based marker system, single nucleotide polymorphisms (SNPs) and other derived marker systems along with their genetic nature and relative comparison.

2. Why DNA markers?

The greater utility of molecular markers lies in following the inherent properties that distinguish them from morphological markers (Powell *et al*. 1994, Morin *et al*. 2004, Black *et al*. 2001):

- Number of alleles at molecular loci is very high compared to morphological features.
- The phenotypes of morphological and most of the protein-defined markers are specific to instar or stage of development. Being genomic features, molecular markers are independent of life processes and even stored DNA samples can be used for marker assay.
- Unlike molecular markers, most phenotypic features of insects are epistatic in nature — interdependent and influenced by other characters and the environment. It is possible to generate and monitor a large number of polymorphic loci, as effects such as epistasis and pleiotropy are seldom observed.
- Morphological markers available for study in a set of individuals are usually few and limited. DNA markers are many, with a search option, to discover and develop markers for a specific purpose.
- PCR methods have low ascertainment and per genotype cost.
- Except for RFLP and AFLP, most of the marker systems have rapid ascertainment and assay development methods.
- Alleles at morphological loci inherit in a dominant-recessive manner that limit the identification of heterozygotes, but molecular markers (except RAPDs) are co-dominant permitting genotypic identification of individuals in any segregating population.

Application of molecular techniques in entomology range from insect diagnostics, variability, identification of pathogens carried by them, systematics and population dynamics. Issues such as species dispute, origin of metapopulations, pestiferous locusts, dynamics of resistance to insecticides, etc. can be studied using these tools. Application of molecular markers for genetic studies of insects has been highly diverse.

3. Choice of molecular markers

Recent developments in molecular biology have resulted in several tools and techniques to analyze genomic variation at both individual and population level (Black *et al*. 2001). In areas such as molecular ecology, molecular entomology, molecular systematics, population dynamics and diagnostics, DNA-marker systems such as RFLP, RAPD, AFLP, SSRs, SNPs, etc. have profound uses (reviewed in Morin *et al*. 2004). Molecular markers differ with respect to several key features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, nature of inheritance and technical requirements apart from financial concern. The choice of a molecular marker system depends on a number of factors including the availability of markers, level of polymorphism desired, genomic information available in a given species, technical skills, availability of protocols, problem to be solved, etc., and thus it is often difficult to categorically state the superiority of a specific marker system over another. For example, where simple differentiation between two individuals of a species or two geographical populations of the same species is necessary, neutral markers such as RFLP, RAPD, SSRs or AFLP are the markers of choice. However, if one wants to discriminate environmentally influenced characters, non-neutral marker systems like isozymes or complex micro-arrays would be a better choice (Morin *et al*. 2004). For population dynamics, insect systematics, fingerprinting of individuals and diagnosis of resistance to xenobiotics, the neutral marker systems are appropriate. Therefore, choice of a marker system for a given application and the problem to be addressed is a key decision. RFLP, AFLP and micro-array for instance are not only

Table1. Comparison of DNA-based marker systems.

Feature	RFLPs	RAPDs	SSRs	AFLPs	SNPs
Technical basis	Sequence specific endonuclease restriction, southern blot and hybridization	DNA amplification with random decamer primers	DNA amplification of simple sequence repeats of different length using specific primers	Endonuclease restriction use of adopters and selective primers	Sequence comparison and analysis
Type of polymorphism	Single base changes; insertions, deletions	Single base changes: insertions, deletions	Changes in repeat length	Single base changes, deletions	Single base changes
Loci analyzed per primer/attempt	1–4	1–50	1–4	20–100 or more	1
Detection	Radio labeled DNA probe	Ethidium bromide staining	Ethidium bromide or silver staining and rarely labeled DNA probe	With or without radio labeled DNA probe	Sequencing DNA fragments
Inheritance	Co-dominant	Dominant	Co-dominant	Dominant	Dominant
Ease of use	Cumbersome	Easy	Easy	Not cumbersome	Easy
Need for sequence information	No	No	Yes	No	Yes
Reproducibility	High	Unreliable	High	High	High
Development cost	Very high	Low	High	High	High
Cost per analysis	High	Low	Low	Moderate	Low
Amenable to automation	Low	Moderate	High	Moderate	High
PCR-based	No	Yes	Yes	Yes	Yes
DNA required	10 µg	0.02 µg	0.05 µg	0.5–1.00 µg	0.05 µg
DNA quality required	High	Low	Moderate	High	High

expensive but also demand greater technical skill. Brief information on each molecular marker is provided in Table 1.

3.1. Restriction Fragment Length Polymorphisms (RFLPs)

RFLP is a class of polymorphism that arises due to differences in nucleotide base sequence at positions called 'recognition sites' of a specific 'restriction endonuclease' (RE) on the DNA, resulting in varied size of DNA fragments. These are inherited like any other Mendelian trait. Alternative RFLP phenotypes at a given locus are determined as length polymorphisms, following electrophoresis of genomic DNA digested with one or more REs. In DNA strands the changes in base sequence at RE sites are brought about by DNA rearrangements that occur due to the evolutionary processes, point mutations within the restriction site sequences, insertions or deletions within the fragments and unequal crossing over. DNA can be cut using one or more REs that recognize corresponding sites on the DNA. A number of restriction enzymes that recognize and restrict four and six base pair sequences are available commercially. REs recognizing much longer sequences (such as 16 base pairs, bp) and restricting in more complex but specific patterns are also available.

The restricted DNA fragments can be visualized under UV-light using ethidium bromide stained agarose or polyacrylamide gels. Based on the size of the fragments generated, the type and concentration of the gel is selected. In eukaryotes

where size of the genome is generally big and several fragments of different sizes are generated, specific DNA fragments incorporated with radioactive bases (called probes) are used to detect RFLP alleles on the gel blot (Fractionated DNA on gels is transferred to a DNA binding membrane) by DNA–DNA hybridization (Bruford *et al*. 1991, Frischauf 1991, Avise 1994). The probes themselves are developed separately. Hence, the hybridizing probes and REs used specify the RFLP. Generally, the probes are generated by cloning the fragments of the target genomes into a suitable vector or could also be adopted from other systems-called heterologous probes.

The ability of RFLP probe to detect analogous DNA on both members of a pair of homologous chromosomes yield codominant, non-epistatic Mendelian markers that provide greater genetic resolution. RFLPs have been widely used in entomological research: genetic linkage maps in *Heliothis* (Lu *et al.* 1992, Heckel *et al.* 1998), apple maggot fly *Rhagoletis pomonella* (Walsh) (Roethele *et al.* 1997), *Bombyx mori* (Linnaeus) (Shi *et al.* 1995, Tan *et al.* 2001), diamond back moth *Plutella xylostella* (Linnaeus) (Heckel *et al.* 1999), Colorado beetle *Leptinotarsa decemlineata* (Say) (Hawthorne 2001), Colias butterflies (Wang & Porter 2004), population genetics studies (Lu *et al.* 1992, Haymer *et al.* 1992, Hall 1990), determination of male and female sexes in honey bee (Hall 1990), phylogenetic studies in mites and ticks (reviewed in Cruickshank 2002) and gene flow studies (reviewed in Black *et al.* 2001). However, when compared to the PCR based methods this technique demands high quality and large quantity of DNA (in μg), the involvement of radioactive material, toxic reagents and relatively high technical skills, which makes this technique less attractive over others.

3.2. Randomly Amplified Polymorphic DNAs (RAPDs)

This is a PCR based marker system where genomic segments are amplified using oligo-nucleotide primers. Generally, random decamer primers are used to prime the synthesis of DNA from homologous sites on the test DNA in PCR. These primers bind to homologous sequences along the genome and PCR amplification occurs only where identical primer sites occur within the range of amplification by *Taq* polymerase. DNA sequences up to 3,500–4,500 bp, where primer-binding sites define the ends, are amplified in an exponential fashion in about 30–40 cycles of DNA synthesis in a thermal cycler. Because there can be a number of sites homologous to the primer in the genome, several amplicons of varying length are produced. Length of primer used and size of the genome determine the number of amplicons produced. Amplified fragments are fractionated on agarose gels using ethidium-bromide staining and visualized under VU-light. Within a population sample, mutations influence the base sequence of primer binding sites on the DNA, allowing polymorphism to be detected (Williams *et al*. 1990). Owing to the shorter length of the primer and its binding kinetics to the target genomic DNA, the non-repeatability of the amplicon profiles has been the major concern in the use of this technique. However, consistent RAPD marker is as reliable as any other marker system. A RAPD band could also be eluted from the gel and purified for use as a probe or sequenced to develop more specific primers for sequence characterized amplified regions (SCARs) (Paran & Michelmore 1993, Garner & Slavicek 1996). SCARs are more definitive especially in a situation where the amplicon is used as proxy (= linked marker) for a definite trait such as insecticide resistance or any other phenotypic trait for which the obvious association has been established (Fakrudin & Patil 2005).

RAPD markers have been extensively used in entomological investigations which include molecular fingerprinting (Fakrudin & Patil 2005), phylogenetic analysis (Zhou *et al*. 2000, Fakrudin *et al*. 2004), genetic diversity studies of agricultural insect pests such as *Helicoverpa armigera* (Hübner) (Zhou *et al*. 2000, Fakrudin *et al*. 2004), taxonomy and population genetics of aphids, moths and parasitoid detection (Black *et al*. 1992, Puterka *et al*. 1993, Stevens & Wall 1995, Garner & Slavicek 1996, Vaughn & Antolin 1998), social behavior in honey bees (Hunt & Page 1992), tracing the phenotypic variation in aphids (Lushai *et al*. 1997), maternal contribution (Hooper & Siva-Jothy 1996) and genetic linkage

map construction in *Tribolium castaneum* (Herbst) (Beeman & Brown 1999), *Apis mellifera* (Linnaeus) (Hunt *et al.* 1995), bumble Bee *Bombus terrestris* (Linnaeus), and *Heliothis virescens* (Heckel *et al*.1998). RAPD marker based molecular maps have been used along with the other available maps in *Bombyx mori* (Hwang *et al*. 1998, Promboon *et al*.1995).

3.3. Microsatellite markers

Microsatellites, also known as short tandem repeats (STR) or simple sequence repeats (SSRs), are ubiquitous in eukaryotes. These are sequence units, generally of 2–7 bp tandem repeats up to certain length. These are scattered throughout the genome and the number and composition of microsatellite repeats differ in animals and plants (Jacobs *et al*. 1996). The frequency of repeats longer than 25 bp has been estimated to occur every 6 kb in mammals unlike plants where it is found to occur every 33 kb (Wang *et al*. 1994). The variation in SSR loci is due to differences in the number of repeats, which primarily arise as errors during DNA replication. Using specific forward and reverse primers, designed for sequences homologous to the flanking sequences of the repeat units, length variation in the repeat can be detected by PCR. Nucleotide changes may occur at a high rate in non-coding regions, compared to regions that are functionally active (Loxdale & Lushai 1998). These are useful to monitor gene flow, discrimination of parent-offspring, forensic and genome divergence studies, construction of physical maps and a number of other applications in entomology. They are highly reproducible and amenable for multiplexing; hence, high-throughput approach is possible. They are co-dominant (except null allele) and can detect variation within and between insect populations. Often the SSR amplicons are separated on polyacrylamide sequencing gels to detect polymorphism that could be due to a few base differences. Silver-staining procedure is generally adopted to detect them on acrylamide gels whenever ethidium bromide stained agarose gels do not resolve the amplicons. Whenever sequence information is available, radioactive probe based detection is also possible. SSR markers developed for one organism could also be used in other closely related species.

The primary application of SSRs has been in genetic diversity and genetic linkage map construction. SSR markers have been successfully used in paternity studies of Hymenoptera (Estoup *et al*. 1995) and genetic sexing of lepidopteron insects (Ananthakrishnan 2005). In aphids, hymenopteran insects, mosquitoes, moths and butterflies these markers have provided useful information on genetics of populations (Black *et al*. 2001). SSRs have also been used in social wasps (Strassmann *et al*. 1997, silk worm (Reddy *et al*. 1999), *Drosophila melanogaster* (Hackel 2003), Hymenoptera (Estoup *et al*. 1994), and moths and butterflies (Palo *et al*. 1995). Lehmann *et al*. (1997) used these markers in mosquitoes for genetic studies at population level. The greater advantage of this marker system is its repeatability, stability and provide time-scale dimension in ecological and evolutionary studies of insects. In addition to nuclear genome, they are also known to occur in organelle genome (Soranzo *et al.* 1999). However, the major disadvantages include the requirement to screen several loci for adequate information and availability of primers in the target species.

3.4. Amplified Fragment Length Polymorphism (AFLP)

AFLP combines the advantage of both PCR and RFLP techniques. AFLPs are fragments of DNA that have been amplified after restriction digestion of genomic DNA (Metthens *et al*. 1998). Genomic DNA is cut with selected restriction enzymes and then short oligomers called adapters are ligated to the ends of the DNA-fragments to facilitate PCR amplification using adapter and adjacent restriction site-specific primers, selective nucleotides are added to the 3' ends of the primers to enable only a subset of the restricted fragments to be amplified. Generally, combination of a rare cutter and a frequent cutter are used to generate fragments for subset amplification. As many as 50–150 restricted fragments are known to be co-amplified and detected in a typical AFLP reaction, facilitating detection of differences between very closely related individuals.

The advantage of this method lies in the visualization of polymorphism by PCR without the knowledge of nucleotide sequence and ability to co-amplify a high number of restriction fragments (Vos *et al*. 1995, Wang & Porter 2004). Rapid generation, high reproducibility and high frequency polymorphism makes DNA analysis using AFLP an attractive tool for identifying genetic polymorphism, constructing genetic linkage maps, and detecting linkage by analyzing individuals in an appropriate segregating population. AFLPs are especially suited for genetic studies in closely related taxa. Important requirements for the use of AFLP include hands-on proficiency and the choice of restriction enzyme (Ridount & Donini 1999) and primers (Mohan *et al*. 1997, Kardoless *et al*. 1998). In recent years, AFLP has been gaining importance in place of RAPD and RFLP owing to its repeatability, robustness and low laboratory errors (Jones *et al*. 1998). However, AFLPs are still expensive to generate and use routinely as the fragments (bands) are detected by silver staining, fluorescent dyes or radio-labelling. Genetic diversity (Blears *et al*. 1998), insect systematics in mosquitos (Vos *et al*. 1995), genetic linkage map construction in mosquito (Severson *et al*. 1993), *Rhagoletis pomonella* (Roethele *et al*. 1997), *Heliothis virescens* (Fabricius) and *Helicoverpa armigera* (Heckel *et al*. 1998), *Plutella xylostella* (Heckel *et al.* 1999), Colarado potato beetle (Hawthorne 2001) and economically important insects, such as the silk worm (Shi *et al*. 1995), are examples where AFLP has been used.

3.5. Single Nucleotide Polymorphisms (SNPs)

SNP is a nucleotide site in a DNA sequence where more than one nucleotide (G, A, T or C) occurs in the population. SNPs are regarded as ideal molecular markers for genetic linkage map construction and in detection of genetic disorders. Due to their propensity, they are highly suited for high-resolution genotyping. The microsatellite (and mitochondrial DNA) sequences have been the tools of choice in molecular ecology and evolution of insect species. These markers are informative for answering population level questions as they integrate time-scale component for studying evolution. Most of the marker systems including microsatellites suffer from non-detection of null alleles and mutation patterns, leading to ambiguity in interpretation (Morin *et al*. 2004). Most of the mutations that do not influence change in the marker pattern go undetected by the use of most of the DNA markers. The genetic changes observed as single nucleotide polymorphism are abundant and spread in the coding and non-coding regions of genomes of many species (Vignal *et al*. 2002). However, SNPs can also be sparse in some genomes as in mites (Navajas *et al.* 1998)

In organisms where multiple individuals have not been (completely) sequenced, SNP discovery has to be done through laboratory sequencing of regions of the genome of interest from several individuals of the population. It is possible by designing primers in the conserved regions of loci and PCR amplification from the relevant individuals. It has been estimated that for every 200–500 bp a SNP occurs (Brumfield *et al*. 2003). In situations where loci of interest cannot be sequenced in a particular target organism (species), a random sequencing approach or a method based in AFLP is adopted (Morin *et al*. 2004). Generating SNP data is costly and laborous, depending on the number of loci and approach adopted. However, SNP technology presents a unique opportunity to implement and standardize the method and bioinformatics (analysis) system that will facilitate the most economical and informative use of SNP markers in insect systematics, evolution, applied research and population dynamics.

SNPs have been used in mites for discriminating species and subspecies (Navajas *et al*. 1998), phylogeography (Brumfield *et al.* 2003), biodiversity assessment (Van Tienderen *et al*. 2002), linkage disequilibrium analysis (Akey *et al*. 2003), population genetic parameters (Kuhner *et al*. 2000) and in ecology, evolution and conservation biology (Morin *et al*. 2004).

3.6. Ribosomal DNA internal transcribed spacers (rDNA-ITS) and mitochondrial DNA (mt DNA) markers

This category of markers is especially useful in evolution and phylogenetic analysis, as the re-

gions involved are generally under little or no selection pressure and tend to respond (~ evolve) quickly. It includes introns of nuclear genes, internal transcribed spacer regions of nuclear ribosomal genes and mitochondrial control regions (Zhang & Hewitt 1997, Loxdale & Lushai 1998, Friesen 2000, Cruickshank 2002). These marker systems are good for differentiating individuals of the same species or very closely related ones. rDNA is a multi-gene family with nuclear DNA copies in higher eukaryotes. These are arranged in nucleolar organizer region in a tandem fashion, where each unit consists of genes coding for 18S (small) and 28S (large) rRNA subunits. ITS1 and ITS2 are spacer regions that separate 5.8S gene from 18S and 28S subunits. The size of these regions considerably varies across organisms (Zolodos *et al*. 1999). For a PCR based approach, primers that can amplify ITS regions have been designed that work well across taxa, and generally such primers are designed in more conserved regions. Moreover, ITS1 and ITS2 vary in their relative suitability, revealing desired information from species to species.

Mitochondrial DNA sequences have been widely used for studies on population and molecular systematics of insects. Maternal inheritance of mtDNA has been of particular use in closely-related species and race-level differentiation for the mitochondrial genes that code for both enzymes/protein and ribosomal RNA, and mutation rate in the nucleotide sequences has been reported to be 20 times faster than that of nuclear DNA, making them more useful in phylogenetic and evolutionary investigations (Simmon *et al*. 1994). Ribosomal genes, 16S rDNA and 12S rDNA and a number of other specific genes are generally used in combination for establishing phylogenetic relationships.

Both ITS and mitochondrial markers have been used in *Apis* species (Anderson & Truemann 2000, Toda *et al*. 2000), *Ixodes pecificus* (Cooley & Kohls) (Kain *et al*. 1999), *Dermacentor* species (Crosbie *et al*. 1998), mites (Navajas *et al*. 1992, Cruickshank & Thomas 1999), ticks (Rich *et al*. 1997), phytoseiids (Yli-Mattila *et al*. 2000), population biology (Simon *et al*. 1994), genetic variation (Chapco *et al*. 1994, Fakrudin & Patil 2005), fruit flies (Powers *et al*. 1989), leaf hoppers (Fang *et al*. 1993, Chapco *et al*. 1994) lepidopterans (Brown *et al.* 1996) and genetic linkage mapping in *Drosophila melanogaster* (Berger *et al*. 2001, Hoskins *et al*. 2001).

3.7. Other marker systems

3.7.1. Sequence Tagged Sites (STS)

STS is referred to any unique fragment of the genome amplified by the primers derived from any genomic sequence, including RAPD marker or RFLP probe. Hence, STS overcomes the problem of background bands, making desired amplicon clearer and specific to score. This technique also overcomes tedious hybridization procedures involved in RFLP analysis. Polymorphism is visualized as length variation if portion of the intervening regions vary due to insertion/deletion or as presence or absence of a band. Whenever polymorphism is not revealed, a frequent (4 bp) cutting restriction enzymes is used to detect differences. A number of RAPD markers used in mapping have been converted in to STS markers in *Athalia rosae* (Linnaeus) (Nishimori *et al*. 2000).

3.7.2. Allele-Specific PCR (AS-PCR)

AS-PCR is a PCR based technique involving short oligo-nucleotide primers, essentially to amplify DNA sequence variants (alleles) of a specific locus. This is achieved by designing primers at one or both ends of the sequence in such a way that they partially overlap the portion of sequence that differs between alleles. Generally, stringent annealing temperatures are employed to achieve specificity and polymorphism is visualized as differences in size of the amplicon. It is also possible to use restriction enzymes for further detection of polymorphism between alleles if not directly achieved with the use of designed primers. AP-PCR amplicons are generally fractionated on acrylamide gels and detected by silver staining.

3.7.3. Expressed Sequence Tag (EST) markers

ESTs are obtained by the partial sequencing of randomly chosen cDNA clones made from cytosolic mRNA. This marker system, like microarray, exclusively represents the coding part of

the genes. Thus, these are useful in understanding interactions of individuals or populations with the environment. As the ESTs are representatives of the mRNA, it is possible to specifically point out functional differences between alleles of a locus. However, in genetic diversity investigations EST markers are less useful as they seldom detect variation (Black *et al*. 2001). However, once generated, they are useful in cloning specific genes of interest and synteny mapping of functional genes in related organisms. ESTs are popular in full genome sequencing and genome mapping programs, as they serve as good initial information for full-length gene isolation through modified PCR approaches. These are of unique interest in identification of markers linked to insecticide resistance in agricultural pests and to clone genes involved in such a phenomenon. Application of these markers in entomology is for deeper understanding of insecticide resistance and integrating the results into insecticide resistance management (IRM) programs. In beneficial species such as *Bombyx mori*, pestiferous species such as *Heliothis* and aphids (in cotton), disease and virus carrying vectors such as mosquito and whiteflies, honey bees, spiders, cattle ticks, model insect organisms such as *Drosophila* and *Tribolium* a number of ESTs have been established and in some cases the whole genomes have been sequenced (Horn *et al*. 2003).

3.7.4. Single Strand Conformation Polymorphism (SSCP)

This technique requires DNA sequence information but is a powerful and rapid technique for gene analysis, particularly for detection of point mutations and of DNA polymorphism. However, it can only be used with relatively short DNA fragments. The secondary and tertiary structural differences in DNA fragments that arise for whatever reason are made of use using this technique in detecting difference. SSCP can identify heterozygosity of DNA fragments of the same molecular weight and can even detect changes of a few nucleotide bases as the mobility of the single-stranded DNA changes with its GC content due to conformational changes. SSCP alleles are generally fractionated on non-denaturing acrylamide gels, usually at low temperature conditions of electrophoresis. SSCP markers have been used in constructing genetic linkage maps in mosquito (*Aedis aegypti* and *Aedis albopicture* (Antolin *et al*. 1996a, Mutebi *et al*. 1997) and *Bracon hebetor* (Say) (Antolin *et al*. 1996b).

4. Applications of molecular markers

1. Assessment and monitoring of genetic diversity in insect species: genetic diversity estimation and monitoring serves several uses in both basic and applied entomology. In the context of widespread resistance to insecticides and biopesticides among economically important insect pests such as *Helicoverpa*, *Plutella* etc., understanding the genetic diversity of different populations is important in understanding the response variation among individuals.
2. Identification of markers linked to genes conferring resistance to insecticides: such markers will help in monitoring the frequency of resistance allele in different populations of target insect species. Such results are crucial for insecticide-resistance management (IRM) programs. A RAPD marker linked to cypermethrin resistance in Raichur strain of cotton bollworm *Helicoverpa armigera* in the south Indian cotton ecosystem was found useful in this endeavor (Fakrudin & Patil 2005).
3. Identification of species-specific molecular markers: such markers will help in accurately estimating relative abundance and dynamics of related species in space and time. Such information is useful in the management of pest insects such as *Helicoverpa*/*Heliothis* species.
4. Population DNA fingerprints. Development of population specific DNA fingerprints, specific to each geographical population helps in monitoring the migration of specimens from region to region. Such information vis-à-vis insecticides resistance inventory would be useful in developing insecticide-resistance management strategies. Recently, we have developed 12 SCAR markers specific to each geographical population of cotton bollworm of the south Indian cotton ecosystem (Fakrudin & Patil 2005).

5. Identification of differences among strains that are difficult to differentiate using morphological characters: even the cryptic differences among strains or races could be determined adopting suitable DNA marker systems.
6. Fingerprinting and diversity assessment of biocontrol agents, predators and parasites to keep track on variable insect populations.
7. Identification of genetic difference and fingerprinting of entomo-pathogenic viruses and other biocontrol agents: markers can be developed for their quality control.
8. DNA-based markers can be used in identification of insects at species, sub-species, biotype and population levels.
9. Survey of natural populations of insects: DNA based variability can be used to define population structure and as a measure of gene flow between populations. This approach has been considered as advantageous over traditional morphometric characters.
10. Use of molecular marker data can enhance our understanding of insect biosystematics and evolutionary trends that have enabled e.g. some insects to rapidly achieve pest status in the recent past.
11. Comparative genomics: With the relative ease of discovering genes and determining gene functions using model species, comparative genomics has become an important strategy for extending genetic information from model species to more complex insect pests under field conditions. Over the past few years, wealth of genomic information generated in mosquito and *Drosophila melanogaster* as model insect species has indeed helped our understanding of a number of genetic phenomena that can be extended to a number of pest species.
12. Development of genetic linkage maps using a variety of molecular maps and identification of chromosome regions associated with traits of interest help in marker-assisted approaches and cloning of specific genes. In a few economically important insect species such efforts have been initiated, successfully demonstrated, and used.
13. Insect population genomics involve simultaneous sampling of a large number of variable loci in a genome for locus specific inferences (Black *et al*. 2001)
14. Genetic sexing of insects. Such efforts in silkworm have been usefully employed (Ananthakrishnan 2005)

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