# Genetic Diversity of Greek Wild and Cultivated Pomegranate (*Punica granatum* L.) Genotypes and Cultivars Using Molecular Markers

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**Abstract**

**In this study, different pomegranate genotypes and cultivars were discriminated and identified using the Random Amplified Polymorphic DNA (RAPD) and the Inter Simple Sequence Repeats (ISSR) molecular markers. 38 different pomegranate genotypes and 2 cultivars were discriminated and identified using RAPD and ISSR molecular markers. Genomic DNA was extracted from young leaves using the CTAB method. 35 decamer RAPD primers and 15 ISSR primers were tested from which 10 RAPD and 10 ISSR primers, which gave the most polymorphic bands, were used. Amplified products of PCR were separated by electrophoresis in 3% (w/v) and 5% (w/v) agarose gel equivalent. NTSYS software, version 2.02, was used to estimate genetic similarities using Jaccard's algorithm while the dendrogram was constructed by UPGMA and Neighbor Joining methods. High genetic similarity between the samples collected from the agricultural area of Pella, and the samples collected from the Institute of Pomology of Naoussa (>0.70), suggests that they might be clones of some cultivars. The Greek cultivar 'Ermioni' showed high genetic similarity (95%) with cultivar 'Wonderful' (95%). RAPD and ISSR molecular markers were useful for studying the genetic relationships between pomegranate genotypes and cultivars, with the ISSR markers generating more polymorphic bands.**

## INTRODUCTION

Pomegranate (*Punica granatum* L.) is widely cultivated throughout Iran, India, South Africa, Europe, China and America (Jbir, 2008). In Greece, pomegranate is cultivated in many different parts of the country (Drogoudi, 2005). Its successful adaptation to the Mediterranean climate has led to the creation of a multitude of new forms in time, which are sometimes grouped under the same denomination (Melgarego, 2009).

There is a growing interest in pomegranate fruit since it is considered to be a product of great benefit for the human diet. Pomegranate is believed to be related with cancer prevention, LDL oxidation and atherosclerosis, and improvement of menopausal syndromes. Its beneficial effects may be related with its high antioxidant activity, resulting from the presence of a variety of biologically compounds (Aviram, 2002).

Morphological characteristics of the fruit for identification of pomegranate genotypes are useful, however, these traits are mostly affected by environmental and cultivation techniques (Sarkhosh, 2009). Different cultivars can be identified using different genetic techniques. Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) molecular markers have been successfully used for DNA fingerprinting and population genetic studies (Gupta et al., 2008).

In the present study, two molecular markers technics have been used to discriminate and identify Greek pomegranate genotypes and cultivars. The two techniques were also compared according to their discrimination ability.

## MATERIALS AND METHODS

### Plant Material and DNA Extraction

A total of 40 pomegranate genotypes were used in this study. 21 pomegranate genotypes were sampled from a collection at the Institute of Pomology of Naousa, 17 samples of 5 different genotypes were collected from the agricultural area of Pella, and 2 pomegranate cultivars were collected from the Pomology Station of Poros (Table 1). Leaves were stored at -20°C. Genomic DNA was extracted from young leaves using the CTAB method of Doyle and Doyle (1987).

### Amplified Reactions

For the RAPD markers 35 decamers primers (Operon Technology Inc. Alameda, CA, USA) were tested from which 10 were finally used (Table 2). PCR amplification reactions were carried out in 25 μl final volume of reaction mixture containing 1× PCR buffer, 200 μM dNTPs, 1.75 mM $MgCl_2$, 0.5 μM primer, 1 U Taq DNA polymerase (PROMEGA) and 25 ng of total genomic DNA. The amplification was performed in a Sensoquest Labcycler Standard thermocycler. The temperature profile consisted of an initial 4 min denaturation step at 94°C, followed by 35 cycles of: denaturation at 92°C for 1 min, primer annealing at 37°C for 1 min and extension step at 72°C for 2 min. The final elongation step was at 72°C for 5 min. Amplification products were separated in 1.5% agarose gel by electrophoresis stained with ethidium bromide in 1× TAE buffer. The RAPD bands were visualized under UV light and photographed with a digital camera. The DNA Molecular Weight Marker 100 bp (New England BioLabs, USA) was used as a standard molecular weight size marker.

For the ISSR markers, fifteen primers (University of British Columbia, Canada) were tested from which ten were finally used (Table 2). PCR amplification reactions were carried out in 20 μl final volume of reaction mixture containing 1× PCR buffer, 200 μM dNTPs, 1.5 μM $MgCl_2$, 0.3 μM primer, 1 U Taq DNA polymerase (PROMEGA) and 25 ng of total genomic DNA. The temperature profile consisted of an initial 5 min denaturation step at 94°C, followed by 40 cycles of: denaturation at 94°C for 40 s, primer annealing at 50°C for 45 s and extension step at 72°C for 90 s. The final elongation step was at 72°C for 5 min. Amplification products were separated in 2.5% agarose gel and treated similarly to the RAPD reactions.

### Data Analysis

Genetic similarities for the RAPD and ISSR data were calculated using the Jaccard's (Sneath and Sokal, 1973) similarity coefficient. Phylogenetic trees were created using the UPGMA (Unweighted Pair Group Method with Arithmetic mean) and N.J. (Neighbor-Joining) methods. The correlation among all genetic similarity matrix was checked using the Mantel (Mantel, 1967) test. The analysis was performed using the NTSYS pc 2.02 (Rohlf, 1998).

## RESULTS AND DISCUSSION

A total of 38 RAPD polymorphic bands (47%) out of 81 were scored for the 38 pomegranate genotypes and 2 cultivars. The total number of bands per RAPD primer ranged from 5 ($RI_3$) to 12 ($OPBA_3$) (Fig. 1) with a mean of 8.1 fragments per primer. Primers $OPB_{11}$ and $OPAH_{17}$ yielded the highest percentage of polymorphic bands (71%), while primer $OPBB_4$ (17%) yielded the lowest percentage of polymorphic bands. The ISSRs primers amplified 119 bands, of which 84 were polymorphic (71%). The total number of bands per primer ranged from 8 ($UBC_{861}$) to 16 ($UBC_{842}$ and $UBC_{844}$) (Fig. 2) with a mean of 11.9 fragments per primer. Primers $UBC_{856}$ yielded the highest percentage of polymorphic bands (90%), while primer $UBC_{861}$ (12%) yielded the lowest percentage of polymorphic bands (Table 2).

Results of the similarity matrix showed that for RAPD markers, the genetic similarity among the 40 Greek accessions ranged from 0.66 to 0.97. The lowest similarity

(0.66) was found between genotypes '11041' and 'Thiriopetra-9' and the highest similarity (0.97) between genotypes '11026' and 'Polykarpi-2'. Sarkhosh et al. (2006) concluded that there was a high genetic relationship (60%) among the 24 Iranian genotypes they studied, with the Jaccard's coefficient ranging from 0.29 to 0.89. Jbir et al. (2008) also found genetic similarities ranging from 0.1 to 0.86, by studying 34 Tunisian pomegranate cultivars. For the ISSRs markers, the genetic similarity ranged from 0.61 to 0.96. The lowest similarity (0.61) was found between genotype '11019' and cultivar 'Wonderful' and the highest (0.96) between genotypes 'Thiriopetra-2' and 'Thiriopetra-9'. Combining the two methods, RAPD and ISSRs, the genetic similarity ranged from 0.69 to 0.93, were the lowest (0.61) was found between genotypes '11019' and 'Thiriopetra-9' and '11019' and 'Thiriopetra-2' and the highest (0.93) between genotypes 'Thiriopetra-2' and 'Thiriopetra-9' and 'Chrisi' and 'Megalokarpi'.

The derived UPGMA dendrograms (Figs. 3 and 4) clustered the 40 samples studied according to their geographical origin. The 2 cultivars 'Ermioni' and 'Wonderful' seem to be genetically very close, since their genetic distance is very low. The cophenetic correlation coefficient was high for RAPD-UPGMA (r=0.71), ISSR-UPGMA (r=0.77) and RAPD and ISSR-UPGMA (r=0.80) revealing a good fitness of the genetic similarity matrices to the obtained phenograms.

## CONCLUSIONS

In the present study, the Greek pomegranate genotypes and cultivars were differentiated successfully, using the 2 different molecular marker techniques, RAPD and ISSR, which gave a sufficient number of polymorphic bands between pomegranate genotypes and cultivars. The combination of the 2 molecular methods gave a better picture of the genetic relationships between pomegranate genotypes and cultivars.

The samples that were collected from the agricultural area of Pella and from the Institute of Pomology of Naoussa seem to be genetically close (>0.70), suggesting that they might be clones derived from a single cultivar. Also, the two cultivars, 'Ermioni' and 'Wonderful', seem to be very closely genetically, reaching 95% similarity, suggesting having a common genetic background. In order to characterize Iranian germplasm of pomegranate at the molecular level, Talebi Baddaf et al. (2003) applied 13 RAPD primers on 28 pomegranates and observed a high level of polymorphism. Similar conclusions were reported by Sarkhosh et al. (2006) studying Iranian pomegranate genotypes while Ghobadi et al. (2005) found a high level of genetic similarity (65%) among the 24 Iranian cultivars they studied using 6 ISSR markers.

According to our results, the Greek pomegranate genotypes showed a low genetic diversity suggesting that the initial Greek plant material originated from a smaller "gene pool". It will be useful to compare Greek genotypes with Iranian ones, from where pomegranate is originated, as well as to import some new pomegranate genotypes or cultivars with different genetic background in order to increase the genetic diversity of pomegranates in Greece.

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## Tables

Table 1. Denomination and geographical origin of the 40 Greek pomegranate genotypes and cultivars studied.

| Genotypes | Origin | Genotypes | Origin |
|---|---|---|---|
| 11002 | I.PN. | Thiriopetra 1 | Pella |
| 11003 | I.PN. | Thiriopetra 2 | Pella |
| 11005 | I.PN. | Thiriopetra 3 | Pella |
| 11006 | I.PN. | Thiriopetra 4 | Pella |
| 11009 | I.PN. | Thiriopetra 5 | Pella |
| 11010 | I.PN. | Thiriopetra 6 | Pella |
| 11011 | I.PN. | Thiriopetra 7 | Pella |
| 11012 | I.PN. | Thiriopetra 8 | Pella |
| 11014 | I.PN. | Thiriopetra 9 | Pella |
| 11015 | I.PN. | Polykarpi1 | Pella |
| 11016 | I.PN. | Polykarpi 2 | Pella |
| 11018 | I.PN. | Polykarpi 3 | Pella |
| 11019 | I.PN. | Polykarpi 4 | Pella |
| 11020 | I.PN. | Chrisi | Pella |
| 11021 | I.PN. | Megaplatanos | Pella |
| 11022 | I.PN. | Apsalos1 | Pella |
| 11025 | I.PN. | Apsalos 2 | Pella |
| 11026 | I.PN. | Ermioni | P.S.P. |
| 11028 | I.PN. | Wonderful | P.S.P. |
| 11029 | I.PN. | | |
| 11041 | I.PN. | | |

Table 2. Primers used for RAPD and ISSRs analyses: total number, polymorphic bands and % of polymorphism obtained.

| Primer | Sequence 5'→3' | Total bands | Polymorphic bands | % Polymorphism |
|---|---|---|---|---|
| RAPD | | | | |
| OPB – 11 | GTAGACCCGT | 7 | 5 | 71 |
| OPB – 1 | GTAGACCCGT | 5 | 2 | 40 |
| OPBA – 3 | GTGCGAGAAC | 12 | 7 | 58 |
| OPBB – 4 | ACCAGGTCAC | 6 | 1 | 17 |
| OPAH – 17 | CAGTGGGGAG | 7 | 5 | 71 |
| OPH – 18 | GAATCGGCCA | 10 | 5 | 50 |
| OPH – 13 | GACGCCACAC | 8 | 3 | 37 |
| OPA – 9 | GGGTAACGCC | 10 | 4 | 40 |
| RI – 3 | GTCCGTGAAC | 5 | 2 | 40 |
| RI – 16 | GTCGCCGTCA | 11 | 4 | 36 |
| Total: | | 81 | 38 | 47 |
| ISSR | | | | |
| UBC – 818 | $(CA)_8G$ | 9 | 5 | 56 |
| UBC – 823 | $(TC)_8C$ | 11 | 9 | 82 |
| UBC – 825 | $(AC)_8T$ | 14 | 10 | 71 |
| UBC – 826 | $(AC)_8C$ | 10 | 8 | 80 |
| UBC – 842 | $(GA)_8YG$ | 16 | 9 | 56 |
| UBC – 844 | $(CT)_8RC$ | 16 | 13 | 81 |
| UBC – 850 | $(GT)_8YC$ | 13 | 10 | 77 |
| UBC – 856 | $(AC)_8YA$ | 10 | 9 | 90 |
| UBC – 861 | $(ACC)_6$ | 8 | 1 | 12 |
| UBC – 889 | $DBD(AC)_7$ | 12 | 10 | 83 |
| Total: | | 119 | 84 | 71 |

## Figures

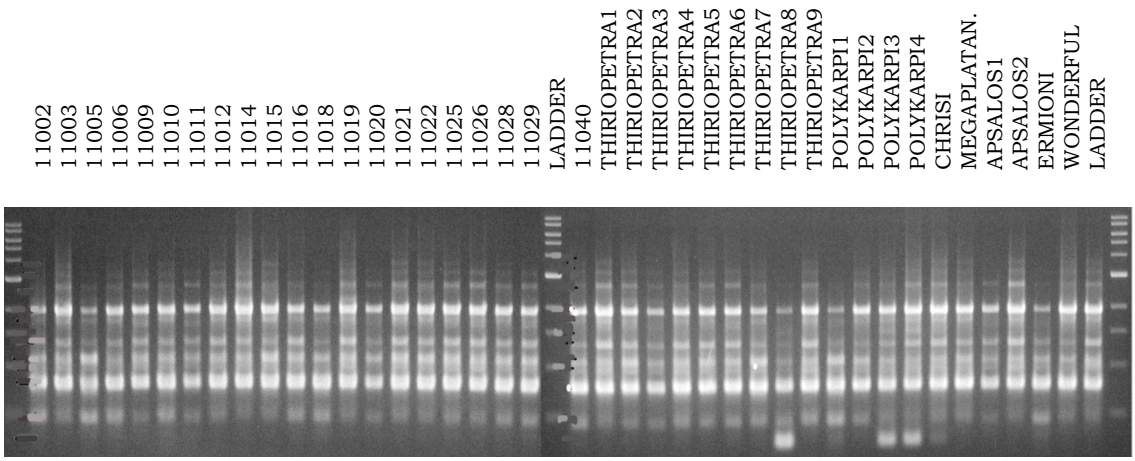


Fig. 1. Pomegranate samples with RAPD primer OPBA-3 analyzed in 1,5% (w/v) agarose gel, staining with ethidium bromide.

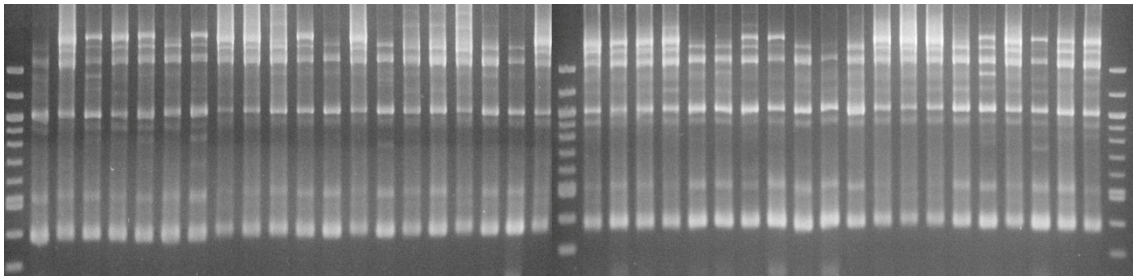

Fig. 2. Pomegranate samples with ISSR primer $UBC_{844}$ analyzed in 2,5% (w/v) agarose gel, staining with ethidium bromide.

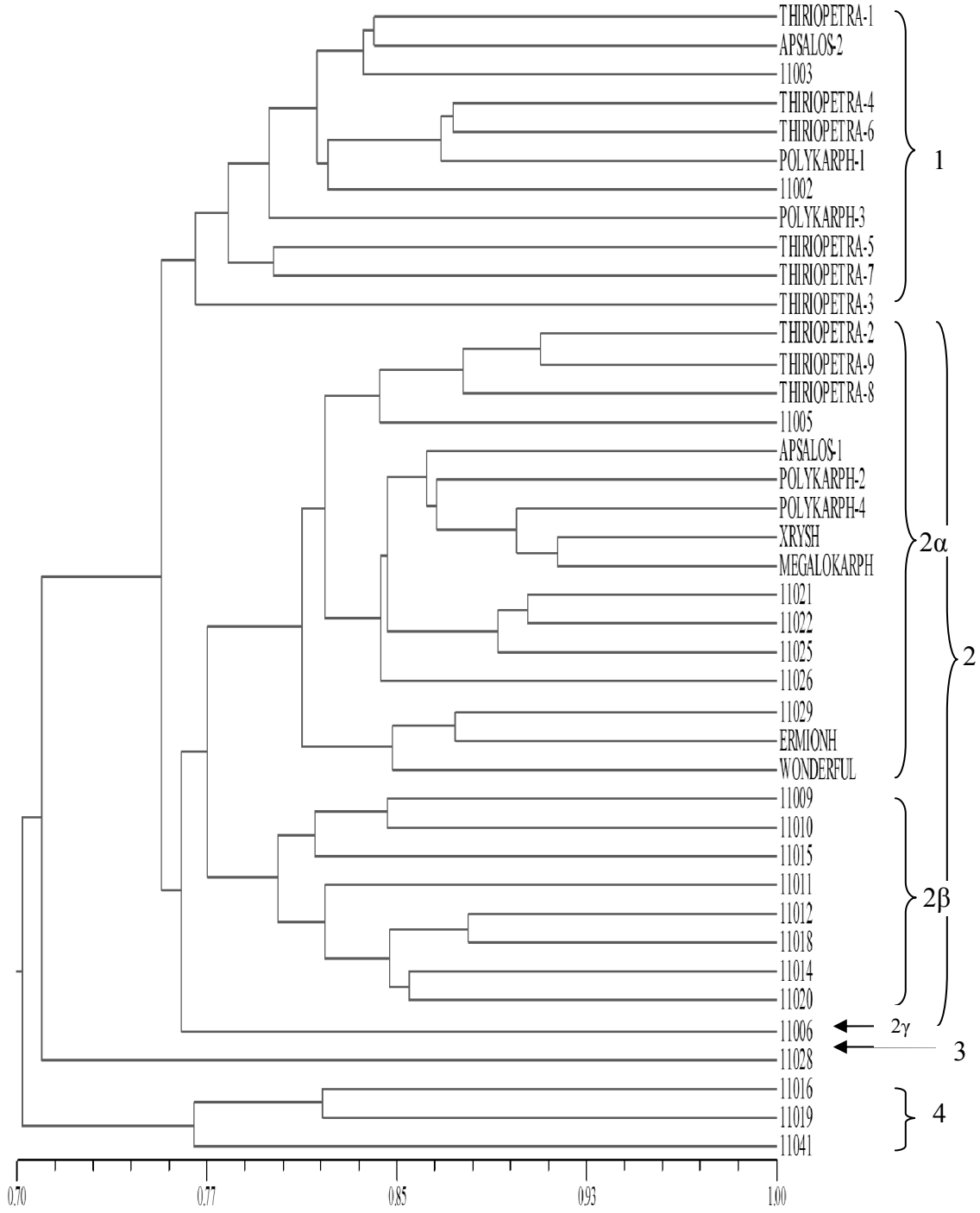


Fig. 3. Genetic relationships among 40 Greek pomegranate genotypes using RAPD and ISSR primers.

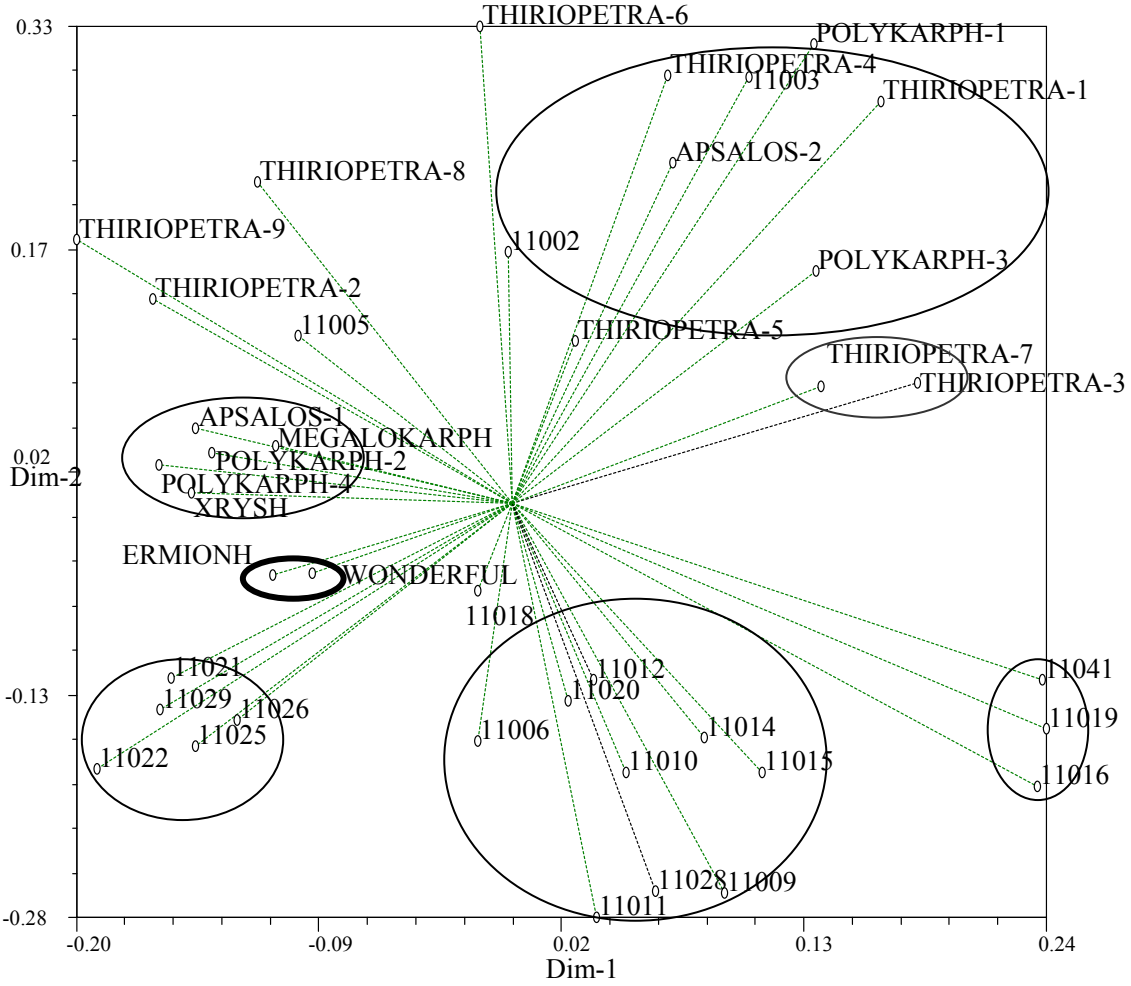


Fig. 4. Genetic relationships among 40 Greek pomegranate genotypes using RAPD and ISSR primers, in 2 dimensions (PCOORDA).