

## **DNA TECHNIQUES TO PROVE (NON)-DISTINCTNESS**

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### **Introduction**

GESLIVE is an association of breeders dedicated to the management and defence of plant breeders' rights to plant variety rights or other types of intellectual property affecting plants.

The enforcement of plant breeder' rights is a particularly difficult work in Spain, in the first place in the absence of a culture of respect for such rights, as revealed in studies run by our organisation based on official statistics, which show use of illegally reproduced plant material in excess of 50% in many crops (particularly cereals, fruit trees and cut flowers).

In investigating and confirming a breach of plant breeder' rights, many difficulties are encountered in practice, arising in the first place from the exact identification of the variety which the plant material suspected of being illegally reproduced belongs to. As you know well, the current EU legislation (Council Regulation 2100/94) faithfully according to the terms of the 1991 Act of the UPOV Agreement, distinguishes a variety (Art. 7: "Distinctness") "by reference to the expression of the characteristics that results from a particular genotype or combination of genotypes, from any other variety whose existence is a matter of common knowledge ...". In other words, varieties are characterised by their phenotype in terms of a regulated morphological description detailed in the UPOV technical protocols, in all cases involving a technical examination (Art. 55 et seq. of the base Regulation) in terms of the field assays growing the variety and observation of the relevant morphological characteristics.

Confirmation of the identity of a variety by this procedure in case of suspected breach thus involves technical examinations similar to those run by the Examination Offices. Such technical examination, by cultivation of the variety, raises no major drawbacks with some species such as cereals, with an annual cycle and whose reproduction material coincides with that variety's harvested product. However, for many asexual reproduction species like fruit trees or certain species of flowers, it is not possible, in practice, to implement these technical examinations:

- Because the growing cycle required to verify the morphological characteristics may run for several years;
- Because the reproductive material and the harvested product (where the variety's added value is normally found) don't coincide.

With these crops, it's often not possible to access the presumably illegal plantings of a protected variety without a Court order, being in closed greenhouses or properties. The only material from these crops easily accessed for verification as to the variety is the harvested product, which is normally commercialised and, after investigation, can be found and acquired on the market.

However, except for a few varieties whose end product has such particular characteristics that they can be differentiated from others, it is normally hard to distinguish the variety from others just from that end product (think, for example, about the number of very similar varieties of red nectarines or red carnations on the market). In the case of asexually reproduced varieties such as fruit trees, that product cannot be used to grow them, since neither uniformity nor stability are guaranteed.

In these circumstances, it was a primary aim for GESLIVE to find techniques making it possible to distinguish the plant varieties in these asexually reproduced species, to defend our member's rights.

## **Research on molecular markers at IRTA (the Institute for Food and Agricultural Research and Technology)**

At GESLIVE, we were fortunate because one of our most significant partners is the IRTA (The Institute for Food and Agricultural Research and Technology), a state-owned company of the Government of Catalonia, dedicated to the scientific research and technology transfer in the area of agriculture.

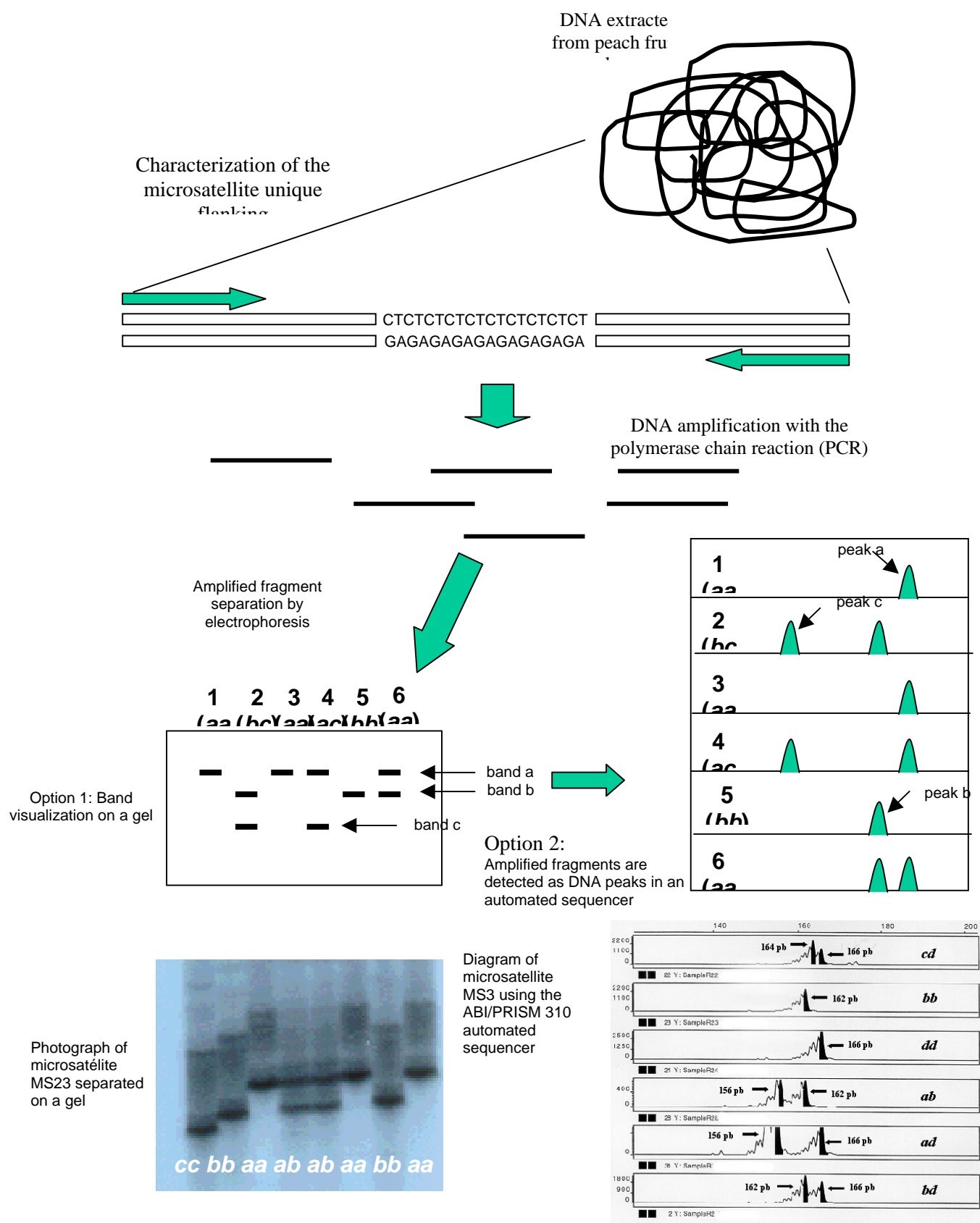
IRTA's Plant Genetics Department (PGD) have worked on markers applied to plant breeding for the last 19 years. A significant part of this research has been devoted to the fruit crops of the genus *Prunus*, particularly to peach and almond. The group has coordinated the first European *Prunus* mapping project and is one of the leading teams in this area at the international level. Recently, they have assessed the efficiency of different molecular markers for peach cultivar identification using leaf and fruit extracts. This work revealed that microsatellites were clearly more adequate than the rest of available markers for this purpose, and developed its own new set of microsatellites. Currently IRTA has a database of microsatellite-identified peach and nectarine cultivars that include more than 200 entries.

### **What are microsatellites?**

**(Source: IRTA)**

Microsatellites are short DNA sequences made by 1 to 6 nucleotide motifs that are tandemly repeated many times (usually 10 or more). These simple DNA sequences are highly variable, abundant in the plant and animal genome, and can be studied with fast and relatively simple methods (see Figure 1). Microsatellite markers are also known as SSRs (Simple-Sequence Repeats).

**Figure 1. Schematic representation of the microsatellite detection method.**



### **How is a marker-based identification test performed?**

**(Source: IRTA)**

Basically, first DNA is extracted from the sample to be studied. DNA can be obtained from leaf or fruit tissues and it can be used to analyze many different microsatellites. Each marker gives a specific banding pattern, usually one or two bands (or peaks when using an automated DNA sequencer) of different sizes. The sum of the information produced by various microsatellites (15-25 of them usually give sufficient resolution) produce a joint banding pattern specific of each cultivar. This banding profile is like a fingerprint or bar code that can be used to identify this cultivar from the rest.

### **How sure a marker-based identification test is?**

**(Source: IRTA)**

If we have the fingerprint of an unknown sample and want to know if it corresponds to that of a known cultivar, we will have to compare them. If the fingerprints are different, the cultivars are different.

Otherwise, either they correspond to the same cultivar or to another one that by chance has the same marker profile for the markers used. To discard one of these two options, we can calculate the probability to find an identical fingerprint to that of the known cultivar with the set of microsatellites used for its characterization. If this probability (that we call “I”) is very small, we can reasonably discard the hypothesis that we have found another cultivar with the same marker profile, and accept that the two samples correspond to the same cultivar. This probability decreases when the number of markers used increases.

In a recent study carried out at IRTA laboratory, they analyzed 24 peach and nectarine cultivars with 38 microsatellites (Aranzana et al., 2001). The mean “I” value was of  $5 \times 10^{-16}$ , meaning that the average probability that one can find by chance the same marker profile in a different cultivar is of 5 in 10,000 trillions. This probability is

so low that we can discard with an almost complete certainty this hypothesis and accept that the two samples correspond to the same cultivar.

In the same publication, only 3 microsatellites were needed to individually identify all 24 cultivars. The remaining 35 markers were used to obtain minute "I" values. The calculation of "I" was done as the product of the probabilities to find the observed marker profile in each microsatellite. This probability has been obtained with data of more than 100 cultivars of the IRTA database.

Another research of IRTA allowed, after marker analysis of 18 peach seedlings, to group them for their provenance from three different crosses. The parents of these crosses were also identified with the decisive help of only 4 microsatellites. This analysis was done considering 37 possible parents, meaning that parents were chosen from a total of 703 possible pairs. The fact that microsatellites are codominantly expressed was critical for the establishment of pedigree relationships, in a much more efficient way than using other dominant markers.

Microsatellite data can be obtained with DNA that can be extracted at any point of the distribution chain with a sample of 4-5 fruits. Results may be rapidly available (one week after) and can be re-examined when needed because the DNA can be kept for years.

### **Creation of the Genetic Data Base for the Identification of Plant Varieties:**

To ensure the efficient and rigorous application of this technology in the defence of plant breeders' rights, in 2003 GESLIVE and IRTA concluded an "Agreement for the development of a genetic data base for plant variety identification".

The aim is to identify the genetic profile of all the plant varieties managed by GESLIVE, by developing specific molecular markers and systematising all this

information in a data base belonging to it. The BDGIVV currently incorporates some 80 varieties of *prunus*, especially peach and nectarine, plum, apricot, etc., and the incorporation of varieties of other species such as grapes, apples, strawberries or carnations is being finalised. Ninety percent of these varieties are protected in the European Union in front of the CPVO.

A very, very important aspect in terms of the legal value which can be assigned to all this information is the procedure for sampling to obtain the marker profiles of all the varieties in the BDGIVV. To ensure this value and reduce potential error, the samples are not delivered by the breeders but are taken directly from the official reference collections held by the Examination Offices, once clearance has been secured for that from the Community's Plant Varieties Office, and the holder of the rights. The samples are taken by IRTA technical personnel in the presence of the officer responsible for the collection, and written certification is drawn up, and signed by both parties.

### **Admission of DNA identification by the Courts:**

The creation of the IRTA database has enabled GESLIVE to secure information of the greatest value in the investigation of possible breaches of plant breeders' rights. Every season, several hundred samples are taken by our inspectors on the market or, when legally possible, from leaves or other plant material from plantings in the field, to find out whether their genetic profile corresponds to that of any of the varieties in the BDGIVV.

Until now, GESLIVE has made use of the genetic identification technique of plant varieties in sixteen (16) court cases in all, brought against plantings identified as protected varieties, for which the plants were reproduced and exploited without a licence or authorisation from the holder. The varieties affected were from the species *Prunus persica* (peach-nectarine), fourteen cases; gypsophila, one case; and *cicer arietinum* (chickpea), one case.

The procedure in general has been as follows: after confirming the varietal identity from samples of fruit taken on the market, or detecting a suspicious planting by other means, the Court has been asked to allow preliminary proceedings for the verification of the facts in civil jurisdiction, involving the taking of samples from the plants by court officials, and varietal identification analysis of those samples. The suitability has been argued of using the technique described of genetic identification by molecular markers, because of its speed and economy, using for this all the data available in the BDGIVV created in the agreement with the IRTA.

The Courts have in all cases accepted that these analyses be carried out, and their full evidential value, even on several occasions with the intervention of independent specialists appointed by the Court. In just three cases has a defendant questioned the independence or neutrality of the IRTA, as a GESLIVE member (along with the holders of the varieties which were the subject of the procedures). In two cases, the Appeal Court has found that, given the technical complexity of the matter, the IRTA evidence has full legal value, although the defendant is able to seek or carry out a comparative analysis in the main proceeding.

In the sixteen (16) cases using genetic identification data evidence of the variety, ten (10) are already over, with a decision finding that the protected variety defended by GESLIVE and the variety exploited by the defendant coincide. The others are at various stages, although a majority have been settled in out-of-court agreements between the parties. There has been a final decision in one case, ordering the producer responsible for the planting to dig up the protected variety plants cultivated in breach of its holder's rights and to indemnify them with the royalties proportional to the period of time that the operation lasted.

Attached is the model of the report submitted by the IRTA in these cases, containing and evaluating the genetic identification analyses run on the varieties. As can be seen, the analysis gives a summary description of the technology and methodology used and, of particular importance, the findings on the differences or the identity of the samples analysed. If negative (different markers), the varieties are



deemed to differ. If on the contrary there is no distinction, the conclusion is always drawn statistically as **“the probability  $P$  of finding at random another variety of the same species with the same DNA profile, with the markers and the methodology used”**. In all the cases considered, this probability is hugely reduced (one of several trillions), enabling the courts to decide that the variety to which the allegedly illegal plants belong is the same as the protected variety with which it was compared.

### **Defence of Plant variety Rights through DNA identification:**

As we have said, the IRTA database has permitted GESLIVE systematically control possible breaches of plant breeders' rights both at the field and market, taking hundred samples of fruit or leaves to find out whether their genetic profile corresponds to that of any of the varieties in the database.

We must emphasise that, as a consequence of the court proceedings described above and market controls made, part of the Spanish fruit and cut flower production sector has decided to regularise its situation, on a massive scale, with the conclusion of general regularisation agreements involving the competent public authorities. These processes (Andalusia 2003; Murcia 2005/2006) have required the creation of Land Registers detailing existing plots, including the plant variety, with all parties agreeing to use genetic analysis technology using molecular markers as a simple and economic means of identification.

This has allowed large-scale use of this technology (more than 400 samples in the regularisation implemented in Andalusia) and an adequate evaluation of its results, while also making it possible to define some of its current limits, referred to subsequently.

### **Conclusions**

- Distinctness or identification of plant varieties using DNA-based techniques may prove an essential tool for the effective enforcement of plant breeders' rights in many species.
- The validity of these techniques as evidence has been accepted in several cases by the Spanish Courts, based on statistical calculation of the probability of error.
- However, given the current state of the technology, such evidence is most uneven in its reliability and value for different species, very high for the *prunus* genus, high for other rosaceae (*malus*, *fragaria*, *rosa*) and species like *Dianthus*, *Vitis*, etc.; reduced for *Citrus* (orange, tangerin, etc.), and of limited value for species sexually reproduced as cereals.
- Even for species where these analyses provide a high standard of reliability (as *prunus*), errors cannot be discarded as a consequence of the extreme proximity of varieties as a result of mutations, very close lines, retro-crossing, etc. Some of these have arisen in practice in GESLIVE, so that the IRTA has had to develop specific molecular markers to try to fix differences between such close varieties, or use additional morphological and phenotypic tests to distinguish varieties.
- However, the reality and utility of these techniques, and growing difficulties in the handling of reference collections comprising living plants, point unquestionably to their serious consideration by the UPOV and the European authorities. As the technique is perfected, offering greater precision and guarantees in its results, the legislation in place must include such advances, incorporating these techniques for DUS evidence, particularly in distinctness between varieties.

**Thank you very much for your attention**

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