# Identification and characterisation of citrus biodiversity: traditional and molecular approaches

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# Identification and characterisation of citrus biodiversity: traditional and molecular approaches

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**SUMMARY** An account is given of the techniques used to identify genetic biodiversity in citrus by traditional and molecular means. Concerning the molecular approach, PCR and non-PCR methods are shortly described. The interests and limitations of these techniques are highlighted, thus suggesting the integration of several methods as the best approach for the identification of genetic biodiversity in citrus.

**Key words**: citrus, biodiversity, morphology, physiology, phenology, agronomy, molecular techniques, PCR, MAAP, AFLP, SSRs, isoenzyme, RFLP

***RESUME*** *Dans ce travail on parcourt les techniques utilisées pour identifier la biodiversité génétique des agrumes par les méthodes traditionnelles et moléculaires. Parmi ces dernières, La PCR et les autres méthodes sont brièvement décrites. Les avantages et les limites de ces techniques sont mis en évidence, en suggérant une intégration de plusieurs méthodes comme meilleure approche pour l'identification de la biodiversité génétique des agrumes.*

***Mots clés*** *agrumes, biodiversité, morphologie, physiologie, phénologie, agronomie, techniques moléculaires, PCR, MAAP, AFLP, SSRs, Isoenzyme, RFLP.*

## Introduction

The conservation and use of genetic diversity have become major issues of international concern since they are essential to the world ecology and to the long-term survival of the human race.

Citrus is one of the world's major fruit crop and it is widely grown in the Mediterranean area. Citrus and many related genera hybridise readily and have done so naturally for centuries. Moreover, many species reproduce via nucellar embryos, which permit the continued asexual existence of a species or a hybrid. These factors, coupled with the ability to propagate citrus by grafting, have led to the selection of more desirable traits by humans and the perpetuation of "elite" germplasm lines, making citrus genetically vulnerable.

The techniques used to identify genetic biodiversity in citrus, as in any crop, require first of all identification of its extent and distribution. The techniques used to identify genetic biodiversity can be distinguished into two broad categories, the traditional and the molecular ones.

The *traditional methods* are based on morphological, physiological, phenological and agronomic characters such as leaf, flower and fruit parameters, susceptibility or resistance to biotic and biotic factors etc. These data are of the greatest importance as regards the use and management of genetic material and the establishment of a standard type of language. However, the use of these characters is limited by the following factors:

1) the variation of these characteristics is highly dependent on the expression of gene combinations interacting with the environment and may be difficult to be measured;
2) highly heritable traits often show leaf phenotypic variation;
3) it is impossible to determine at a given time the characteristics that will be important in the future;
4) the evaluation of these traits is subjective.

With *molecular techniques*, many of the complications related to environmental effects can be avoided by looking directly at the variation controlled by genes or by looking at the genetic material itself. The molecular markers can provide basic information on varietal characterisation, heterozygosis, intra- and inter-specific polymorphism and phylogenetic relationships within and between different species. The molecular markers can be distinguished into two general types: the protein-based and the DNA-based markers (Ayad *et al.*, 1995; Karp *et al.*, 1996).

The most commonly protein-based markers used for citrus are isozymes (isoenzymes). Isozymes can be separated by electrophoresis on starch polyacrylamide, or agarose gels, or by bioelectric focusing. Although polyacrylamide gels theoretically have a greater resolving power for individual enzyme systems, starch gels are more appropriate for analysis of large number of individuals, and their isolation serves many purposes. Several enzyme systems have clearly been resolved on starch gels, and the use of a larger number of *loci* will usually compensate for the lower resolving power of starch gels. Agarose gels are generally similar to starch gels, but they have not been used much in plants. lsoelectric focusing has still greater resolution, but is more expensive, time-consuming, and seems to be more able to resolve bands determined by non-genetic causes. In citrus, a large number of enzyme systems have been studied, including phosphatase,

esterase, peroxidase etc. (Esen and Soost, 1977; Protopapadakis, 1987; Roose and Traugh, 1988).

The reliability of isozyme analysis is determined by the skill of the technician, the choice of plant material, and the enzyme systems used. It is very important to choose enzymes whose mode of inheritance is well defined, and which are little influenced by the environment.

Over the last 15 to 20 years, a whole range of different techniques has been developed which detect polymorphism at the DNA level. Indeed, this wide array falls into two broad categories: (A) Non-PCR based approaches and (B) PCR-based approaches.

A brief description of the most widely used methods, of their advantages and disadvantages is given below.

## Non -PCR techniques

### *Restriction Fragment Length Polymorphism (RFLP)*

The RFLP technique consists in DNA isolation, digestion of DNA with restriction enzymes, separation of the restricted DNA fragments by gel electrophoresis, transfer of the separated fragments to a filter membrane, detection of individual restriction fragments by hybridisation with labelled cloned probes, and scoring of bands by direct observation of autoradiograms. Differences in band patterns reflect genetic differences such as point mutations, deletions or insertions.

RFLP technique is highly reproducible, it distinguishes heterozygotes from homozygotes and can be applied immediately for diversity screening in any system since no sequence-specific information is required. However, it is time-consuming, needs a good supply of probes that can reliably detect variation below the species level, requires relatively large amounts of good-quality DNA and is expensive.

## PCR- based techniques

Polymerase chain reaction (PCR) is a powerful but simple technique for amplifying DNA. Following the advent of PCR, new techniques have become available and they may overcome many of the difficulties encountered with probe-based hybridisation RFLP method. The most commonly used techniques of this category are the following:

1. ***Multiple Arbitrary Amplicon profile (MAAP)***

   MAAP techniques are closely related and they involve the use of single "arbitrary" primers in a PCR reaction, whose result is usually the amplification of many discrete DNA products (Caetano-Annollès, 1994). The most commonly used is RAPD (Randomly Amplified Polymorphic DNA), in which the primers are usually 10 or 20 mer and 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 (Arbitrarily primed PCR) and DAF (DNA Amplification Fingerprinting) differ from RAPD mainly in primer length and method of separation and detection of DNA fragments (Ayad *et al.,* 1995). RAPD is fast, simple, relatively inexpensive and does not require radioactive probes for hybridisation. However, RAPD shows the following limitations:

   1) it can be difficult to be reproduced between, and even within laboratories;

   2) the quality of data is poor since it gives dominant markers, while the presence of a band of apparently identical molecular weight in different individuals does not indicate that the two individuals share the same homologous fragment and single bands can sometimes be the result of several co-migrating amplification products.

2. ***Amplified Fragment Length Polymorphism (AFLP)***

   This technique combines restriction digestion (RFLP) and PCR (RAPD) and is very efficient in revealing diversity below the species level. Furthermore, it is as reproducible as RFLP, but it detects a higher number of polymorphisms per assay. It requires more DNA and is more technically demanding than RAPD and it has the same problems as RAPD regarding data quality.

## PCR-targeted techniques

The opposite approach to arbitrary amplicon profiling consist in designing primers to amplify specific regions of the genome. The targeted amplified product can be compared on an agarose gel to the corresponding product from another individual, but only changes involving many base pairs in length will be detected. In order to resolve all the possible differences, it is necessary to sequence the entire fragment.

The main advantage of PCR-targeted approaches is that they produce high-quality data and information since the fragment in which polymorphism is studied has a known identity, and this makes it possible to avoid the ambiguities of analysing RAPD and AFLP bands. These approaches have the following limitations:

1) unless the frequency of variants is high enough for detection by sensitive gel assays, sequencing is required which necessitates adequate resources and experienced researcher;
2) the coverage of the genome is highly restricted, often to only one sequence;
3) although chloroplast and mitochondria primers are available, there are currently few nuclear sequences that can be used below the species level and the rate at which sequences vary also differs between genomes;
4) contamination of DNA, detection of multiple gene copies and pseudogenes are also observed.

### Simple Sequence Repeats (SSRs) or Microsatellites

Simple sequence repeats (SSRs) or Microsatellites are highly mutable and may be present at many sites in a genome. Since the flanking sequences at each of these may be unique, if SSR are cloned and sequenced, primers to the flanking regions can be designed to define the sequence-tagged microsatellite (STMS) (Beckman and Soller, 1990).

## Considerations for choosing appropriate techniques

It is important to mention that molecular techniques have considerably advanced since new techniques are continuously developed and new information about pre-existing methods is provided. Furthermore, all techniques have some limitations and the best results may be obtained by combining more than one approach. While choosing the appropriate technique, the following questions/factors should be taken into account:

1) *What kind of information is needed?* It is important to choose the right technique in order to obtain the right information. For example, concerning information on how many different diseases are present, any technique can be used and the final choice will be determined by other factors. As for information on how the different classes are related, data on sequence or restriction sites should be obtained.

2) *What is the anticipated level of variation?* This will depend upon the taxonomic level of the material under study. The closer the relationship, the more discriminatory techniques should be considered such as AFLP or SSR.

3) *Reproducibility.* RFLP, SSR, and PCR sequencing display high, AFLP medium and RAPD low reproducibility.

4) *Cost.* Isozymes are the least expensive, RAPD and RFLP have an intermediate cost, whereas AFLP is the most expensive.

5) *Speed*. PCR-based methods give undoubtedly rapid results if primers are available, whereas hybridisation methods are slower.

6) *Expertise.* Techniques involving hybridisation /autoradiography or sequencing are technically demanding. RAPD is the least demanding and the cost per assay is low.

7) *DNA availability***.** Most PCR-based methods require limited amounts of DNA, while RFLP analysis requires larger amounts and finally, for sequencing, the highest amounts are needed.

8) *Mode of inheritance.* RFLP, isozymes and microsatellites show co-dominant inheritance (heterozygotes can be distinguished from homozygotes) whereas RAPD and AFLP show dominant inheritance.

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