Identification of Sour Orange Accessions and Evaluation of Their Genetic Variability by Molecular Marker Analyses

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Abstract. A collection of 18 accessions of sour orange (Citrus aurantium L.) coming from Sicily and other countries was investigated by two polymerase chain reaction (PCR)-based DNA marker technologies. Ten inter-simple sequence repeat (ISSR) primers and fifteen randomly amplified polymorphic DNA (RAPD) primers were used to identify and to evaluate the genetic variability and relationships of accessions. A total of 111 ISSR and 145 RAPD amplified fragments were used to estimate the Dice’s coefficient of similarity for cluster analysis using an unweighted pair-group method using an arithmetic averaging (UPGMA) algorithm. The genetic relationships identified using ISSR and RAPD markers were highly concordant, such that the correlation between ISSR and RAPD genetic distance (GD) estimates was \( r = 0.93 \). The ISSR and RAPD analysis of 18 sour orange accessions found a high grade of genetic diversity in foreign accessions, while a low variability was detected in local accessions. Sicilian accessions could be grouped in two distinct clusters, including indiscriminately plants from three origin regions. Some markers could be linked to the different growing areas. The ISSR and RAPD molecular reference system seems to be suitable for a fine identification of tightly related plants and the obtained results can form the basis for future setting up of Citrus rootstock genetic improvement projects.

Sour orange (Citrus aurantium L., \( 2n = 2x = 18 \)) is one of the most widely used Citrus rootstocks in the world. Despite its decreasing cultivation because of spreading tristeza virus (CTV), sour orange is still cultivated in many countries, in particular in the Mediterranean Sea coasts. Its importance is due mainly to its capability of growing in calcareous and saline soil and being tolerant to several serious Citrus diseases (such as phytophthora, exocortis, and xylorosepsis viroid and blight). Furthermore, sour orange guarantees consistent yields, good fruit quality, and ability to import cold hardiness to the scion (Castle, 1987). In nurseries, sour orange cultivation is represented by local populations seed-propagated and maintained over generations by selection based on morphological traits (Herrero et al., 1996). As with other polyembryonic Citrus species, seedling populations of sour orange are genetically quite uniform because they arise from apomictic seed through nucellar embryony (Castle, 1987; Esan, 1973; Xiang and Roose, 1988), allowing clonal propagation. However, sour orange produces seeds that contain both nucellar and zygotic (sexual) embryos, and therefore uniformity is almost complete. Off-type seedlings are generally eliminated by nurseriesmen, but zygotic seedlings are sometimes difficult to identify and they could develop plants with very different agronomic traits. Currently, in southern Italy, there are several sour orange populations with interesting agronomical traits, but no information is available about their variability, and no type characterization has been done yet. Because the rootstock can greatly affect performance of trees budded onto them (Roose and Traugh, 1988), an improved understanding of sour orange genetic diversity is desirable to select lines suitable for increasing the productivity of Citrus cultivations. It also would permit to isolate, within the best local selections, individuals amenable for setting up new breeding programmes for genetic improvement. These breeding programmes aim to constitute mainly new CTV-resistant hybrid rootstocks having the same good qualities of sour orange (Grosser et al., 2004).

Polymerase chain reaction (PCR)-based DNA marker technologies have represented a useful tool to characterize closely related plants (Barcaccia et al., 2003; Imazio et al., 2002) and to evaluate the genetic homogeneity and purity, respectively, of inbreeds and hybrids (Bellant et al., 1996; Scaran et al., 2002). They were also used to investigate the phylogenetic relationship between plants and populations belonging to the same genus (Burstin et al., 2001; Rossetto et al., 2002).

In particular, PCR methods using arbitrary primers have become very popular among researchers since 1990 (Williams et al., 1990), as they do not require any information about DNA sequences. Of these techniques, randomly amplified polymorphic DNA (RAPD), using 10 base pairs random primers, is the most widely applied (Lanham et al., 1995; Mailer et al., 1994; Stiles et al., 1993), principally due to the easiness of the procedure and the very low amount of DNA required for analysis. In Citrus, RAPD markers have been used previously for studies about genetic diversity (Coletta Filho et al., 1998), hybrid and mutant identification (Deng et al., 1995; Elisirario et al., 1999), mapping (Cai et al., 1994), and linkage analysis (Cheng and Roose, 1995; Gmitter et al., 1996; Ling et al., 2000).

Inter-simple sequence repeat (ISSR) technique also uses arbitrary primers composed of a microsatellite sequence repeated in tandem (core) and a flanking sequence of two to four degenerate nucleotides (anchor). Because ISSR primers require high-anealing temperature, this technique guarantees high reliability and repeatability, in addition to cost- and time-effectiveness (Fang and Roose, 1997), especially if compared with RAPD. A few genetic studies using ISSR markers have been carried out on Citrus and Citrus-related species mainly to characterize closely related commercial varieties (Fang et al., 1997; Fang and Roose, 1997), experimental somatic hybrids (Scaran et al., 2002), and to discriminate zygotic and nucellar plants (Lambardi et al., 2004).

In the present study, phenotypically diverse sour orange accessions collected from different parts of Sicily and other foreign countries were analysed by RAPD and ISSR methods to detect genetic polymorphisms useful for setting up a molecular reference system that would allow a precise identification. This information, with morphological and phenological descriptors, could be useful for assessing the basis of breeding programmes aimed at the genetic improvement of sour orange. Data obtained from this study may also be used to provide useful information about genetic relationships among the accessions examined.

Materials and Methods

Plant materials and DNA extraction. A total of 18 accessions of Citrus aurantium L., including 15 Sicilian accessions and three foreign clones were used in the investigation. Plants different for morphological and physiological traits (Tusa et al., 1979) were collected from several sites located in the three major Citrus diffusion areas of Sicily: northwest (province of Palermo), northeast (province of Messina), and southeast (province of Siracusa-Ragusa). They were introduced in the germplasm collection in the Lascari field station (38ºN, 14ºE). The Sicilian accessions were ‘Barcellona 1’ (BC1), ‘Barcellona 2’ (BC2), ‘Barcellona 3’ (BC3), ‘Palermo 1’ (PA1), ‘Palermo 2’ (PA2), ‘Palermo 3’ (PA3), ‘Palermo 4’ (PA4), ‘Siracusa’ (SR), ‘Noto 1’ (NT1), ‘Noto 2’ (NT2), ‘Rosolino’ (RSL), ‘Ispica 1’ (IS1), ‘Ispica 2’ (IS2), ‘Sco- glutii’ (SC0) and ‘Campofilice’ (CM), according
DNA was quantified and stored at –80 °C. The samples were concentrated in three growing areas. Northwest (province of Palermo): ‘Palermo 1’ (PA1); ‘Palermo 2’ (PA2); ‘Palermo 3’ (PA3); ‘Palermo 4’ (PA4). Northeast (province of Messina): ‘Barcellona 1’ (BC1); ‘Barcellona 2’ (BC2); ‘Barcellona 3’ (BC3). Southeast (province of Siracusa-Ragusa): ‘Noto 1’ (NT1); ‘Noto 2’ (NT2); ‘Ispica 1’ (IS1); ‘Ispica 2’ (IS2); ‘Scoglitti’ (SCO); ‘Siracusa’ (SR); ‘Rosolini’ (RLS). The accession ‘Campofelice’ (CM) was found in an intermediate localization between Palermo and Messina areas.

The three foreign clones were selected for their interesting agronomical characters. The clone ‘Argentina’ (ARG) is a typical Citrus rootstock of Argentina. The clone ‘Smooth flat Seville’ (SFS), reported also as ‘Australian sour orange’, is a possible sour orange hybrid less susceptible to CTV (Castle, 1987). The clone ‘Gou Tou’ (GOU) is a CTV-resistant Chinese selection (Castle, 1987; Garnsey, 1992). The method used to isolate genomic DNA from leaves was as described by Doyle and Doyle (1987). The leaves were collected from mature trees present in the germplasm collection and they were carefully washed, as advised to avoid insects and fungal contaminations (Fang and Roose, 1997), frozen in liquid nitrogen and stored at –80 °C. The samples were ground in a mortar with liquid nitrogen. DNA was quantified by measuring OD260 as described by Sambrook et al. (1989) and diluted for PCR to 5 ng µL⁻¹.

ISSR analysis. A total of 10 primers—i.e., (AC)YG, (AG)YC, (AC)YA, (AC)YT, (AG)YT, (GT)YG, (TCC)RY, (GA)YC, (CA)RG, and (GA)YG (reported by Fang and Roose, 1997)—were used to amplify the DNA. The primers were purchased from Life Technologies, Gaithersburg, Md.

Each amplification was performed in a 25 µL reaction volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM MgCl₂, 800 µm dNTP (dATP:dTTP:dCTP:dGTP in 1:1:1:1 ratio), 0.5 µm of each primer, 1 U of Platinum Taq polymerase (Life Technologies) and 30 ng of template DNA. The amplification was performed in a MJ Research thermocycler (Genencor) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 4 min at 94 °C, followed by 36 cycles at 94 °C for 30 s (denaturation), 50 °C for 45 s (annealing) and 72 °C for 120 s (extension), followed by a final extension step at 72 °C for 7 min. PCR-amplified DNA fragments were separated on a 1.5% agarose gel at 100 V. The gel was then visualized with a UV transilluminator at 300 nm. To confirm the reproducibility of the banding patterns, all analyses were repeated three times.

RAPD analysis. Fifteen arbitrary decamer primers—i.e., OPH04, OPAT14, OPH15, OPM04, OPO14, and OPN14 (reported by Coletta Filho, 1998) and UBC219, UBC234, UBC237, UBC239, UBC247, UBC251, UBC264, UBC266, and UBC272 (reported by Wang, 1999) were used for the amplification of DNA sequences. The primers were purchased from Life Technologies, Gaithersburg, Md.

DNA amplification reactions were performed in a volume of 25 µL with 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 800 µm dNTP, 0.4 µm of each primer, 1 U of Platinum Taq polymerase (Life Technologies) and 30 ng of template DNA. The amplification was performed in a MJ Research thermocycler (Genencor) equipped with a Hot Bonnet under the following cycle program: initial denaturation step for 90 s at 94 °C, followed by 40 cycles at 94 °C for 1 min (denaturation), 35 °C for 2 min (annealing) and 72 °C for 2 min (extension), followed by a final extension step at 72 °C for 10 min. PCR-amplified DNA fragments were visualized as described above. To confirm the reproducibility of the banding patterns, all analyses were repeated three times.

Data analysis. Amplified bands from each primer were scored as present (1) or absent (0) for all the accessions studied. Only those bands showing consistent amplification were considered; smeared and weak bands were excluded from the analysis. Dice’s (1945) coefficient of similarity (Dij) was determined between each pair of accessions. Dice’s coefficient has been recommended for the evaluation of genetic similarities when using RAPD data (Lamboy, 1994). The genetic distance (GD) between two samples was calculated as: GD = 1 – Dij.

The degree of concordance between ISSR and RAPD markers was determined by visual appraisal of graphic depictions generated from the analysis of GD values and correlation analysis (Pearson rank correlation) using Microsoft Excel 2000. The estimates of similarity between accessions were then used for cluster analysis by unweighted pair group method of arithmetic average (UPGMA) (Sneath and Sokal, 1973) using the NTSYS package version 2.02 for windows (Rohlf, 1994).

Additional statistics were computed to estimate the grade of polymorphism among plants studied. The average marker allele frequency (p) for each primer and among all primers was calculated for each single accession and among all accessions. Genetic diversity (H) of Nei (1973) and Shannon Index (S) (Lewontin, 1972) were used to sumarize the data for molecular markers and their standard deviations (St. Dev.) were indicated. The percentage of polymorphisms (P) was given as number of polymorphic loci/number of total loci, regardless of allele frequencies. The fixation index (Gst) was computed as proportion of genetic diversity expressed between accessions coming from different geographic regions. From this, the gene flow (Nm) was derived and interpreted according to McDermott and McDonald (1993). All calculations and analyses were conducted using the software POPGENE version 1.31 (Yeh et al., 1999).

Results

ISSR. A total of 10 ISSR primers were used for analyzing the patterns of 18 (15 Sicilian and 3 foreign) selected sour orange accessions. These ISSR primers had been already used successfully in Citrus characterization studies by Fang and Roose (1997).

A total of 11 well-resolved band classes were observed. The amplified fragments ranged from 180 bp [primer (GA)YG] to 2.3 kb [primer (CA)RG] in size. The number of ISSR bands obtained for each primer varied from 7
All the primers screened revealed marked polymorphism in foreign plants. In particular, the ISSR primer (AC)YT amplified unique markers for the clones ‘Argentina’, ‘Smooth flat Seville’ and ‘Gou Tou’. Six primers [(AC)YG, (AG)YT, (AC)YT, (AG)YT, (GT)Y, (CA)RG] were also able to identify differences in Sicilian accessions and the ISSR primer (CA)RG amplified a unique fragment for the Sicilian accession ‘Ispica 1’. These data are already an indication of the presence of moderate diversity among the studied accessions. Out of 111 amplified bands, 33 ($P = 29.7\%$) were polymorphic. As shown in Fig. 2, the primer (AG)YT gave the highest number of polymorphisms (7) and high values of Nei’s gene diversity ($H$) were obtained for its 600 bp ($H = 0.483$) and 2200-bp-long ($H = 0.498$) amplified bands.

The total number of amplified fragments generated per primer had no correlation with the proportion of polymorphic bands. For example, the primers (GA)Y and (AG)YT amplified about the same number of bands (14 and 13 respectively), whereas the primer (GA)YC showed only 1 polymorphic band ($P = 7.1\%$), whereas the primer (AG)YT revealed 7 ($P = 53.8\%$).

The polymorphisms identified were used to generate the GD matrix (above the diagonal of Table 1). The average GD among Sicilian accessions was extremely low (0.017); the single values were never higher than 0.045. The accessions ‘Barcellona 3’, ‘Siracusa’, ‘Scogliiti’, and ‘Palermo 2’ showed minimal GD values. The GDs between the foreign clones ‘Argentina’ and the Sicilian accessions ranged from 0.065 (with ‘Palermo 1’ and ‘Noto 2’) to 0.107 (‘Ispica 1’), whereas the GDs with ‘Smooth flat Seville’ and ‘Gou Tou’ were higher (0.170 and 0.224 respectively). The GD between ‘Smooth flat Seville’ and ‘Gou Tou’ was lower than expected (0.111).

RAPD. The RAPD analysis was conducted using six RAPD primers (reported by Coletta Filho, 1999) previously used in Citrus analysis, and nine UBC RAPD primers (reported by Wang, 1999) previously used only in Vitis vinifera studies. All 15 RAPD primers produced a total of 145 well-resolved band classes, 50 of these ($P = 34.5\%$) were polymorphic. The RAPD primers OPN14, OPH14, and UBC239 were able to amplify unique markers for the clones ‘Argentina’, ‘Smooth flat Seville’ and ‘Gou Tou’. The total of the RAPD bands were used to create a GD matrix (below the diagonal of the Table 1). When ISSR and RAPD GD estimates were compared, the RAPD values were generally higher than those of ISSR. The average GD among Sicilian accessions was low (0.03) but almost twice than that obtained from ISSR analysis. GDs among accessions ‘Barcellona 3’, ‘Siracusa’, ‘Scogliiti’, and ‘Palermo 2’ ranged from 0.01 to 0.042. As observed with ISSR data derived GDs, the clone ‘Argentina’ showed GD estimates lower with the Sicilian accessions (from 0.095 to 0.055) than with the foreign clones ‘Smooth flat Seville’ and ‘Gou Tou’ (0.132 and 0.287 respectively). The GD between ‘Gou Tou’ and ‘Smooth flat Seville’ (0.3) was three times higher than that obtained from ISSR analysis.

The 9 UBC RAPD primers previously used in Vitis studies gave 86 band classes (59.3% of the total RAPD bands obtained), with an average of 9.6 bands per primer. These bands ranged from 430 bp (primer UBC 219) to 2.3 kb (primer UBC 219). These results are comparable to data obtained in Vitis accessions by Wang (1999). On the contrary, the capability to discriminate the accessions was lower, only 9 amplified fragments ($P = 10.5\%$) were polymorphic and the diversity was mostly linked to foreign clones.

Best results were obtained with the 6 RAPD primers chosen from Citrus characterization literature. These primers gave 59 band classes (with an average of 9.8 bands per primer), ranging from 300 bp (primer OPN14) to 3 kb (primer OPAT14). These constituted only the 40.7% of the total RAPD band classes obtained but 32 bands (54.2% of fragments amplified from these 6 primers) were polymorphic, that is the 64% of the total polymeric RAPD bands. In particular, as shown in Fig. 3, the primer OPH115 amplified 12 bands, out of these 9 were able to identify polymorphisms. These polymorphisms were able to discriminate tightly associated accessions. High Nei’s gene diversity ($H$) was obtained with the 1400-bp-long OPH04 band ($H = 0.498$) and with 850 bp and 500-bp-long fragments obtained respectively with the primers OPM04 and OPAT14 ($H = 0.488$).

Genetic relationships among Citrus aurantiurn accessions analyzed with ISSR and RAPD markers. Pair-wise comparison of GD values was used to determine the concordance between ISSR and RAPD markers. The correlation between ISSR and RAPD markers was high ($r = 0.93$), and the regression line equation describing this relationship was $y = 0.819 + 0.002$. Because of the concordance between markers, a joint analysis was performed using all the available marker data. Dice’s similarity was used to carry out the cluster analysis and to generate a dendrogram showing the relationship among the selected accessions, as
shown in Fig. 4. This dendrogram possessed four main branching nodes, with the Sicilian plants closely clustered and well separated from the foreign clones. At node 1, the ‘Gou Tou’ clone clustered with the remaining sour orange accessions examined, but was genetically quite distinct from them (similarity of 0.78). At node 2 and 3, respectively the ‘Smooth flat Seville’ and ‘Argentina’ clones could be distinguished from the Sicilian accessions, having a similarity of 0.85 and 0.92. At node 4, the Sicilian accessions could be clearly assigned to two groups with similarity of 0.96. Plants belonging to these two groups came indistinctly from three different regions of Sicily: northwest (province of Palermo), northeast (province of Messina) and southeast (province of Siracusa-Ragusa).

The four accessions, ‘Barcellona 1’, ‘Ispica 1’, ‘Scoglitti’, and ‘Palermo 2’, formed a subgroup with minimal genetic differences, while the accession ‘Campofelice’ was the most genetically distinct. The accessions ‘Siracusa’, ‘Noto 2’, and ‘Rosolini’ were coming from the same region (Siracusa-Ragusa) and constituted another subgroup.

The total Nei’s genetic diversity ($H_i$) was 0.122 ± 0.173; it was four times lower if calculated only for the Sicilian accessions (0.029 ± 0.111). An additional measure for genetic variation was obtained by Shannon index ($S$), a genetic diversity index suitable when dominant markers are used (Dawson et al., 1995). $S$ value was 0.191 ± 0.249 for all the analyzed accessions and it was 0.044 ± 0.157 when only Sicilian accessions were considered.

An analysis of gene diversity of Sicilian sour orange was carried out in accessions present in three different geographic areas (Palermo, Messina and Siracusa-Ragusa). The accession ‘Campofelice’ was found in an intermediate localization between two areas (Fig. 1) and its genetic profile appeared equally distant from both (GDs = 0.03). For this reason, it was considered separately.

GD between accessions coming from Messina area and Siracusa-Ragusa area was the highest (0.011), whereas the GDs between Palermo area accessions and Messina and Siracusa-Ragusa areas accessions were respectively 0.003 and 0.008. In Table 2, listed some genetic diversity parameters of the three collecting geographical areas calculated separately for ISSR and RAPD analysis and their cumulative effects. Here, the higher contribution given from RAPD analysis for identification of genetic diversity appears clearly, specially for the accessions of the province of Messina. Low gene diversity was found in all three regions analyzed, with greater value in area of Palermo ($H_i = 0.025 ± 0.106$). The fixation index ($G_{ST} = 0.463$) showed that as observed genetic variability could be attributed to within-area accession differences as due to differences among the three area accessions. As expected from the common propagation procedure, the estimated gene flow was extremely low ($N_{m} = 0.58$).

Some markers could be associated with the different areas. The 2200-bp-long band amplified from ISSR primer (AG)YT was found in all the accessions coming from the Messina region. The 740-bp-long fragment amplification from the RAPD primer OPN14 was tightly linked to the Siracusa-Ragusa zone accessions. The 1350-bp-long fragment from RAPD primer OPH04 was present in all the accessions of Palermo and Messina regions, whereas it was fairly uncommon in the Siracusa-Ragusa zone accessions.

Discussion

In the present study, reliable DNA fingerprints were identified by specific patterns in different accessions of sour orange commonly employed in Sicily and in other countries. The use of multi-locus PCR-based markers ISSR and RAPD allowed efficient differentiatation of tightly linked accessions. Foreign clones could be identified by unique fragments amplified from several ISSR and RAPD primers. In particular, the ISSR primer (AC)YT and the RAPD primers OPN14, OPH15, and UBC239 amplified unique markers for the clones ‘Argentina’, ‘Smooth flat Seville’, and ‘Gou Tou’. The ISSR primer (CA)RG amplified a unique

<table>
<thead>
<tr>
<th>Geographical area</th>
<th>Accessions (no.)</th>
<th>Total loci (ISSR + RAPD)</th>
<th>Percent polymorphic loci (ISSR + RAPD)</th>
<th>Nei’s gene diversity ($H_i$) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palermo</td>
<td>4</td>
<td>86</td>
<td>195</td>
<td>0.019 ± 0.092</td>
</tr>
<tr>
<td>Messina</td>
<td>3</td>
<td>86</td>
<td>195</td>
<td>0.009 ± 0.066</td>
</tr>
<tr>
<td>Siracusa-Ragusa</td>
<td>7</td>
<td>86</td>
<td>195</td>
<td>0.025 ± 0.099</td>
</tr>
</tbody>
</table>
fragment for the Sicilian accession ‘Ispica 1’. Despite the relatively low level of genetic variation, most Sicilian accessions could be uniquely identified by markers obtained from crossing analysis of two or three primers.

The great potential of the ISSR method for differentiating closely related Citrus cultivars is well documented. In particular, ISSR is the best molecular-marker system to distinguish sweet orange (C. sinensis L.) cultivars and the first one that successfully differentiated certain grapefruit (C. paradisi Macf.) cultivars (Fang and Roose, 1997). Likewise, the efficiency of the RAPD method to identify tightly-linked accessions was demonstrated in many studies in several accessions systems (Bhat et al., 1999; Shimada et al., 1999; Vidal et al., 1999). In Citrus, RAPD markers have been used to study genetic relationships principally in mandarin (C. reticulata Blanco) cultivars (Coletta Filho et al., 1998; Elisíario et al., 1999; Machado et al., 1996).

Our data confirmed that ISSR and RAPD methods are useful tools for identifying closely related accessions. Their advantages consist principally in detecting simultaneously many loci randomly distributed within the genome in a simple and cost-effective manner, requiring no previous knowledge of genome sequence as other methods, e.g., SSR.

Furthermore, the combination of ISSR and RAPD methods guarantees some additional benefits. The use of different types of molecular markers, which analyse different regions of the genome, permits a better and deeper analysis of genetic variations, as strongly recommended in several somaclonal variability studies (Martins et al., 2004; Palombi and Damiano, 2002).

RAPD primers quickly scan the whole genome detecting point mutations and insertion-deletion events (Milbourne et al., 1997). ISSR markers detect the same kind of variations at hypervariable sites, such as DNA repetitive regions. In these sites, variations occur more frequently than other parts of the genome. In particular, because of a specific mechanism named DNA-polymerase slippage, mutations in lengths of repeats can happen frequently during DNA replication. These kinds of variations are detectable by ISSR analysis (Fang and Roose, 1997), but not by RAPD analysis.

The reliability of the RAPD method deserves additional discussion. Wide-scale application of molecular marker techniques by a network system is necessary to guarantee a coordinated action of laboratories requiring highly standardized and reproducible methods. While the reproducibility of RAPD technique was deeply questioned (Hanssen et al., 1998; Jones et al., 1997; Karp et al., 1997), ISSR markers repeatability was confirmed in 99% of amplified fragments (Bornet and Branchard, 2001; Fang and Roose, 1997). Actually, our ISSR and RAPD markers gave a high concordance value ($r = 0.93$), allowing a joint analysis of data. Moreover, the accession-specific markers were found to be consistently stable in 98% of cases.

The molecular investigation of 18 selected sour orange accessions has also allowed the evaluation of the degree of genetic diversity reached despite of the past phenotypic-based variation carried out from nurserymen against variability (Herrero et al., 1996). Our results estimated a moderate level of genetic diversity among sour orange accessions coming from different countries. This variability was almost comparable to that identified from Coletta Filho et al. (1998) in mandarins (C. reticulata Blanco), a wide Citrus group evolved from sexual hybridization among a great number of species and intraspecific hybrids. In particular, the low similarity grade (0.78) of the Chinese selection ‘Gou Tou’ with all the other analysed genotypes seemed to confirm its supposed natural hybrid origin (Castle et al., 1989; Müller et al., 1996) until now corroborated only by a close inspection of its fruits and isozyme profiles (H.P. Medina Filho, R. Bordignon, G.W. Müller, unpublished data; Herrero et al., 1996) and few molecular markers data (Nicolosi et al., 2000). Likewise, the similarity grade showed from ‘Smooth flat Seville’ (0.85) would support the hypothesis of its hybrid origin. On the contrary, a very high grade of similarity was detected in accessions located in near geographical regions. All the analysed Sicilian accessions appeared genetically quite similar ($H = 0.748 \pm 0.111$) but, as showed in the dendrogram, it was possible to group them into two different clusters. Also if the accessions coming from all the collecting regions were indistinctly present in both groups, a small nesting of the accessions ‘Siracusa’, ‘Noto 2’ and ‘Rosolini’, coming from Siracusa-Ragusa area, was noted. More interesting aspects about it come from the analysis of genetic diversity of Sicilian accessions present in the three collecting geographical areas. In fact, accessions coming from Siracusa-Ragusa area appeared more genetically different also considering GD values (0.011 with Messina area and 0.008 with Palermo area). On the contrary, accessions coming from Palermo and Messina areas had a lower GD value (0.003). Accessions coming from Siracusa-Ragusa area also showed the most tightly linked marker identified in this study; a 740-bp-long fragment amplified from the RAPD primer OPN414 was present in 100% of the cases, whereas it was present only in the 50% of accessions of other areas.

All our data reflects the fact that few programs of sour orange type-selection and new genotypes research have been set up. The populations of sour orange have a conserved gene pool, as confirmed by the rather low estimate of gene flow. Most of their genetic variability is therefore due to zygotic seedlings not recognized by nurserymen and propagated by mistake.

The high value of the fixation index ($G_{ST} = 0.463$) may be explained by taking in account three factors. First, the reproductive characteristics of sour orange: most of the seedlings develop from apomictic seed through nucellar embryony, frequently the zygotic embryos die (Xiang and Roose, 1988). Second, the lack of breeding programs keeps genetic complements apart. Third, the conservative selection criteria of offsprings and mother plants to be used for seed production applied by nurserymen does not support gene exchange.

In conclusion, the molecular investigation carried out in this study allowed an evaluation of the degree of genetic differentiation reached from local and foreign sour orange accessions. The analysis of sour orange genetic variability and the set-up of a fine molecular characterization system can form the basis for future genetic breeding programs aiming to develop new and improved Citrus rootstocks. The combination of ISSR and RAPD methods has proven to be a powerful tool to separate closely related accessions and we propose it as a suitable system for evaluation of genetic diversity in plants.

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