

**9th ICABR International Conference  
on**

**Agricultural Biotechnology: Ten Years Later**

**Ravello (Italy), July 6 to 10, 2005**

**Genetic traceability along the agrofood chain: the example of fig cultivar “Fico Bianco del Cilento”**

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In recent times, the accurate and timely traceability of products and activities in the supply chain has become a new factor in food and agribusiness. Increasingly, consumers in many parts of the world demand for verifiable evidence of traceability as an important criterion of food product quality and safety. In this study, we propose the genetic traceability of “Fico Bianco del Cilento” as an experimental model since it has an important role in the agricultural economy of the Campania region of Southern Italy, the major Italian geographic area for the production of edible figs. We developed a procedure to extract and amplify by PCR DNA obtained from fig leaves, dried figs and fig jam.

Key words: RAPD-PCR, genetic traceability, fig, jam, dried fig, molecular markers, agrofood chain.

## INTRODUCTION

There is an increasing demand for traceability in agrofood products. The ability to discriminate different plant varieties is of great importance for quality control and the goal will be to identify possible molecular markers as a fast and efficient tool for such analysis. Thanks to modern techniques in genetics, researchers are now able to produce the molecular identity cards of different agricultural varieties. Molecular markers are of extreme importance to traceability since they allow the fate of a given item to be monitored at each stage in the food chain from the farmer to the shop in order to certify the origin and quality of products on the market and prevent fraudulent commercial activity.

In the past few years, we focused our efforts on developing a technique that could be used to facilitate the genetic traceability of farm produce. Current research builds on our experience in the extraction of DNA from diverse plant materials (Galderisi et al., 1998; Galderisi et al., 1999); we decided to develop an experimental protocol to extract and analyze DNA from fig leaves, dried figs and fig jam using as experimental model the “Fico Bianco del Cilento”. Campania is the most important region in Italy for the production of edible figs. The fig cultivar ‘Bianco del Cilento’ has unique characteristics that make it particularly suitable for drying and syrup production, and is still used in herbal and dietetic preparations for its medicinal and therapeutic virtues (D'Alessandro, 1991). Protection of the cultivar is of major importance to the Campania Region, and thus DNA fingerprint techniques are of great interest for its proper identification. The more widespread clonal variants of ‘Bianco del Cilento’ are designated clones 151, 250 and 356. These originated in different Campania areas and present several morphological and/or phenological differences, such as fruit dimensions and flowering time. Some other fig cultivars that occur in the region include ‘Dottato’, ‘Luminedda’, ‘Melagrana’, ‘Petrelli’ and ‘Zecola’. Although these cultivars do not have as great a commercial value as ‘Bianco del Cilento’, they also have clonal variants.

Standards for the identification of fig cultivars are primarily based on plant morphological traits. These, however, are generally unreliable indicators of plant genotype, and are influenced by environmental factors. It is now feasible to identify variations at the DNA level that are not necessarily expressed phenotypically. Such

variations can be identified by RAPD (Random Amplification Polymorphic DNA) (Hattemer, 1991; Kaneko et al., 1986; Powell et al., 1991; Sigurgeirsson et al., 1991), a DNA fingerprinting technique used to detect genomic polymorphisms. In the RAPD method, genomic DNA extracted from fresh leaves is amplified by polymerase chain reaction (PCR) under low stringency conditions using a single, short oligonucleotide primer of arbitrary sequence. The low stringency PCR conditions allow the primer to anneal to multiple sites on the genome, resulting in an array of amplified DNA fragments. Polymorphisms among individuals or closely related strains, such as different cultivars of a specie, are detected as differences in banding patterns either on an agarose or polyacrylamide gel (Caetano-Anolles et al., 1991; Castiglione et al., 1993; Demeke et al., 1993; Mulcahy et al., 1993).

To make a genetic analysis of these cultivars we had to confront a number of difficulties encountered in the extraction and purification of DNA. A large number of plant species produce secondary metabolites such as tannins, alkaloids, flavanoids, etc., that are present in the extract and can interfere with DNA analysis such as DNA restriction, amplification and cloning (Loomis, 1974; Porebski et al., 1997; Weishing et al., 1995). In addition, also for foodstuffs, in this case dried fig and fig jam, contain high levels of plant secondary metabolites or polysaccharide and polyphenol components due to some manufacturing processes.

## MATERIALS AND METHODS

### DNA extraction

DNA samples were extracted from fresh leaves, dried fig, and fig jam. Extraction procedure was repeated at least three times on each sample.

*Plant material.* In this study fresh leaves of *Ficus carica* clones analyzed belong to the cultivars 'Bianco del Cilento' (clones 151, 250, 356), 'Dottato' (clones 71, 156, 4, 240), 'Luminedda' (clones 383, 74), 'Melagrana' (clone 137), 'Petrelli' (clone 176) and 'Zecola' (clones 344, 264). All were from the collection of Vivaio Forestale di Foce Sele, Salerno, Campania region, Italy. Each clone derives from a tree population that was obtained by vegetative propagation. The different populations are located in separate areas of the region.

Samples of dried figs and jams from three different factory farm producers were used. Dried figs were obtained from ripe fruit dried naturally. We analyzed two kinds of fig jams differing only in sugar content: one was free of added sugar, the other was prepared with 150 g cane sugar per kg of figs. Different procedures were tested to improve extraction yield and to remove Taq polymerase inhibitors.

Fresh leaves (0.5 g) were ground to powder in liquid nitrogen. The powder was dissolved in homogenization buffer (1.4 M NaCl, 2% w/v CTAB, 200 mM Tris-HCl pH 7.5, 20 mM EDTA pH 8.0, 2% 2-mercaptoethanol) and incubated at 60°C for 30 min. The mixture was then extracted with an equal volume of chloroform: butanol (24:1), and the aqueous phase was precipitated with 100% isopropanol at -20°C. To achieve further purification, the resulting DNA pellet was dissolved in homogenization buffer again and the above procedure was repeated at least once. The resulting DNA was solubilized in bi-distilled water and treated with 5 g/ml of RNase A for 60 min at 37°C.

To obtain DNA from 0.5 g of dried fig and jam the same procedure was used with a key change: 60 µl of N-phenacylthiazolium bromide (PTB) 10% solution, a reagent that cleaves macromolecule-derived protein cross-links (Poinar et al., 1998; Vasan et al., 1996), was added to the extraction mixture to release DNA that might be trapped within sugar-derived condensation products.

As sugar molecules can migrate in agarose gels and can interact with ethidium bromide, mimicking the behavior of DNA molecules, we needed to verify that the bands

visualized on the agarose gel were in fact DNA. To do this, we treated extracted DNA samples with DNase I before loading on agarose gel. DNase I treatment completely removed the bands on the gels demonstrating that they were DNA (data not shown).

#### DNase I treatment

1µg of extracted DNA was treated with 1.5 units of DNase I for 10 minutes at 37°C in 50 µl of a buffer containing 40mM Tris-HCl (pH 7.9 at 25°C), 10mM NaCl, 6mM MgCl<sub>2</sub>, 10mM CaCl<sub>2</sub>.

RAPD DNA amplification The twenty designed primers (U1-U20) tested in this study are shown in table 1.

The PCR reaction was performed in 50 µL solution containing 10 mM Tris-HCl pH 8.3, 10 mM KCl, 3 mM MgCl<sub>2</sub>, 200 µM each of dATP, dCTP, dTTP, dGTP, 20 pmols of the primer, 2.5 U of Taq DNA polymerase, and 10 ng of fig DNA. Reactions were incubated at 94°C for 3 min and DNA amplification was performed for 45 cycles. Each cycle was at 94°C for 1 min, at 40°C for 1 min, at 72°C for 1 min. Each PCR analysis was repeated from three to five times; only reproducible amplification patterns were used for further analyses.

#### DNA amplification of 5S ribosomal spacer region

The 5S-ribosomal spacer region was amplified by PCR using the following primers:

Upper primer 13 5' TGTTTATCTTATCCGTTTTT 3'

Lower primer 83 5' GTAGCCGCGTTCGTGGGTCC 3'

PCR amplification was performed in 50 µl containing 10 mM Tris-HCl pH 8.3, 10 mM KCl, 3 mM MgCl<sub>2</sub>, 200 µM each of dNTP, 20 pmol of the primer, 1.25 U of Taq DNA polymerase and 2-5 µl of DNA. The mixture was assembled at 0°C, then transferred to a Perkin Elmer Cetus DNA Thermal Cycler, precooled at +4°C. Following incubation at 94°C for 3 min, DNA amplification was performed for 42 cycles. Each cycle was at 94°C for 45 sec, at 49°C for 45 sec, at 72°C for 45 sec. 1 µl of PCR product

was re-amplified under the same conditions for 20 cycles for sequencing. Re-amplified products were eluted from 1.2% agarose slices and directly sequenced using cycle sequencing dye terminator technology.

## RESULTS AND DISCUSSION

We have described here a molecular method which can be used to detect and to analyse genome traits of a given horticultural item at each stage from farm to market.

Initial problems were encountered in the extraction of sufficiently pure DNA from fresh leaves. The extraction methods based on single step of purification in the extraction buffer resulted in DNA not suitable for PCR, and in fact no amplifications were obtained.

Consequently the DNA pellet was dissolved again in homogenization buffer and the above procedure was repeated at least once in order to eliminate different molecules, such as tannins, alkaloids and flavones present in the extract. These compounds could interact with the double stranded DNA, interfering with PCR amplification (Bohm, 1987; De Jong, 1991).

High molecular weight DNA (>20 Kb) was obtained from fig leaves with the extraction protocol described in the method section. These DNA samples were free of Taq DNA polymerase inhibitors and could be amplified by RAPD-PCR reaction, as we have demonstrated.

Polymorphisms were observed among the 6 cultivars of *Ficus carica* using the RAPD procedure. Although the primers U8, U12 and U18 produced conserved amplified DNA fragments common to all the cultivars, other primers, such as U1, U3, U4, U11 and U14 yielded RAPD bands useful for identification (data not shown). The number of amplified DNA products was cultivar and primer dependent. A minimum of one (primer U13) and a maximum of 15 (primer U2) DNA fragments were observed. The size of amplified DNA bands ranged from approximately 200 to 1400 base pairs. The combination of primer U1, U3, U4, U11 and U14 allowed us to distinguish all the cultivars of *Ficus carica* analyzed (data not shown). In particular, 'Bianco del Cilento' was successfully identified by the RAPD fingerprint obtained with the primers U4 and U1. With primer U1 the 'Bianco del Cilento' clones 151, 250 and 356 (Fig. 1 Panel A, lanes *b*, *c* and *d*) gave identical RAPD profiles, while the clones of the other cultivars, such as 'Luminedda' 383 and 74 (Fig. 1 Panel A, lanes *i*, *k*) were quite different from each other. With the primer U4, the 'Bianco del Cilento' clones 151, 250 and 356 (Fig. 1 Panel B, lanes *b*, *c*, *d*) produced different amplification patterns. Their profiles were also

different from that of the remaining clones (lanes *a* and *e* to *m*). Other clonal variants were also identified with this primer. For example, the ‘Luminedda’ clones 383 and 74 (Fig. 1 Panel B, lanes *i*, *k*) were different from each other and from the clones of the other cultivars. Likewise, the ‘Zecola’ clones 344 and 264 (Fig. 1 Panel B, lanes *l*, *m*) were distinguishable from each other and from the other clonal variants. ‘Bianco del Cilento’ appeared to be the most homogenous cultivar.

We also established a procedure to extract and amplify by PCR, DNA from complex matrixes, such as fig jam and dried figs. We used the same procedure to extract DNA from dried figs and fig jams. However, at our first attempt we were unable to obtain DNA from tested samples (Fig. 2 A lane 3).

We believe that this failure could have been due to the presence of Maillard’s products (Poinar et al., 1998; Vasan et al., 1996). In fact, the high temperatures of industrial processing could allow the condensation of carbonyl groups of reducing sugars with primary amines (Maillard’s reaction). This may result in extensive cross-linking of macromolecules producing “tangles” of proteins and nucleic acids that prevent DNA extraction and/or amplification (Poinar et al., 1998; Vasan et al., 1996).

This problem was overcome with the presence of PTB in the extraction mixture and allowed the recovery of DNA from dried figs and fig jams (Fig. 2A and Fig. 2B). No differences were observed between the two different fig jam preparations (data not shown).

DNA extracted from these samples was highly fragmented, probably the result of the various industrial processing phases. The average size was about 500 bp (Fig. 2A and Fig. 2B).

As sugar molecules can migrate in agarose gels and can interact with ethidium bromide, mimicking the behavior of DNA molecules, we needed to verify that the bands visualized on the agarose gel were in fact DNA molecules. To do this we treated extracted DNA samples with DNase I before loading on agarose gel: DNase I treatment completely removed all the bands on the gels demonstrating that they were DNA (data not shown).

We performed PCR reactions on the extracted DNA samples to evaluate if they were free of contaminants, which could inhibit Taq DNA polymerase. RAPD method is

very useful to discriminate cultivars when DNA extraction is performed on raw material (leaves, seeds, roots).

This technique is not suitable on fragmented DNA extracted from complex matrixes, since reproducibility of RAPD patterns is highly dependent on intact genomic DNA. Moreover, DNA solution must be free of byproducts that could originate from industrial manufacturing.

For the above reasons, in order to estimate the amplificability of DNA extracted from complex matrixes (dried fig, fig jam), we carried out PCR reactions on genomic fig DNA with primers encompassing a known sequence. We amplified a fragment of intergenic region of 5S-ribosomal genes. These genes (approximately 120 bp in size) repeat in tandem with the non-transcribed spacer of about 200 bp.

The primers that we selected encompass a tract of 5S-ribosomal spacer region and annealing them to complementary regions in the spacer repeats should give rise to several amplicons with a progressive increase in size (laddering profile fig. 3).

PCR amplifications performed on DNA from fig leaves produced two bands, the sizes of which were 90 bp and 383 bp, respectively (Fig. 4 lanes 1, 3 and 5). We did not detect bands of higher molecular weight. The absence of amplicons greater than 383 bp could be due either to the PCR reaction profile and/or to intrinsic properties of the Taq DNA polymerase we used. PCR reactions performed on dried fig and fig jam DNA produced only 90 bp amplicons (Fig. 4 lanes 2, 4 and 7). This is probably related to the degradation of DNA induced from some manufacturing processes; therefore only low molecular weight DNA can be obtained from these matrixes and this restricts the targets that can be amplified. In fact, PCR reactions carried out on DNA obtained from jam and dried figs produced only small size amplicons.

Nevertheless, the extracted DNA molecules are “sufficiently pure” to be amplified by Taq DNA polymerase enzymes.

In conclusion the use of molecular methods to detect the genome traits of horticultural products offers the possibility to univocally identify a given item and follow its fate during the production phases.

Our future research objective is to identify molecular markers which could specifically and univocally discriminate between different fig cultivars. In this way, the

genetic traceability procedure we described would allow each stage of the fig jam and dried fig production process to be monitored and would also verify if a given product originates from a specific cultivar.

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- **FIGURE LEGENDS**

**Figure 1** - Agarose gel electrophoresis of DNA fragments obtained by RAPD amplification of different *Ficus carica* cultivars and clones with the primer U1 (panel A) or U4 (panel B). *a* - 'Dottato' clone 71; *b* - 'Bianco del Cilento' clone 151; *c* - 'Bianco del Cilento' clone 250; *d* - 'Bianco del Cilento' clone 356; *e* - 'Dottato' clone 156; *f* - 'Dottato' clone 4; *g* - 'Dottato' clone 240; *h* - 'Petrelli' clone 176; *i* - 'Lumincedda' clone 383; *j* - 'Melagrana' clone 137; *k* - 'Lumincedda' clone 74; *l* - 'Zecola' clone 344; *m* - 'Zecola' clone 264; *W* - 100 base pair ladder as molecular marker.

**Figure 2** - Agarose gel electrophoresis of genomic DNA extracted from complex matrixes. Panel A - lane 1: 100 bp molecular marker; lanes 2 and 3: DNA from fig jam extracted with and without PTB reagent, respectively.

Panel B - lanes 1 and 2: DNA from dried fig extracted with PTB reagent; lane 3: 1 Kb molecular marker.

**Figure 3** - Outline of 5S rRNA gene locus showing some of the tandem repeat units. The PCR primers that are complementary to specific regions of the intergenic spacer produce several amplicons with a progressive increase in size (in the example a 90 bp and 383 bp products).

**Figure 4** - Agarose gel electrophoresis of PCR reactions to amplify 5S rRNA gene repeat region. Lanes 1, 3 and 5: amplicons from fig leaves genomic DNA; lanes 2, 4 and 6: amplicons from dried fig genomic DNA; lane 7: amplicon from fig jam genomic DNA; lane 8: 100 bp molecular marker.

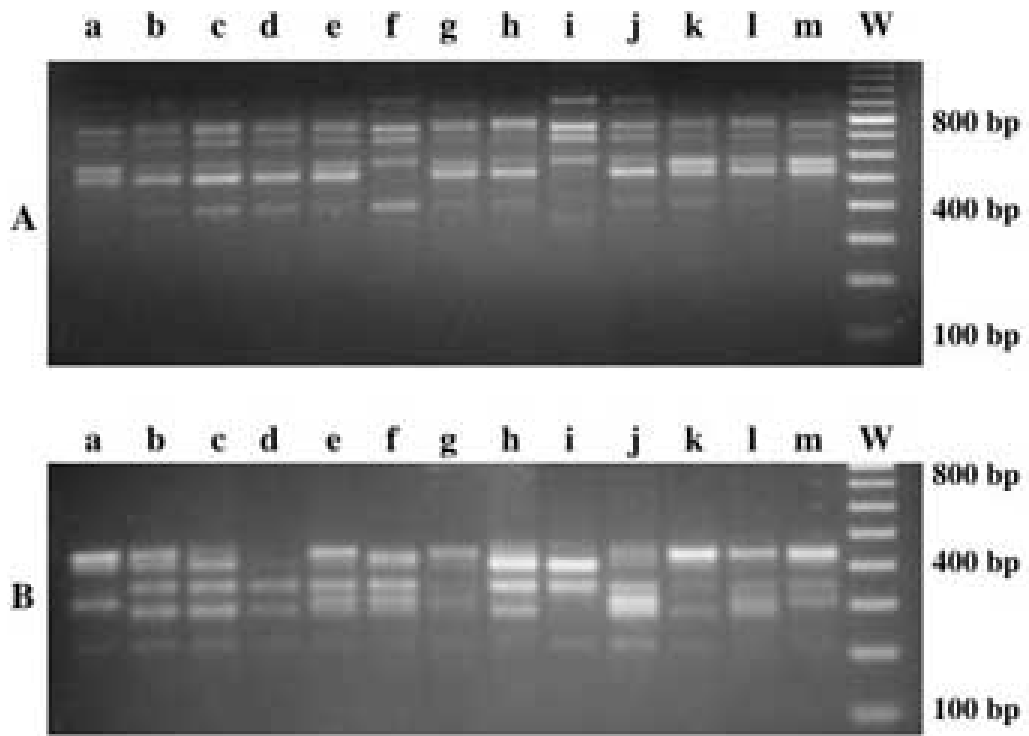


Figure 1

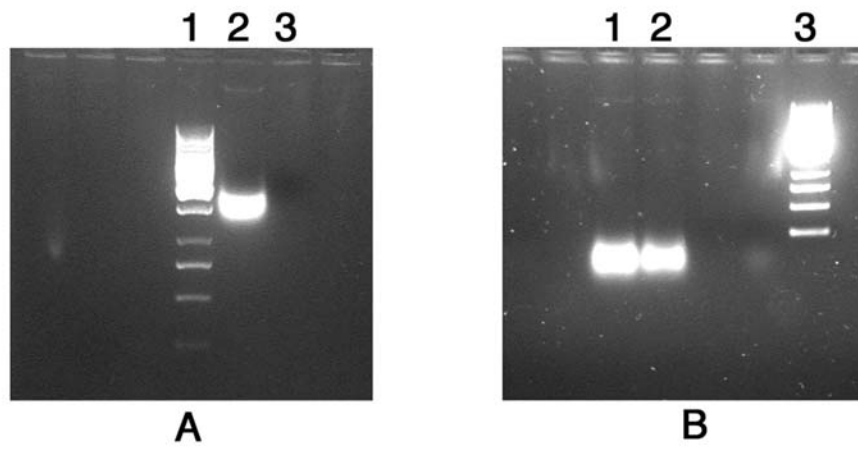


Figure 2

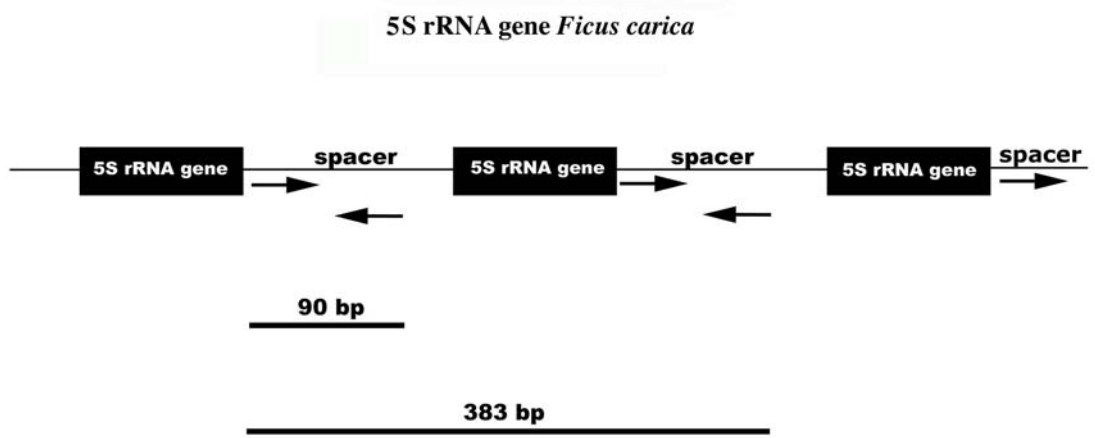


Figure 3

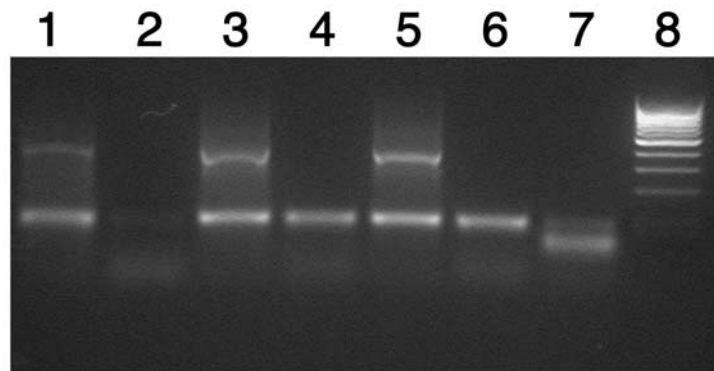


Figure 4