

DNA Isolation and AFLP Fingerprinting of Nectarine and Peach Varieties (*Prunus persica*)

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Abstract. Traditional identification of peach and nectarine varieties relies on the assessment of agronomic traits of the adult plant. This leads to a significant delay of time, constraints to breeders in the surveillance of germplasm and a risk for fruit growers and exporters. We describe a method for rapid assessment of peach and nectarine varieties based on AFLP fingerprinting and extraction of high quality DNA. The best primer pairs were selected from 64 primer combinations that reliably distinguished 8 peach and 6 nectarine varieties. A graphical representation of the detected polymorphisms was shown to simplify the analysis.

Key words: Amplified fragment length polymorphism, genetic markers, nectarine, peach

Abbreviations: AFLP, amplified fragment length polymorphism; APS, ammonium persulfate; CTAB, hexadecyltrimethylammonium bromide; f.wt., fresh weight; T4 PNK, bacteriophage T4 polynucleotide kinase; *Taq*, *Thermus aquaticus*; TBE, tris-borate-EDTA; TE, tris EDTA; TEMED, N,N,N',N' tetramethylethylenediamine.

Introduction

Fruit export is a very important area of Chilean agriculture. The market abroad has become increasingly difficult to access, due to competition with other countries, especially with others in the Southern Hemisphere. The ability to offer a large number of varieties would provide a good positioning in the international markets. The introduction of new varieties is supported by the

incorporation of Chile to the 1978 Act of the UPOV (Union for the Protection of New Varieties of Plants) on the 5th of January of 1996 under the decree law 19.342 that regulates the rights of the plant variety breeders.

Peaches and nectarines are important crops in Chile, some varieties having high economic value. These registered varieties are certified by the National Agricultural and Livestock Service (Servicio Agrícola y Ganadero, SAG) and in turn, the certification produces a bonus for exporters. Traditional methods of identification (field testing) rely on phenotypic traits that can be measured only at plant maturity and therefore take a long time to complete (3 to 5 years). Molecular analyses of genotypic markers offer an attractive alternative or complement to identification based on phenotypic characters and can be performed reliably at any stage of maturity, avoiding long study periods. We chose AFLP because it produces a larger number of markers than RAPD (Vos et al., 1990; Lin and Kuo, 1995; Lin et al., 1996) and also it requires no previous information about markers or genomic sequences. AFLP markers have been used to study populations, ecotypes and to construct linkage maps of numerous plant species (Alonso-Blanco et al., 1998; Waugh et al., 1997; Mackil et al., 1996)

Analysis of genomic variation requires unsheared, pure DNA as a starting material. We therefore developed a procedure based on critical aspects of published protocols (Doyle and Doyle, 1990; de la Cruz et al., 1997) that uses mature, healthy leaves to obtain high quality DNA.

Materials and Methods

Plant materials

Sampling of the selected varieties was carried out in different locations of the VI Region (Central Chile). Sample plants were collected from the Seed Department, Agricultural and Livestock Service, corresponding to 8 varieties of peaches (Rich Lady, Ryans Sun, September Sun, JRB, Fortune (FOR), Everst, Flavor Crest and Queen Crest) and 6 varieties of nectarines (Diamond Jewell, Artic Snow, Red Glenn, Royal Glow, Sparkling May and Ruby Diamond). Six shoots with mostly new leaves were taken from the four orientations from adult trees of each variety and kept at 8 °C during transport to the lab. The leaves were then separated from the branches and quickly frozen at -20 °C until use.

Solutions

- lysis buffer: 100 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 4% (w/v) SDS, 0.3 M NaCl, 0.14 M β -mercaptoethanol.
- CTAB buffer: 100 mM Tris HCl pH 8.0, 20 mM EDTA, 1.5 M, 8.4% (w/v) solution of CTAB.
- potassium acetate: 5 M potassium acetate, pH 5.2.
- phenol: chloroform: isoamyl alcohol, 25:24:1.
- ethanol 95%.
- ethanol 70%.
- Proteinase K 10 mg/mL (Gibco-BRL).
- Rnasa A 20 mg/mL (SIGMA).
- TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
- sodium acetate 3 M, pH 5.2.
- TBE 5X: 54 g Tris, 27.5 g boric acid, 4.65 g EDTA per liter.
- isopropanol.

DNA extraction

Whole leaves were quickly washed in distilled water, frozen in dry ice and ground with a mortar and pestle until a fine powder was obtained. The plant material was transferred to four Eppendorf tubes and after sublimation of the dry ice 0.6 mL of lysis buffer and 200 μ L buffer CTAB were added to each tube. The suspensions were incubated at 65 °C during 30 min with occasional mixing. After this incubation 160 μ L of 5 M potassium acetate were added to each tube and left on ice during 10 min. The lysed homogenate was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at full speed in a microfuge during 10 min. The DNA was precipitated at -20 °C for 30 min by adding 0.6 volume of cold isopropanol. After centrifugation at top speed for 10 min the pellet of DNA was washed with 70% ethanol, pelleting by centrifugation and dried for 3 min in SpeedVac. Finally, the DNA was resuspended in 50 μ L TE which was facilitated by incubating the solution at 65 °C for 10 min. The RNA was removed by the addition of 0.15 mg of Rnase A to each tube and was incubated at 37 °C during 40 min. 0.2 mg of proteinase K was then added and the solution incubated at 50 °C for 20 min. The fractions were pooled and the DNA was phenol extracted and centrifuged as before. The DNA was ethanol precipitated in a 1/10 volume of 3 M sodium acetate for 1 h at -20 °C. After ethanol 70% washing, the DNA was centrifuged and finally resuspended in 50 μ L double distilled water.

Restricted DNA (digestions were carried out at 37 °C for 2 h) and control DNA preparations were analyzed on 1% agarose gels using 0.5X TBE buffer and stained with ethidium bromide.

AFLP reactions

AFLP products were generated using the AFLP Analysis System I from Gibco-BRL (Gaithersburg, Md., USA), according to the manufacturer instructions. *Taq* Polymerase (Gibco-BRL) was used for all PCR reactions. Primers were radio-labelled with γ -³³P[ATP] (2000 Ci/mmol) from NEN (Boston, USA). AFLP reactions were carried out according to the instructions included in the kit. Control samples of tomato DNA included in the AFLP kit were run in parallel with peach samples during the initial stages of this study. The AFLP products were separated on 5.8% or 6.0% (w/v) acrylamide gels. Aliquots of radiolabelled 25 bp ladder (Gibco-BRL) were loaded on both sides of gels as size molecular markers. The gel was pre-warmed at 50 °C, and 5 μ L samples were loaded and the gel was run at 70 to 80 W (1700–1800 V) in TBE 1.0X on a vertical gel electrophoresis system (model S2001, Life Technologies-Gibco-BRL). After electrophoresis the gel was transferred to chromatography paper, dried on a gel dryer (model 583, BioRad) and exposed to Kodak X-Omat AR film (Eastman Kodak Company, Rochester, USA) during 48 h.

AFLP analysis

Banding patterns of all peach and nectarine varieties were analyzed by scoring the relative migration of all major bands. The 2600 bp band and the 75 bp from the 25 bp ladder (Gibco-BRL) were used as reference points and the relative size of all bands was calculated. Graphical representation of logarithm in base ten of the molecular size marker versus the relative size of the bands was carried out using Microsoft Excel software version 5.0.

Results and Discussion

DNA isolation and purity

DNA extraction was assayed for 10, 5, 1 and 0.5 g of leaves. Yields were optimal when 0.4 to 0.8 g of tissue were processed. Typically the total yield of pure unshered DNA was 20 to 40 μ g g⁻¹ f.wt., which corresponds to one or two leaves. Purified DNA appeared as one symmetrical band of average size approximately equal to the largest marker DNA of λ Hind III (i.e., 23 kb), and with little or no trailing to lower molecular weights.

Table 1. Comparison of two peach varieties using all primer combinations.

	M-AAC	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	☐☐	☐	☐	☐☐	NR	☐☐	☐☐	☐☐
E-AAG	NR	NR	NR	NR	NR	NR	NR	NR
E-ACA	NR	NR	NR	NR	NR	NR	NR	NR
E-ACC	☐	☐	☐	☐☐	☐☐	☐☐	☐	☐
E-ACG	-	-	☐	☐	-	-	NR	NR
E-ACT	NR	☐	☐	NR	NR	☐	NR	NR
E-AGC	☐☐	☐	NR	☐	-	☐	-	☐☐
E-AGG	-	-	-	-	NR	-	NR	☐

NR = No reaction

- = 4 or less polymorphic bands among both peach varieties

☐ = 5 or more polymorphic bands when comparing both peach varieties

☐☐ = primer combinations suitable for the detection of the polymorphic AFLP patterns.




















Before proceeding with AFLP, the suitability for restriction digestion of the different preparations was checked using *EcoR* I (Gibco-BRL). DNA from all cultivars was successfully digested (results not shown).

AFLP analysis

In preliminary analysis, all 64 primer combinations were assayed using two peach varieties (Rich Lady and September Sun) and two nectarine varieties (Red Glenn and Artic Snow) to determine the appropriate primer pair combination. The number of bands produced was highly variable, ranging between 0 and 60 bands. As shown in Table 1, the comparison of two peach varieties produces only 37 combinations of primers that showed banding with both cultivars. Of these, 27 pairs detected five or more clear differences between the two varieties used at this stage. Ten primer pairs detected over 20 differential bands between the two varieties, making these the most suitable for the analysis of peach varieties.


The two nectarine varieties used for the preliminary primer pair selection showed a different AFLP pattern. As summarized in Table 2, all but 5 primer pairs produced AFLP patterns with both tested cultivars, however only 19 primer combinations actually showed 5 or more clearly different bands for both varieties. In general, in nectarines the mean number of bands differentiating the two test varieties was significantly lower than in the tested peach varieties. Therefore only six primer pairs, which detected between 8 and 17 clear differences between the two tested cultivars, were considered to be the most appropriate for the analysis of nectarine varieties.


Table 2. Comparison of two nectarine varieties using all primer combinations.

	M-AAC	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	-		-	-	-	-	-	-
E-AAG	-	-	-	NR				
E-ACA			-			NR		-
E-ACC			-	-	-		-	
E-ACG	-						-	-
E-ACT	-	-	-	-	NR	-	NR	NR
E-AGC	-	-	-	-	-	-	-	-
E-AGG	-	-	-	-	-	-	-	-

NR = No reaction

- = 4 or less polymorphic bands among both nectarine varieties

 = 5 or more polymorphic bands when comparing both nectarine varieties

 = primer combinations suitable for the detection of the polymorphic AFLP patterns.

Determination of polymorphism for different peach and nectarine varieties

Figure 1A shows the results obtained from AFLP analysis of 4 peach varieties using six pairs of primers, which included 5 of the primer combinations that seemed appropriate from the initial selection of primers. All primer pairs showed different banding patterns for these peach cultivars. Figure 1B shows the banding pattern of the four remaining peach varieties. Cultivars FOR and JRB are closely related, since JRB is a stable mutant originally derived from one branch of a FOR cultivar, which presented early ripening of fruits. As indicated by the arrows, both varieties can be easily differentiated by several primer pair combinations, in spite of their close genetic relationship.

In a similar assay, Figure 2 shows the results obtained from AFLP analysis of 6 nectarine varieties using five primer pairs selected from the 19 combinations that showed clear differences at the initial stage of primer pair selection. The 5 primer pairs selected included four of the combinations that appeared most suitable for AFLP analysis of nectarines. Again, all primer pairs showed different patterns for all the nectarine varieties, indicating that the preliminary selection of primers was quite useful as a guideline.

From this analysis a primer pair was selected that permitted the identification of all tested peach and nectarine varieties. The primer pair E-ACC/M-CTC is able to differentiate between the 14 cultivars, and is consequently the preferred pair in our hands for AFLP analysis of this species.

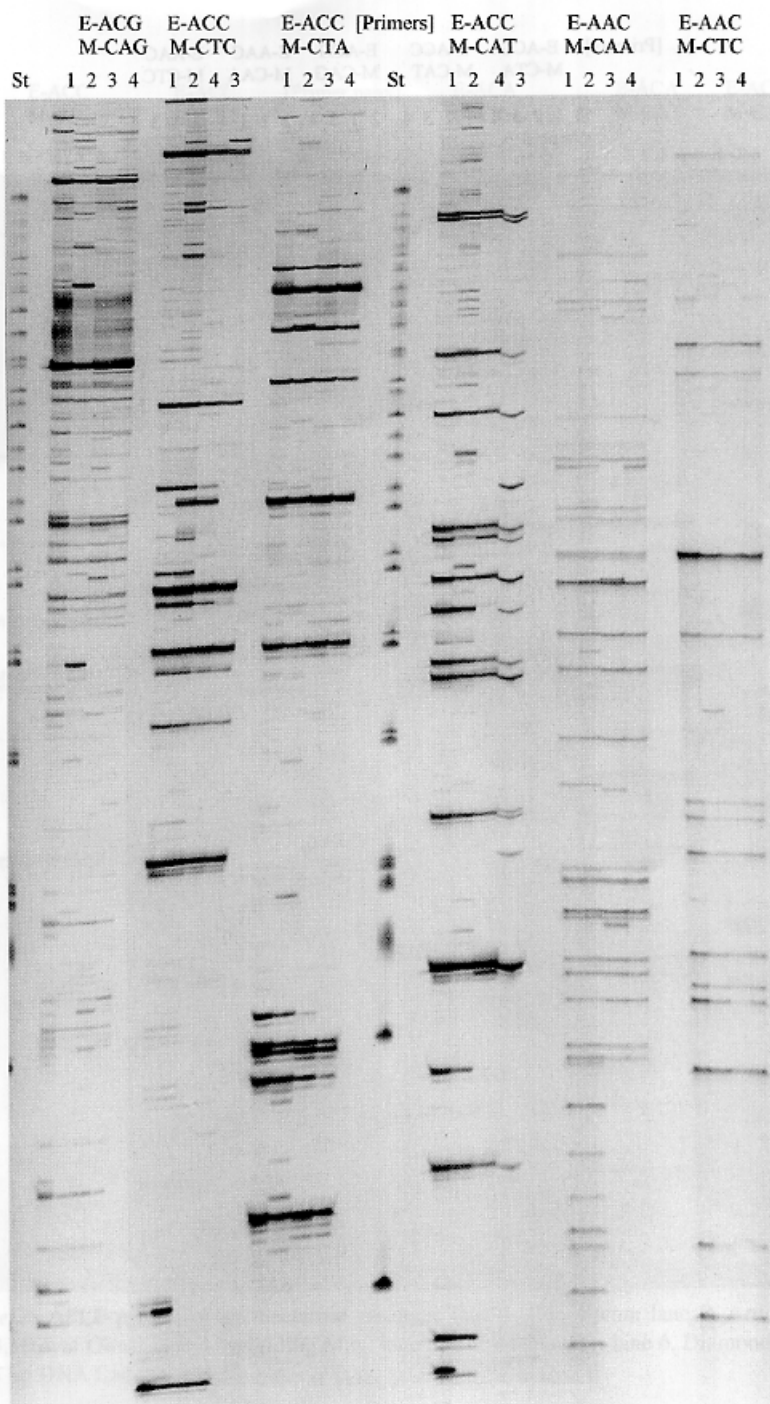


Figure 1a. AFLP patterns obtained from different peach varieties. (A) varieties are: lane 1, September Sun; lane 2, Rich Lady; lane 3, Ryans Sun; lane 4, Queen Crest; St, 25 bp DNA Ladder. (B) varieties are: lane 1, Everst; lane 2, Flavor Crest; lane 3, JRB; lane 4, Fortuna; St, 25 bp DNA Ladder.

[Primers] E-ACC E-ACC E-ACG E-AAC E-AAC
M-CTA M-CAT M-CAG M-CAA M-CTC

St 1 2 3 4 1 2 3 4 2 1 3 4 2 1 3 4 2 1 3 4

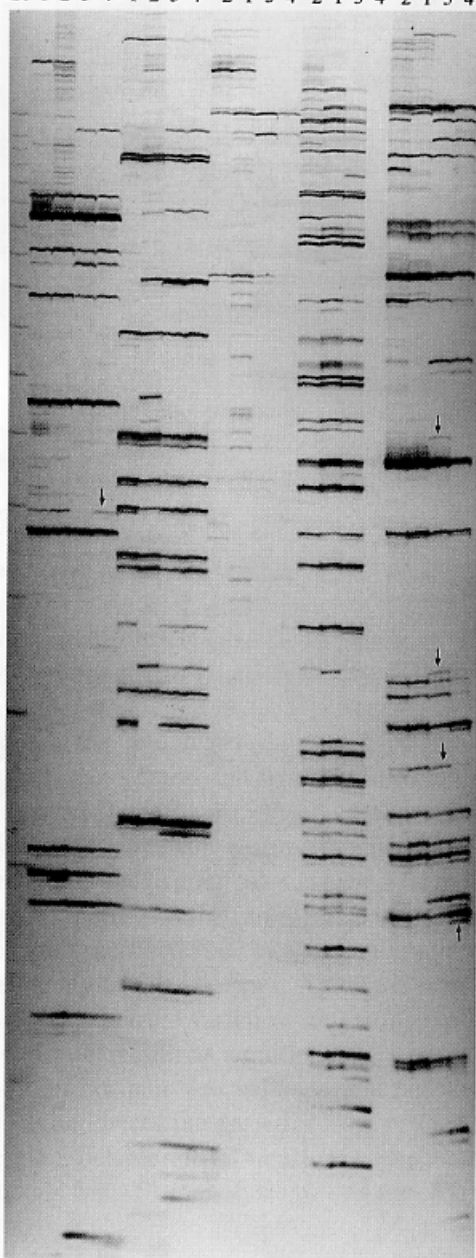


Figure 1b.

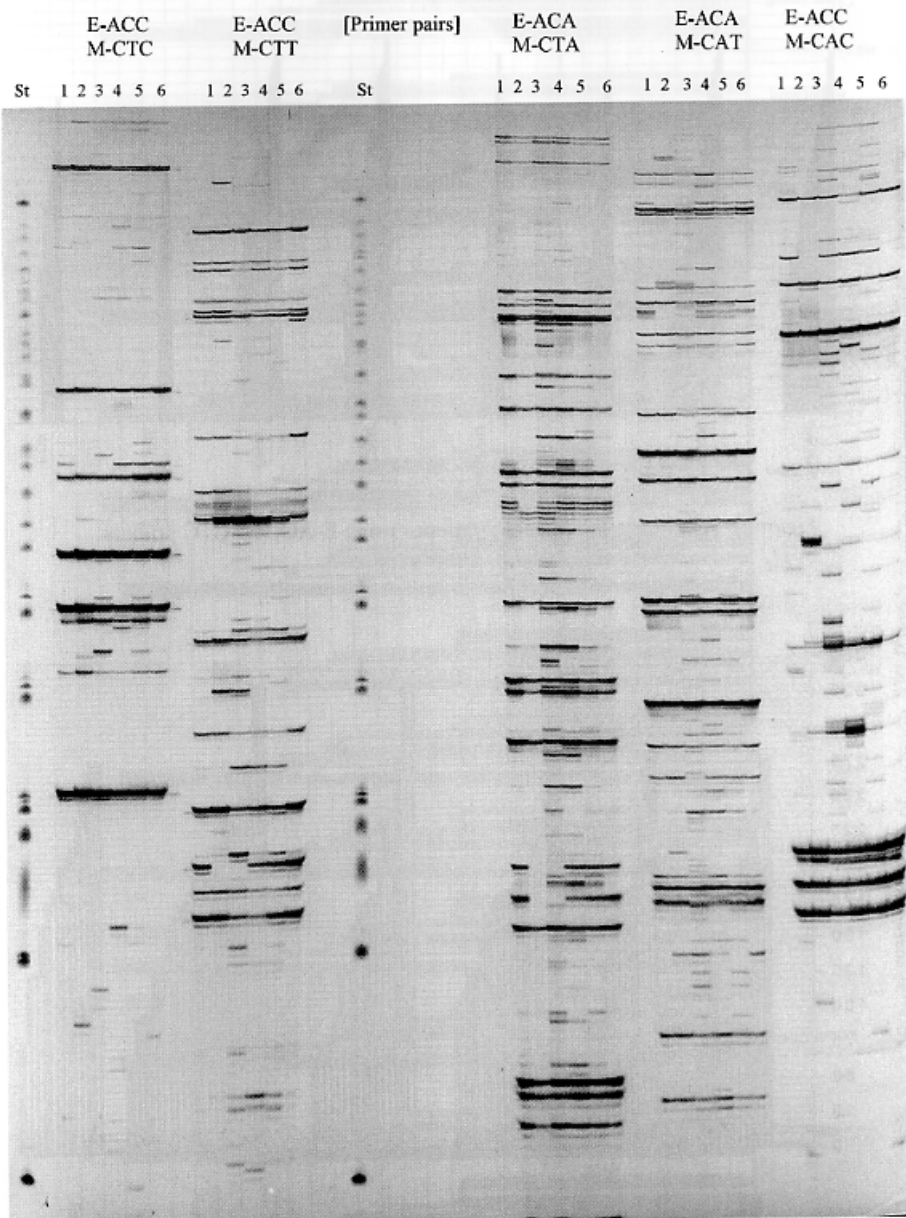


Figure 2. AFLP pattern of six nectarine varieties: lane 1, Red Glenn; lane 2, Artic Snow; lane 3, Royal Glow; lane 4, Sparkling May; lane 5, Ruby Diamond; lane 6, Diamond Jewell; St: 25 bp DNA Ladder.

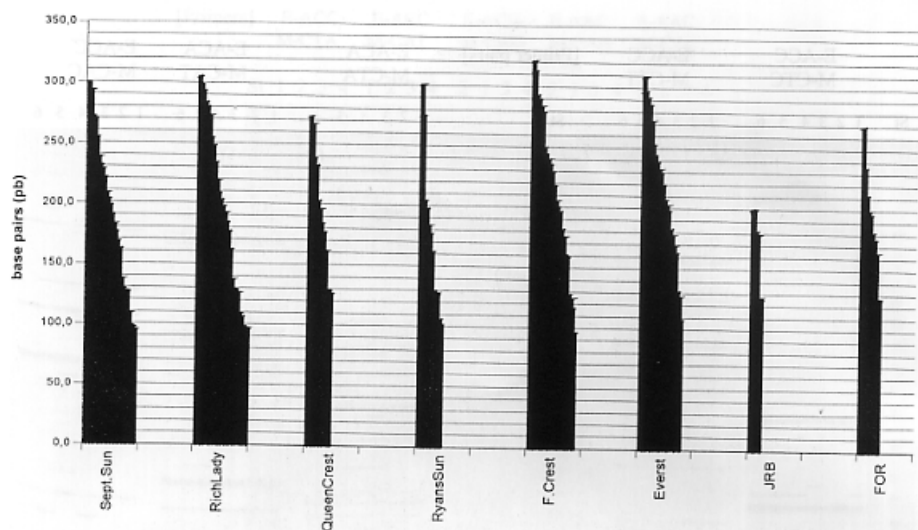


Figure 3. AFLP patterns of peach varieties using E-ACC/M-CTC primers.

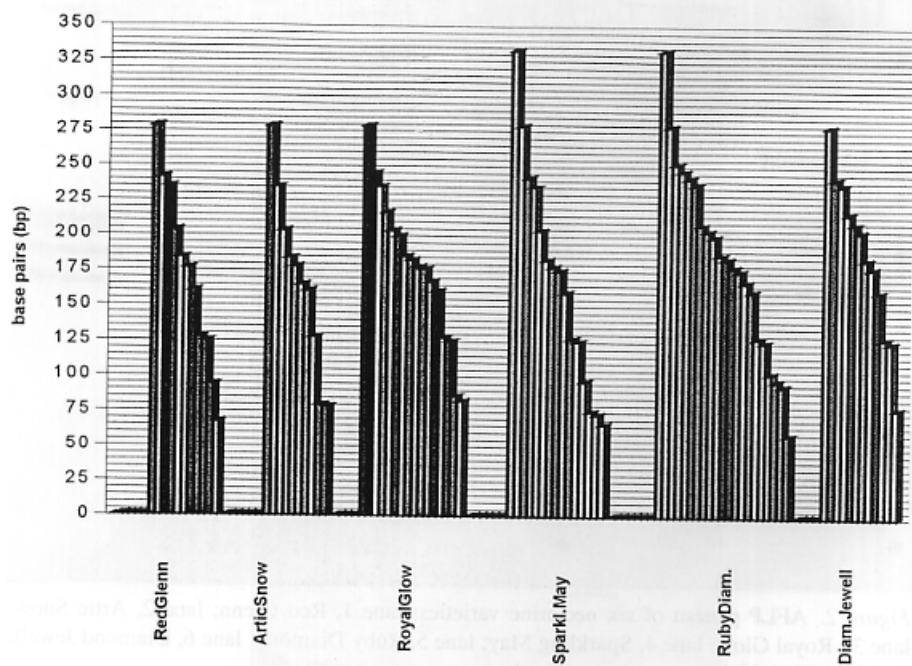


Figure 4. AFLP patterns of nectarine varieties using the E-ACC/M-CTC primers.

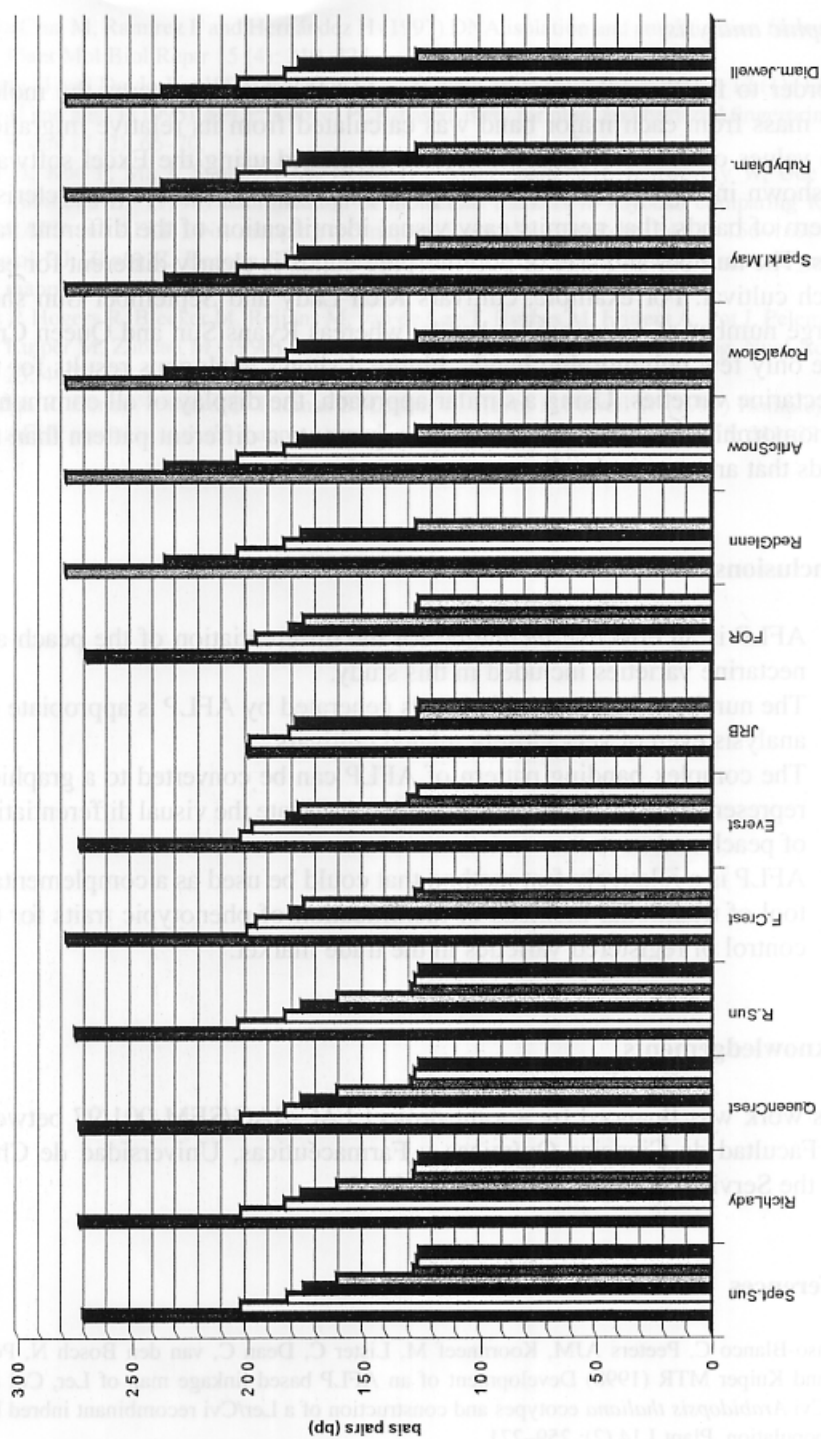


Figure 5. Comparison of common AFLP bands of peach and nectarine varieties.

Graphic analysis

In order to facilitate the analysis of the AFLP banding patterns the molecular mass from each major band was calculated from its relative migration. The values of the molecular mass were displayed using the Excel software. As shown in Figure 3, each of the peach cultivars presents a characteristic pattern of bands, that permits easy visual identification of the different varieties. The number and size of polymorphic bands is clearly different for each peach cultivar. For example, cultivars Rich Lady and September Sun show a large number of polymorphic bands, whereas Ryans Sun and Queen Crest have only few polymorphic bands. Figure 4 shows analogous results for the 6 nectarine varieties. Using a similar approach, the display of all common or monomorphic bands for peach varieties, presents a different pattern than the bands that are present in all nectarine varieties (Figure 5).

Conclusions

- AFLP is an effective technique for the differentiation of the peach and nectarine varieties included in this study.
- The number of polymorphic bands generated by AFLP is appropriate for analysis even of very closely related cultivars.
- The complex banding pattern of AFLP can be converted to a graphical representation of bands or profiles that facilitate the visual differentiation of peach and nectarine varieties.
- AFLP is a relatively fast method that could be used as a complementary tool of traditional methods of identification of phenotypic traits for the control of registered varieties in the trade market.

Acknowledgements

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