AES/CE MAR 84

Workgroup/Department: Pistachio/Pomology

University of California
Division of Agricultural Sciences

Final REPORT

Pistachio Research Board

Project Year 2001 Anticipated Duration of Project 2 years (completed)

Project Leader , Dr. Darush Struss, Dr. Stephen Southwick Location _____

Cooperating Personnel Dr. Louise Ferguson, Dr. Bob Klein, Dr. Riaz Ahmad, Ron Petty,

Project Title Part 1. Development of microsatellite and AFLP markers to determine genetic variation in pistachio fruit for maintenance of product purity. Part 2. Use molecular markers for differentiation of pistachio fruit from different countries of origin.

Keywords Product purity, DNA fingerprinting, genetic markers, breeding, clones
Commodity(s) Pistachio Relevant AES/CE Project No._____

Objectives:

Part 1. Develop molecular markers for pistachio for use in determining variation in roasted and sun-dried pistachio nuts and shell.

Part 2. Utilize markers to reveal genetic variation among pistachio cultivars.

Summary Results:

Microsatellite markers have been developed by constructing a Lambda phage and an enriched library from the genomic DNA of cultivar 'Kerman'. Ten of these markers could distinguish between the cultivar ' Kerman' from California and Iranian pistachio cultivars. An efficient DNA extraction method has been developed to obtain DNA from pistachio nuts and shell. We also developed ISSR markers for cultivar identification in Pistachio.

Isolation of Microsatellite sequences in Pistachio

A Lambda phage library have been constructed for isolating microsatellite sequences from the pistachio genome according to Struss and Plieske (1998). Genomic DNA of cultivar 'Kerman' has been digested with methyl sensitive restriction enzyme *Pst I* . The low molecular weight fraction was isolated from an agarose gel, followed by subsequent digestion with MboI, and cloned into the BamHI site of the Lambda phage vector ZAP Express (Stratagene). The library was screened for the presence of repeats by hybridization with (GA) n and (GT) n which were labeled with 32P-cATP.
Screening of the library has been performed by hybridization under stringent conditions with dinucleotide repeats $(GA)_n$ and $(GT)_n$. A total of 60 clones were sequenced. For the sequences containing a microsatellite locus primers were designed.

An enrichment protocol developed by Hamilton et al (1999) was used with some modification to improve the efficiency of library for microsatellites. Genomic DNA was extracted from leaf tissue of pistachio cultivar 'Kerman' by Qiagen maxi prep according to the manufacturer's protocol. Approximately 15 µg of DNA was digested with restriction enzyme Rsa1 and Nhe1 which resulted in DNA fragments at the range of 300-800bp. These DNA fragments were cleaned with Qia Quick DNA purification column (50ul final elution) and dephosphorylated with Calf intestinal phosphatase (CIP) at 37°C for two hours and re-cleaned with Qiaquick DNA column (Qiagen) with 30ul final elution.

To generate an adaptor, SNX F and SNX R (Phosphorylated) was hybridized in a heating block set at 80°C and cooled slowly over 1hour. Ligation of 99 pmol double-stranded adaptors to 33 pmol blunt ended DNA fragments was done in the presence of restriction enzyme Xmn1 that cuts any SNX linker dimmers. PCR was performed on the linker ligated DNA and run on 1.5% agarose gel. A smear at the range of 300-800 bp showed success in ligation process.
Linker ligated DNA was hybridized to biotinylated repeat oligo $(CA)_n$ and $(CT)_n$. 50µl of streptaviodin coated magnetic beads were prepared according to the manufacture protocol and biotinylated hybridized DNA fragments were added to it. After incubation at 43°C for several hours, magnetic beads were washed twice with 200µl, 2xSSC, 3times with 200µl 1xSSC and 3times with 200µl with 0.5 µl 0.5xSSC. Final wash was performed by shifting magnetic beads to a new tube and washing with 200µl 0.5xSSC solution. Each incubation was for five minutes at 48°C. A high power magnetic stand was used each time to separate magnetic beads and washing solution. After final washing, 30µl low TE buffer was added to magnetic beads and incubated at 98°C for 15 minutes, then quickly centrifuged for a few seconds, put in the magnetic stand and when the magnetic beads were out of the way, TE buffer was removed which became elution 1. The procedure was repeated with 60 µl of TE buffer, which became elution 2. Next 30 µl TE buffer was added to the magnetic beads and stored at -4°C for future use. PCR was conducted on the enriched DNA by taking different dilution. A light smear at the range of 300-800 bp on 1.5% agarose gel showed success in enrichment procedure.
Amplified enriched DNA was cleaned with Qia quick DNA purification kit (Qiagen).To get more efficient transformation with the TA cloning kit (Invitrogen, Carlsbad, CA, USA) , 1µl taq DNA polymerase ,with dATP , 10x PCR buffer was added to the enriched DNA , incubated for 10 minutes and directly added to the vector pCR2.1 Toppo cloning vector (Invitrogen, Carlsbad, CA, USA) and Ecoli competent cells (Top 10, Invitrogen) according to the manufacturer's protocol To check the insert size, white colonies were transferred to tubes containing 20ul of 10mM Tris -HCl pH 8.5 and incubated for 10min at 95°C, and 0.5 µl was used as a template in PCR amplification with two vector primers. At the same time colonies were transfered to LB kanamycin freezing medium. Plasmids were isolated and sequencing was performed by using M13 (-21)F primer. In some cases when the insert was long , re-sequencing was done by using M13R primer. For each unique microsatellite from both the enriched and non enriched libraries that contained sufficient reliable flanking sequence, PCR primers were selected using the primer selection computer program Primer 5.0 (Lander ,Cambridge, Mass,USA).The forward primers were labeled withIR800 to be detected on an automated DNA sequencer (LiCor).

PCR was carried out under the following conditions: 100-150 ng of template DNA, 250 nM of each primer, 200 µM of dNTPs, 1 U of Taq Polymerase, 1.5-2 mM of MgCl 2 . The reaction, depending on the primer pair, was run for 35-45 cycles (denaturing at 94°C for 1 min, annealing at 55°C or 60°C for 1 min, with a 2-min extension at 72°C), followed by a single extension at 72°C for 60 min. The amplification products were detected on 6 % polyacrylamide gels using a Li-Cor DNA sequencer.

DNA extraction from nuts and shell

The nuts and shells were ground to a useable powder for DNA extraction a with a small grinder. Approximately 200 mg of powdered nuts and shell were transferred into Eppendorf tubes. We used two different methods to extract the DNA.
A)- The extraction methods were the same as the extraction method for leaves using CTAB methods with minor differences. 200 mg of pistachio nut powder was transferred into a 1.5ml Eppendorf tube. 500 µl preheated

extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20mM EDTA, 1% PVP-40 and 10mM DTT) was added to the powder. The samples were incubated for 30 minutes at 65°C, extracted with 500µl chloroform-isoamyl alcohol (24:1), centrifuged at 13000 rpm for 5 minutes. The resulting supernatant was transferred into a new 1.5ml Eppendorf tube adding 500µl phenol, incubated for five minutes and centrifuged at 13 K for 5 minutes. The upper phase was removed and the milky phase collected and transferred into a new tube adding 500µl chloroform centrifuging for 5 minutes at 13000 rpm. The aqueous phase was mixed with an equal volume of ice-cold 2-isopropanol and centrifuged for 10 minutes at 13 rpm. The supernatant was discarded and the pellet was washed with 1ml ice-cold 70% ethanol. The dried DNA pellet was re-suspended in 50µl distilled water and stored at 4°C.
B) We also used QIAGENE DNeasy plant mini kit for DNA extraction from nuts following the manufacturer's protocol. With these protocols, we were able to extract DNA from nuts with sufficient quality.

The extracted DNA from nuts and shell was purified by adding 1/10 volume 3M sodium acetate and 2 volumes 100% ethanol by subsequent storage at -20°C for an hour. The samples were centrifuged at 13000 rpm for 15 minutes and the pellets were washed 2 times with 70% ethanol. The pellets were air dried and resolved in 50µl TE buffer. Quantification of DNA was performed with ethidium bromide stained agarose gel (Fig 1).

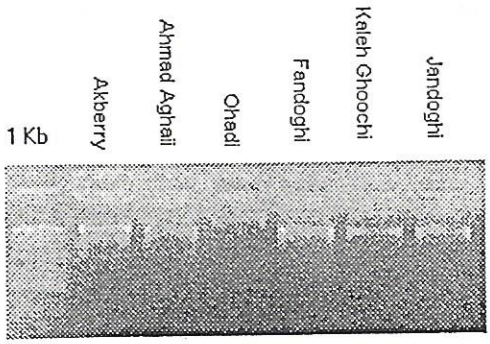

Figure 1: Extracted DNA from six Iranian cultivars.

Results

The extracted DNA from nuts and shell was in sufficient quality to conduct marker analysis. Extraction of DNA from roasted and salted nuts isn't always successful. DNA extraction from the nutshell is clearly a better alternative, since the salt and other ingredients can be washed out by soaking the shells for several hours in water and by occasional changing of the water. The other advantage for using the nutshell is the maternal origin of the nutshell which reflects exactly the same genetic constitution as the female parent. That makes it easy to compare the California pistachio, which is the cultivar 'Kerman' with those of foreign origin. Since generally the male parents of the foreign pistachio remain unknown.
A total of 10 primer pairs flanking microsatellite loci were chosen to analyze the selected cultivars and genotypes. These primers reveal polymorphism among the material tested (Table 1).

Table 1: Polymorphic SSR markers for pistachio, motifs and their expected fragment size.

Name	Motifs	Fragment size (bp)
PIMS1	$(CT)_{19}(AT)_4(GT)_{11}$	195
PIMS2	$(GT)_{15}$	228
PIMS3	$(CA)_{16}$	145
PIMS7	$(GT)_{15}$	193
PIMS9	$(CA)_7$	126
PIMS40N	$(CT)_7$	200
PIMS41N	$(GA)_{11}$	236
PIMS42N	$(CTT)_8$	194
PIMS45N	$(GA)_{18}$	168
PIMS15	$(GA)_{23}$	193

The extracted DNA from the nuts and nutshell were used for DNA analysis using molecular markers. The microsatellite markers could clearly distinguish between cultivar 'Kerman' and the other pistachio samples tested. A total of 17 pistachio cultivars from Iran and also samples from grocery stores were analyzed. For the marker assay, we used the DNA of cultivar 'Kerman' extracted from fresh leaves. The DNA of all other samples was extracted either from nuts or nutshells. The developed SSR markers showed different efficiency. Some could distinguish between few samples (Fig. 2) and some showed a higer rate of polymorphism. Figure 3 shows the DNA fingerprint for 'Kerman' and it differed clearly from those of other samples produced by marker PIMS7 (Fig. 2).

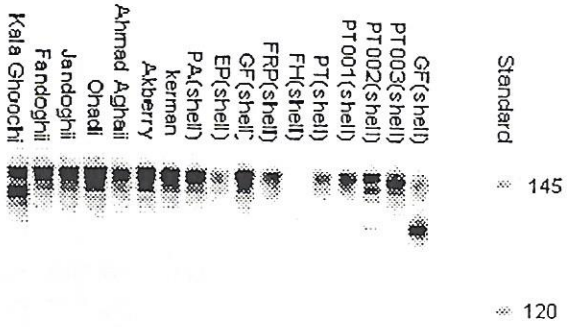

Figure 2: Amplification products of PIMS3 with 17 pistachio cultivars.

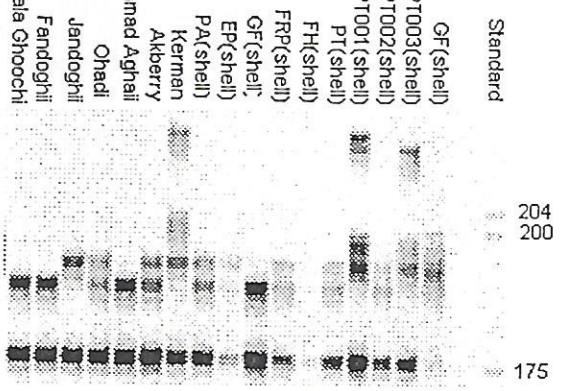

Figure 3: DNA fingerprints of microsatellite marker PIMS7 with 17 Pistachio cultivars. The DNA was extracted either from nuts or from nutshells (shell).

We also developed ISSR markers for cultivar identification in pistachio with high efficiency (Fig. 4).

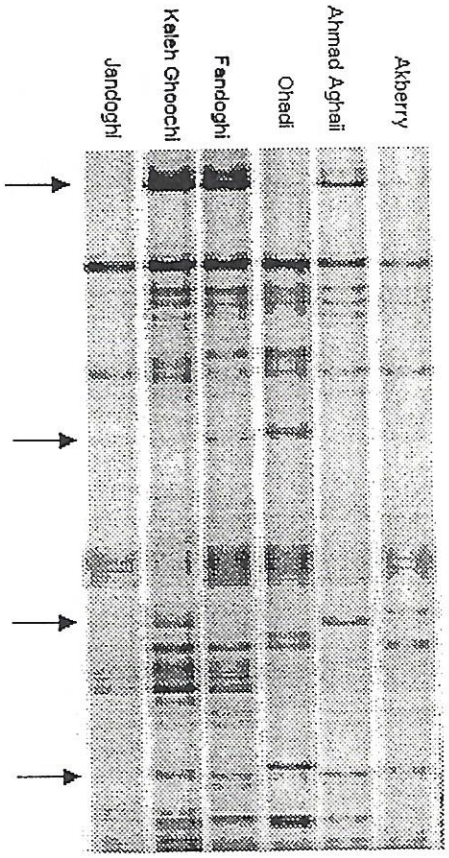

Figure 4: DNA fingerprints of six Iranian pistachio cultivars using ISSR primer 2. Polymorphic bands are shown by arrows.

Conclusion

An efficient DNA extraction method and molecular markers were developed for trueness-to-type in pistachio. With these methods it is possible to reveal the origin of pistachio sold in grocery stores. The DNA fingerprints of California cultivar 'Kerman' are very distinct from the fingerprints of major Iranian cultivars and also pistachio samples collected from grocery stores in Europe. We could not obtain samples of 'Kerman' from Iran, so it is unclear how the California 'Kerman' differs from Iranian one. Nevertheless, the major pollinator for California pistachios is the cultivar 'Peter' and it is unlikely that the same cultivar is being used as a pollinator in Iran. Consequently, it maybe possible to distinguish between Iranian pistachio derived from 'Kerman' and California pistachio.

Selected references:

Hamilton, M.B., E.L. Pincus, A. Di Fiore and R. C. Fleischer, (1999). Universal Linker and Ligation procedures for construction of genomic DNA libraries enriched for microsatellites. Biotechniques 27: 500-507

Struss, D. and J. Plieske, (1998). The use of microsatellite markers for detection of genetic diversity in Barley populations. Theor. Appl. Genet. 97 (1998) 308-315.

Dr. Darush Struss

Dr. Stephen M. Southwick