ISSN: 2321-2152

IJMECE

International Journal of modern electronics and communication engineering

E-Mail
editor.ijmece@gmail.com
editor@ijmece.com

www.ijmece.com

ISSN2321-2152www.ijmece .com
Vol 5, Issue 3 Aug 2017

Evaluation of Plant Diversity Using Molecular Methods

Mr. L.CHAKRAVARTHY
Mr. Ch SATYANARAYANA (Corresponding Author)

Abstract

A variety of different molecular techniques can be used for the study of botanical diversity. Restriction fragment length polymorphism (RFLP), arbitrary primed DNA, amplified fragment length polymorphism (AFLP), variable number of tandem repeats (VNTR), sequence-tagged simple sequence repeats (SSRs) and polymerase chain reaction (PCR) sequencing are briefly reviewed here. These techniques differ in the way that they resolve genetic differences, in the type of data that they generate and in the taxonomic levels at which they may be most appropriately applied. It is imperative to understand the different ways in which the data from the different molecular techniques can be utilized before embarking upon a programme of applying them to any particular diversity study.

Key words: Biodiversity, RFLP, RAPD, AFLP, VNTR, SSR, molecular data, fingerprinting, genetic distances, phylogeny, conservation.

INTRODUCTION

The term ' botanical diversity' conjures up many different images. To some it brings to mind complex natural ecosystems, such as rain forests or grasslands, comprising diverse forms of different species and hybrids. Others may think of living co
llections of botanic gardens, dried specimens in herbaria or row upon row of containers of seed in gene banks. Examples of this kind present the molecular geneticist with contrasting problems in the assessment of diversity which may be investigated using a variety of different DNA technologies.

Traditional approaches to the measurement of diversity rely upon the ability to resolve differences in morphological characters. The range of characters available may be increased by the use of electron microscopy or biochemical and phytochemical assays. Although these approaches are extremely powerful, it is with the new tidal wave of data emerging from DNA-based techniques that this present review is concerned. An obvious advantage of molecular assays is the immense number of characters that they reveal. It would be erroneous to think, however, that because these characters are in the DNA, they are necessarily superior to those revealed by more traditional methods. In fact, molecular techniques vary in the way that they resolve genetic differences, in the type of data they generate and inthe taxonomic levels at which they can be most appropriately applied.

MOLECULAR GENETIC SCREENING TECHNIQUES

In restriction fragment length polymorphism (RFLP), DNA is digested with restriction enzymes, the resultant fragments are separated by gel electrophoresis and blotted onto a filter and then probes are hybridized to the DNA. RFLPs give highly reproducible patterns but variations in fragment lengths between individuals or species can arise either when mutations alter restriction sites, or result in insertions/ deletions between them (Burr et al., 1983) (Fig. 1 A). Because heterozygotes are distinguishable, RFLPs are co-dominant markers. Their technical limitations are: (a) a good supply of probes is needed and, if heterologous probes are unavailable, cDNA or genomic DNA probes must be developed; (b) the blotting and hybridization steps are time-consuming and difficult to automate and; (c) sufficient quantities (e.g. 10 µg per digestion) of good quality DNA are required and RFLPs are, thus, not applicable where very limited amounts of source material or preserved tissue are available.

The development of the polymerase chain reaction (PCR) for amplifying DNA led to a revolution in the

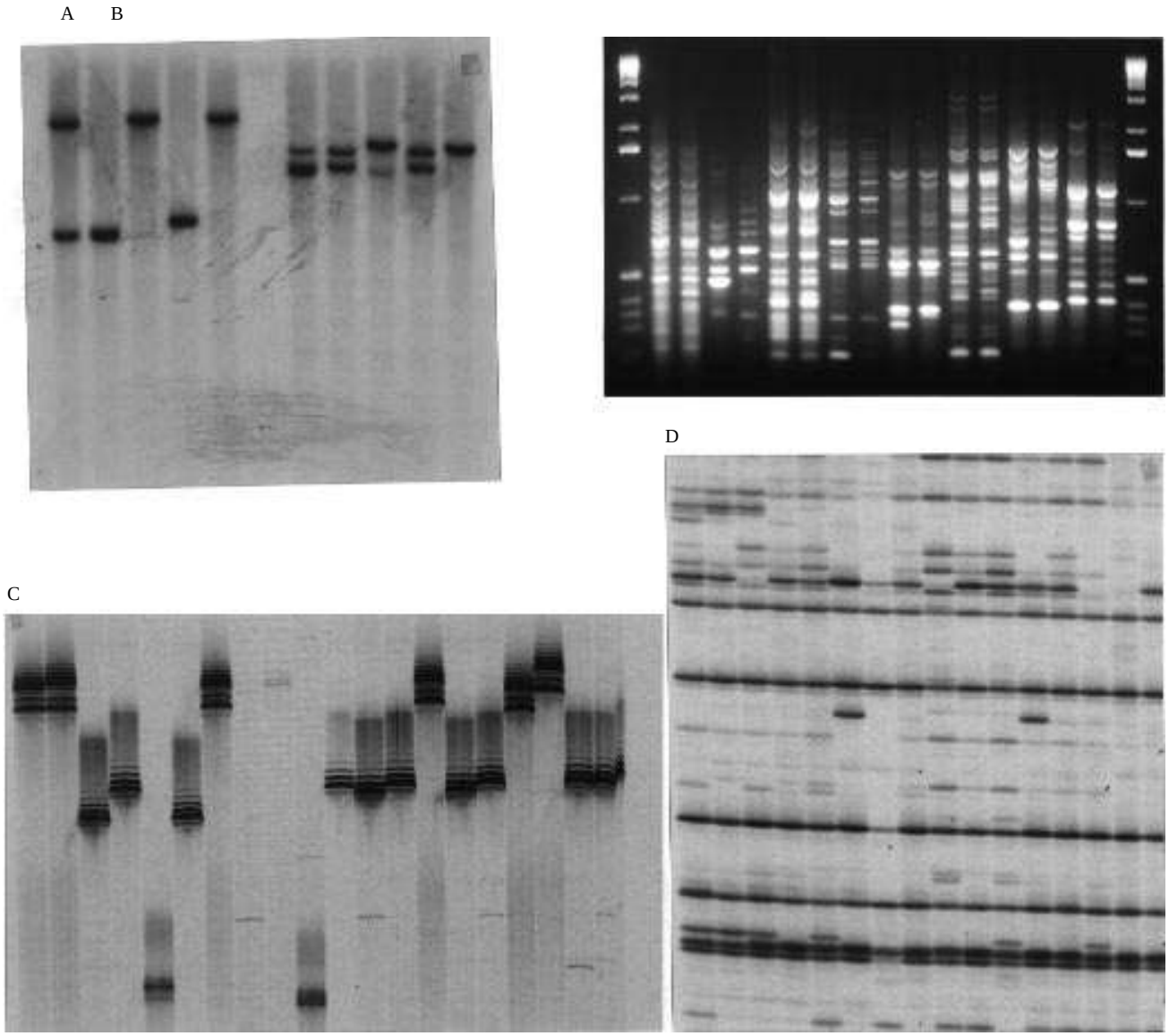

FIG. 1. Four different molecular genetic screening techniques. A, RFLPs. In this example, 10 μg of genomic DNA from five different and unrelated, inbred maize lines have been digested with *Eco*RI (five tracks on the left) or *Hin*dIII (five tracks on the right) and probed with a ^{*1}P- labelled single copy maize sequence. In this example the hybridizing fragments range in size from 3 kb to 8 kb. B, RAPDs. In this example, 10 ng of genomic DNA from two near isogenic lines of maize have been amplified with one of eight different operon primers (giving eight pairs of RAPD tracks). C, SSRs. In this example of sequence-tagged microsatellites (SSRs), 10 ng of genomic DNA from 20 different inbred lines of sunflower has been analysed using a single **P-labelled microsatellite primer pair. The commonly observed 'stutter bands' are clearly visible. In all cases the most intense band was scored as representing the allele present. D, AFLP. In this example, 1 μg of genomic DNA from 16 different breeding lines of sugarbeet have been amplified with a **P-labelled *Pst*I primer and a non-labelled *Mse*I primer.

of molecular methods and a range of new technologies were developed which can overcome many of the technical limitations of RFLPs. A subset of the latter involve the use of a single ' arbitrary' primer, (obtainable from commercial companies) and result in the amplification of several discrete DNA products (Fig. 1 B). Each product will be derived from a region of the genome containing two short segments with sequence similarity to the primer, on opposite strands and sufficiently close for the amplification to work. The most common version is RAPD (randomly amplified polymor- phic DNA) analysis, in which the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light (Williams et al., 1990). AP-PCR (arbitrary primed PCR) (Welsh and McClelland, 1990) and DAF (DNA amplification fingerprinting) (Caetano-Anolle´ s, Bassam and Gresshoff, 1991) differ from RAPDs principally in primer length, primer to template ratio, the gel matrix used and in the visualization procedure. The enormous attractions of these arbitrary priming techniques are: (a) there is no requirement for DNA probes or sequence information for the design of specific primers; (b) since the procedure involves no blotting or hybridizing steps, it is quick, simple and automatable and; (c) very small amounts of DNA (10 ng per reaction)

are required. It is absolutely critical, however, to maintain strictly constant PCR reaction conditions in order to achievereproducible profiles.

The data derived from RAPDs (or AP-PCR and DAF) have their strength in distinguishing individuals, cultivars oraccessions, although the difficulty of achieving robust profiles, particularly in RAPDs, makes their reliability for ' typing' questionable. The presence or absence of bands canbe scored and the data converted into similarity matrices for calculation of genetic distances (Ellsworth, Rittenhouse and Honeycut, 1993). RAPDs have also been used for population studies, molecular ecology (Hadrys, Balick and Schierwater, 1992) and for taxonomy. In using arbitrary priming procedures, it should be realised, however, that: (*a*) the markers are dominant and heterozygotes cannot be detec- ted; (*b*) in the absence of pedigree analysis, the identity of individual bands in the multi-band profiles is not known and there can be uncertainty in assigning markers to specific loci; (*c*) the presence of a band of apparently identical molecular weight in different individuals cannot be taken as evidence that the two individuals share the same homologous fragment, although this assumption is commonly made and; (*d*) single bands on the gel can sometimes be comprised of several co-migrating amplification products.

More recently, a new PCR-based technique has been developed, termed amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), which is essentially intermediate between RFLPs and PCR. The first step involves restriction digestion of the genomic DNA, which is then followed by selective rounds of PCR amplification of the restricted fragments. The amplified products are radioactively or fluorescently labelled and separated on sequencing gels (Fig. 1 D). AFLPs appear to be as reproducible as RFLPs, but they are technically more demanding and require more DNA (1 μg per reaction) than RAPDs. Because of their large genome coverage (on average they give 100 bands per gel compared with 20 for RAPDs), AFLPs are particularly good for mapping and fingerprinting and genetic distances can be calculated between genotypes. They do, however, share many of the limitations, with respect to band homologies and identities as outlined above for RAPDs. Futhermore, AFLPs are a recent technology, about which new information is continually being revealed regarding the identity and distribution of AFLP bands in the genome. This is clearly related to the selected primers being used, but recent data indicate, for example, that AFLP bands may cluster on genetic maps around the centromeres. In using AFLPs for genetic diversity studies, researchers should thus keep a watchful eye on the latest discussions on this technology.

Another powerful technique for studying diversity utilises hypervariable regions of the genome comprised of tandemly repeated simple sequences. These repeats vary in number (and, hence, length) and are, therefore, generally called VNTRs (variable number of tandem repeats), although the terms ' microsatellite' (or simple sequence repeat, SSR) (Fig. 1 C) and ' minisatellite' are used where the basic repeat unit is around two to eight base pairs in length, or longer, respectively. VNTRs may occur at many sites in the genome. When synthetic oligonucleotide probes containing repeatsare hybridized to genomic DNA, multi-band profiles are usually produced. These VNTR fingerprints are generally highly discriminative and are often used to distinguish varieties, or even individuals, and reveal parentage and identity. The multi-locus profiles share many features with RAPDs and AFLPs. Features which are also common to combinations of these methods such as the use of VNTR primers in RAPDs, or SSR primers in AFLPs.

Although VNTRS may be present at several sites in the genome, at each of these sites the flanking sequences may well be unique. If individual VNTRs are cloned, primers to the flanking regions can be designed, thereby converting the VNTR into a sequence-tagged site (Fig. 1 C). Minisatellites are more difficult to use in this way, due to their size. Retrieval of SSRs from new species has hitherto not been easy, due to their relative low abundance in plant genomes, but several methods for highly efficient isolation of SSRs are now available (Edwards *et al*., 1996). The highly mutable nature of SSR loci increases the possibility of having many allelic variants at each locus. Over 30 different alleles have been reported in some instances. Agarose gels can be used to distinguish alleles differing by several base pairs (i.e. several repeats) in length, but polyacrylamide gels are necessary if differences of a single repeat length are to be resolved. Sequence-tagged SSRs give highly reproducible profiles and are co-dominant.

The most precise and informative molecular data on diversity is obtained from sequencing known targets in the genome. Sufficient sequence information must be known initially in order to design primer pairs that will lead to amplification of only the target sequence. How precise this information has to be depends on how conserved the target sequence is. The amplified product can be compared on an agarose gel to the corresponding product from another individual, but only those differences in length that result from many base pair changes will be detected. A number of gel systems, such as TGGE (thermal gradient gel electro- phoresis) (Riesner *et al*., 1992), DGGE (denaturing gradient gel electrophoresis), single strand conformational poly- morphism (SSCP) (Hayashi, 1992) and heteroduplex form- ation (White *et al*., 1992) provide sensitive detection assaysof sequence differences without the need for complete sequencing, but they do require highly controllable condi- tions. In another alternative, termed PCR-RFLP, or CAPS, the amplified product is digested with a specific restriction enzyme and the products directly visualized on the agarose gel after ethidium bromide staining (Akopyanz *et al*., 1992). This simple approach is most informative when the restriction sites are mapped, rather than simply detected as RFLPs, and will not, of course, resolve all differences. Potentially, only sequencing the fragment, either manually, or using an automated DNA sequencer, will resolve all the possible differences between the samples. Sequences derived from protein-coding genes are usually straightforward to align and the interpretation of the data poses few problems. For non-protein coding sequences (e.g. rDNA), however, one of the most difficult and least understood components of sequence analysis—sequence alignment—has to be confronted and data interpretations can, therefore, become very difficult. The PCR-sequencing approach is applicable

to extremely small samples, down to a single pollen grain or tiny fragments and DNA can even be extracted from herbarium specimens or fossils (Herrmann and Hummel, 1992).

Plants possess three different genomes and, therefore, three potential sources of sequences for a PCR-targeted approach. The chloroplast genome (cpDNA) is uniparentally (often maternally) inherited in plants. It is highly abundant in leaves and therefore amenable to isolation in large quantities. The entire chlororplast DNA sequence is known for three species (a liverwort, tobacco and rice) and appears, with a few notable exceptions, to be highly conserved in terms of size, structure, gene content and order. Primers are available that will work either directly, orwith small alterations, across broad taxa (e.g. across all green plants) (Demesure, Sodzi and Petit, 1995). The majority of studies using sequence data from cpDNA have been phylogenetic ones and at fairly high taxonomic levels (intergeneric and above), although, recently, primer pairs for cpDNA have been used for population studies. In contrast, the mitochondrial genome (mtDNA) of plants is less abundant in leaves and more difficult to extract, there is less background knowledge, fewer primers are available and these have been less well characterized. The high rates of structural rearrangements mean that mtDNA analysis using restriction site assays is of limited use at the interfamilial and interspecific taxonomic levels but it can be very useful at detecting variation at the intraspecific and population levels. Primer pairs for conserved regions of mtDNA sequences are available (Demesure *et al*., 1995).

For assays of the nuclear genome, only the ribosomal RNA (rDNA) gene family has been widely used for diversity studies. rDNA genes are located at specific chromosomal loci (NOR, nucleolar organizing regions) where they are arranged in tandem repeats which can be reiterated up to thousands of times. Each repeat unit comprises a transcribed region separated from the next repeat by an intergenic spacer (IGS). The transcribed region comprises: an external transcribed spacer (ETS), the 18S gene, an internal transcribed spacer (ITS1), the 5·8S gene, a second internal transcribed spacer (ITS2) and the 26S gene. Primers pairs have been designed which will enable amplification of the different regions in a wide range of organisms. These regions evolve at different rates and can thus, in principle, be used at all taxonomic levels (Hillis and Dixon, 1991). ITS has proven to be a valuable tool for intergeneric studies in many organisms. Botanists, however, may experience difficulties in detecting sequence variations below the species level.

The advantages of PCR-sequencing approaches lie in their reproducibility, in the quality of their data and in the information engendered. There are disadvantages in the approach, however: (*a*) it is laborious to screen vast numbersof individuals (even with automated sequencing) although pre-screening with CAPS, SSCP, etc can help; (*b*) the coverage of the genome is highly restricted, often to only one sequence; (*c*) there are currently few primer pairs for nuclear genes that vary at rates that will enable detection of polymorphic differences particularly for diversity studies at below the species level, e.g. between cultivars, accessions or populations; (*d*) technical problems of contamination byDNA from other organisms (eg, contamination of plant ITS with ITS from powdery mildew), or of the detection of multiple gene copies (including pseudogenes), can arise when conserved primers are used.

INTERPRETING AND ANALYSING DATA FROM DIFFERENT MOLECULAR SCREENING TECHNIQUES

It is imperative to understand the different ways that data from different molecular techniques can be utilized before embarking upon a programme of applying any one of the techniques to a diversity study (Hillis and Moritz, 1990; Soltis, Soltis and Doyle, 1992; Avise, 1994; Weir, 1996). The most important issue, by far, is whether one is working at or below the species level, i.e. whether the exemplars used in the analysis usually exchange genes or not. Molecular data are collected in two fundamentally different ways, u*iz*. as discrete characters or as continuous characters. Whereas discrete data (e.g. sequence data) relates to individual specimens/species, continuous data (e.g. DNA–DNA-hybridization data) are quantitative pairwise comparisons between specimens or species. Moreover, discrete data can always be transformed into continuous data (distance measures), but the converse is not true. Data gathered by the methodologies described here are all discrete, althoughthey are also of two different qualities. Shared bands (or restriction sites) are scored as presence/absence data, while sequence data are scored as nucleotides (only five permissible values of A,T,C,G and U). Some techniques, like TGGE, DGGE and SSR, are only short-cuts, by-passing sequencing. At the intraspecific level, many problems, such as studies of mating systems, levels of heterozygosity and paternity testing, require discrete genetic markers and information from many individuals at many loci. Studies of geographic variation, gene flow, and hybridization also require discrete genetic markers of this type, but is best done with a more detailed knowledge of the inheritance of the markers, e.g. whether or not they are maternally or biparentally inherited (see, for example, Swofford and Olson, 1990; Queller, Strassmann and Hughes, 1993; Avise, 1994; Weir, 1996). The interpretive tools used at this level range from traditional probabilistic methods (e.g. given two similar banding patterns, what is the chance that they are the results of chance alone) to population genetics (e.g. to be meaningful the data are usually turned into allele frequencies), (Hartl and Clark, 1989).

At the above species level, data are used for the reconstruction of phylogenies and, although it is difficult to find justification for turning discrete data into distances, in principle, both continuous and discrete data may be employed. In the analysis of distance data a matrix of pairwise distances is subjected to a clustering algorithm, like UPGMA (unweighted pair group method using arithmetic averages), or neighbour-joining. Alternatively, the analysis of sequence data can be done using either parsimony based methods or maximum likelihood methods. The aim of parsimony methods is to produce the tree, or group of trees, that require the least amount of change to explain the observed data, while the aim of maximum likelihood

methods is to produce the tree that gives the highest probability of a data set being derived from it. For excellent reviews on this subject see Swofford and Olson (1990) or Forey *et al*. (1992).

The apparently sharp division between the intra- and interspecific levels is not, however, absolute. Algorithms have been developed that attempt to estimate phylogeny from gene frequency data (Swofford and Berlocher, 1987) and the presence or absence of an SSR, for example, is information of potential phylogenetic relevance. Moreover, gene phylogenies, as opposed to species phylogenies, can be made using standard phylogenetic methods provided that the gene in question is located in an uniparentally inherited non-recombining genome, e.g. cpDNA or mtDNA (see, e.g. Avise, 1994). In general, the major theoretical drawback of methods that use the presence or absence of bands as data, is the fact that the characters are not independent. An RFLP-band that has disappeared (e.g. due to the evolution of a new restriction site) may, or may not, depending on the size of the new fragments, be detected and scored as two new bands. This has only limited consequences for rec- ognition of patterns (e.g. in fingerprinting) but may have consequences for determination of gene frequencies, or in reconstructions of phylogeny. Additionally, there is a pronounced asymmetry in the probability of losing and gaining bands (Bremer, 1991 ; Albert, Mishler and Chase, 1992 ; Backelijau *et al.*, 1996).

Seemingly, sequence data are straightforward to understand. In a set of sequences, the characters are the nucleotides sharing positional homology, u*iz*. occurring at the same position. Positional homology is, however, not always easy to interpret. If the aim is to study species phylogeny rather than gene phylogeny, the most important condition that must be met is that the sequence must be orthologous, not paralogous, but even if that is the case, the presence of insertions/deletions may make the assignment of positional homology (alignment) highly ambiguous. The major draw- backs of distance methods, as they are used in reconstruction of phylogenies, are the facts that all sequence data are probably non-additive and that it remains unclear what the distances mean biologically. The main problem related to the use of maximum likelihood methods is the relatively limited number of taxa that they can effectively handle. Furthermore, the underlying evolutionary models may be too simple.

Sequences (and restriction site analyses) are the only molecular markers that contain a comprehensible record of their own history. Hence, appropriate analyses based on sequence data (or restriction site data) can provide hypotheses on the relationships between the different genotypic categories (or species) that they class together. Some would argue that this particular molecular approach is the only appropriate method for taxonomic studies, being superior even to all traditional approaches, but molecular data suffer the same problems (u*iz*. problems of homology) as mor- phological data (see Patterson, 1988 ; Sanderson and Donoghue, 1989). The advantage that they offer is the almost infinite amount of data that can be obtained.

Frequency data from markers such as arbitrary primed DNA, AFLPs and SSRs only provide the means to classify individuals into nominal genotypic categories. This distinction is important to grasp for population studies, particularly when the diversity data are used as a basis for making decisions about conservation. In principle, sequenc- ing will allow the determination of which gene sequences, in samples taken from within or between populations, are the most closely related and hence share a most recent common ancestor. For such genealogical relationships (which may be separate from the genealogy of the individuals carrying the genes) the influences of genetic factors, such as mutation, are independent from demographic factors such as popu- lation size, whereas, in the case of markers that provide only frequency information, these factors are confounded (for an excellent review on this subject, see Milligan, Leebens-Mack and Strand, 1994). This difference is of particular relevance to conservation, where demography (the description and prediction of population growth and age structure) is considered to be as important, if not more important, than genetic factors (Lande, 1988).

In using molecular techniques, it is important to recognise that all molecular diversity data are subject to experimental errors which differ depending on the technique. For example, assays dependent on restriction enzyme digestion are more robust than those that do not (although partial digestion may occur with phenolic and polysaccharide contami- nation); PCR reactions using single primers are more prone to irreproducibility than those using two primers; and so on. Many of the techniques are very new and there are few guidelines on the infinite number of ways in which unexpected results can arise. Reproducibility is the only easy way of assessing the quality of the data. Assessment of reproducibility is most evident when the data are in the form of a sequence (although, even here, it is possible to be misled) and very unclear when it comes to variations in band intensity. Although the probability of differences due to chance (or error) being generated can sometimes be estimated theoretically (e.g. the probability of *Taq* poly- merase making an error in sequencing, see Koop *et al.*, 1993) it is difficult to take account of all possible experimental variables. Most investigators use careful controls and may try to determine whether unexpected bands appear consistently or not, but few have time to determine why they appear. In practice, all researchers use their own judgement as to what is, or is not, significant. One only needs to observe how two investigators score the same RAPD gel to see evidence of this.

A further qualifying issue concerns the interpretation given to molecular data. At the intra-specific level, in particular, the rationale behind the development of molecular tools for screening diversity has generally been the search for polymorphic, ' easy-to-handle' markers, to overcome the main problems of detecting sufficient polymorphisms and of tackling large sample sizes. Molecular markers are thus largely used as arbitrary indicators of diversity. Often the markers are used without knowledge of whether they are located in single or repeated sequences, in coding or non-coding regions nor even, sometimes, whether they are of nuclear or organellar origin. The markers will also be heterogenous in the frequencies of their different variations and, thus, their

rates of evolution, all of which

introduces a potential bias in the interpretation of the variability observed. Whether this variation can be taken as reflecting that which influences future adaptation or individual fitness is debatable (Milligan *et al*., 1994). We have only questionable evidence as to whether the markers are neutral or not. Few molecular markers for fitness are known and there is little conception of what fitness means and little knowledge of how to measure it adequately (Bachmann, 1994).

As our knowledge of different marker systems advances, so our interpretation of their biological significance changes. The neutrality of mitochondrial markers has recently been questioned (Ballard and Kreitman, 1995). VNTR variation, which arises from changes in repeat numbers derived from slippage and/or unequal crossing-over has also beenconsidered ‘ neutral’. A series of human genetic diseases, such as the fragile X syndrome, have, however, now been attributed to changes in the number of SSRs (Sutherland and Richards, 1995). Moreover, long-range correlations in eukaryotes have been shown to exist, comprised of homopurine/homopyrimidine GC/AT containing stretches of variable length (Lio‘ , Ruffo and Buiatti, 1994) of SSR nature (Lio‘ *et al*. unpubl. res.) suggesting that genome variation may also be under ‘ internal constraints’.

CONCLUDING REMARKS

Molecular techniques do have much to offer for the assessment of botanical diversity, but potential users should recognise that this is an emerging, rapidly evolving field in which the technology has advanced faster than scientific understanding. Many exciting opportunities lie before those challenged into bridging the gaps. Just as in systematics, the difference between ‘ gene’ trees and species ‘ trees’ is now widely recognised, so there is a need to calibrate the diversity revealed as polymorphisms by different molecular techniques with traditional measures of botanical diversity at the below species level. In any assessment using molecular markers, attention should first be focused on the specific questions being addressed and on whether or not the choice of marker, sampling strategy and data analysis adequately addresses it. Consideration should also be given to the location of the markers (coding or non-coding regions), their distribution in the genome (where known) and of their potential constraints (i.e. strongly adaptive or near-neutral). Interesting insights may also come from using ‘ novel’ markers in diversity studies, such as physiologically and developmentally relevant genes (Buiatti and Bogani, 1995 ; Bogani et al., 1995) but bridging the gap between the marker per se and the phenotype is a formidable biological problem for the future.

Refference

Akopyanz N, Bukanov N, Westblom TU, Berg DE. 1992. PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen Helicobacter pylori. Nucleic Acids Research 20 : 6221–6225.

Albert VA, Mishler BD, Chase MW. 1992. Character-state weighting for restriction site data in phylogenetic reconstruction, with an example from chloroplast DNA. In: Soltis PS, Soltis DE, Doyle JJ, eds. Molecular systematics of plants. New York: Chapman and Hall, 369–403.

Avise JC. 1994. Molecular markers, natural history and euolution.
London: Chapman & Hall.

Bachmann K. 1994. Molecular markers in plant ecology. The New Phytologist 126 : 403–418.

Backeljau T, De Wolf H, Jordaens K, Van Dongen S, Verhagen R, Winnepenninckx B. 1996. Random amplified polymorphic DNA (RAPD) and parsimony methods. Cladistics 11 : (in press).

Ballard JW, Kreitman M. 1995. Is mitochondrial DNA a strictly neutral marker? Trends in Ecology and Euolution 10 : 473–512.

Bogani P, Simoni A, Scialpi A, Lio‘ P, Buiatti M. 1995. Caratterizzazione di bande RAPD possibili marcatori fisiologica in somacloni di pomodoro. Proceedings of the XXXIX Annual Meeting of Plant Genetics Italy Society, (SIGA), 62.

Bremer B. 1991. Restriction data from chloroplast DNA for phylo- genetic reconstruction: is there only one accurate way of scoring? Plant Systematics and Euolution 175 : 39–54.

Buiatti M, Bogani P. 1995. Physiological complexity and plant genetic manipulation. Euphytica 85 : 135–147.

Burr B, Evola SV, Burr FA, Beckmann JS. 1983. The application of restriction fragment length polymorphisms to plant breeding. In: Setlow JK, Hollaender A, eds. Genetic engineering principles and methods, Vol 5. New York: Plenum, 45–59.

Caetano-Anolle´ s G, Bassam BJ, Gresshoff PM. 1991. DNA amplifi- cation fingerprinting using very short arbitrary oligonucleotide primers. Bio/Technology 9 : 553–557.

Demesure B, Sodzi N, Petit RJ. 1995. A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Molecular Ecology 4 : 129–131.

Edwards KJ, Barker JHA, Daly A, Jones C, Karp A. 1996. Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques (in press).

Ellsworth DL, Rittenhouse KD, Honeycut RL. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. Biotechniques 14 : 214–217.

Forey PL, Humphries CJ, Kitching IJ, Scotland RW, Siebert DJ, Williams DM. 1992. Cladistics : a practical course in systematics. Oxford: Clarendon Press.

Hadrys H, Balick M, Schierwater B. 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Molecular Ecology 1 : 55–64.

Hartl DL, Clark AG. 1989. Principles of population genetics. Sunderland, Massachusetts: Sinauer Associates.

Hayashi K. 1992. PCR-SSCP: a method for detection of mutations.
Genetic Analysis : Techniques and Applications 9 : 73–79.

Hermmann B, Hummel S. 1992. Ancient DNA. New York: Springer Verlag.

Hillis, DM, Dixon MT. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Quarterly Reuiews in Biology 66 : 411–453.

Hillis DM, Moritz C. 1990. An overview of applications of molecular systematics. In: Hillis DM, Moritz C, eds. Molecular systematics. Sunderland, Massachusetts: Sinauer Associates, 502–515.

Koop BF, Rowan L, Chen W-Q, Deshpande P, Lee H, Hood L. 1993. Sequence length and error analysis of sequenase and automated Taq cycle sequencing methods.

Biotechniques 14 : 442–447.
Lande R. 1988. Genetics and demography in biological conservation.
Science 241 : 1455–1460. Lio‘ P, Ruffo S, Buiatti M. 1994. Third codon G+C periodicity as a possible signal for an internal selective constraint. Journal of Theoretical Biology 171 : 215–223.
Milligan BG, Leebens-Mack J, Strand AE. 1994. Conservation genetics: beyond the maintenance of marker diversity. Molecular Ecology 3 : 423–435.
Patterson C. 1988. Homology in classical and molecular biology.
Molecular Biology and Euolution 5 : 603–625.
Queller DC, Strassmann JE, Hughes CR. 1993. Microsatellites and kinship. Trends in Ecology and Euolution 8: 285–288.
Riesner DG, Steger U, Wiese M, Wulfert M, Heibey M, Henco K. 1992. Temperature-gradient electrophoresis for the detection of poly- morphic DNA and for quantitative polymerase chain reaction. Electrophoresis 13 : 632–636.
Sanderson MJ, Donoghue MJ. 1989. Patterns of variation in levels of homoplasy. Euolution 43 : 1781–1795.
Soltis PS, Soltis DE, Doyle JJ. 1992. Molecular systematics of plants.
London: Chapman and Hall.
Sutherland G, Richards RI. 1995. Simple tandem repeats and human genetic disease. Proceedings of the National Academy of Science. USA 92 : 3636–3641. Swofford DL, Berlocher SH. 1987. Inferring evolutionary trees from gene frequency data under the principle of maximum parsimony. Systematic Zoology 36 : 293–325.
Swofford DL, Olsen GJ. 1990. Phylogeny reconstruction. In: Hillis D M, Moritz C, eds. Molecular systematics. Sunderland, Massachu- setts: Sinauer Associates, 411–501.
Vos P, Hogers R, Bleeker M, Rijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23 : 4407–4414.
Weir BS. 1996. Methods for discrete population genetic data. Sunderland, Massachusetts: Sinauer Associates.
Welsh J, McClelland M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18 : 7213–7218.
White MB, Carvalho M, Derse D, O’Brien SJ, Dean M. 1992. Detecting single base substitutions as heteroduplex polymorphisms. Geno- mics 12 : 301–306.
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18 : 6531–6535.