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RESEARCH ARTICLE

# Diversity and structure of a sample of traditional Italian and Spanish tomato accessions

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**Abstract** Italy and Spain are the countries with the oldest record of tomato cultivation in Europe and arguably, with the higher number of traditional and heirloom varieties. In this work we evaluated the genetic diversity and structure in a sample of 26 cultivated accessions belonging to four traditional tomato types, Muchamiel and De la Pera from Spain, and San Marzano and Sorrento from Italy. The $(GATA)_4$ fingerprinting of the 109 genotypes confirmed the ability of this DNA marker to discriminate tomato plants that are otherwise difficult to distinguish. Furthermore, both the estimated population structure and the genetic differentiation statistics were consistent in indicating that subpopulations are more likely to correspond to farmers' breeding efforts and market specialization than to country-specific groups. Our results provide useful information not only for germplasm description and management but also for current breeding programs in both regions.

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## Introduction

The cultivated tomato (*Solanum lycopersicum* L.) was introduced in Europe by Spanish explorers during the sixteenth century. Tomato plants were initially used for ornamental purposes (e.g.: tabletop decoration), but in some Mediterranean countries such as Spain and Southern Italy, the fruits were soon after introduced into the local cuisine. It is interesting that at the time of the diffusion of the tomato in Europe, Southern Italy was part of the Spanish Empire. It is very likely that the commercial relationships between these two countries played an important role in the increase of the tomato cultivation and use.

The immediate acceptance of the tomatoes in Spain and Italy, along with the suitable agro-climatic conditions in both countries, favored the selection of a number of local varieties. Still today, these varieties represent a traditional asset of local farmers. Examples of local varieties or landraces in Spain are Muchamiel from Alicante, Tres cascos from Elche, De la Pera from the Vega Baja del Segura (South of Alicante) and Valenciano from Valencia. Among the traditional Italian varieties, San Marzano, Sorrento and Corbarino, which originated from the Campania region, are among the most popular traditional varieties. Currently, fruits of the traditional cultivars are premium

products in the domestic market. For instance, Muchamiel and De la Pera fruits are sold at a price that is three to six times higher than other tomatoes (Brugarolas et al. 2009; Martínez-Carrasco et al. in press). In 2011, for the production of San Marzano DOP canned tomatoes, fruits were purchased by processing factories at a price that was nearly five times higher than that of other varieties (http://www.consorziopomodorosanmarzanodop.it/).

While traditional tomato accessions are believed to have valuable traits in terms of agro-ecological adaptation (e.g.: yield stability), consumer preference and sensory quality (Ercolano et al. 2008), they are also characterised by high susceptibility to biotic stress (especially viruses) when compared to current commercial varieties (Ruiz and García-Martínez 2009; García-Martínez et al. 2011a). Therefore, there has been considerable interest to introgress genetic resistance to viruses into local accession. In order to achieve this goal, a breeding program has been carried out over the last 10 years at Miguel Hernández University (Spain) (Alonso et al. 2010; García-Martínez et al. 2011b; García-Martínez et al. 2012).

Understanding genetic diversity in traditional tomato accessions is therefore important not only for germplasm management but also for crop breeding. DNA fingerprinting is an essential tool to study genetic variation, to support cultivar identification and to protect names of regional foods (Melchiade et al. 2007; Corrado et al. 2011). A number of DNA markers have been successfully used in tomato such as RFLP (Miller and Tanksley 1990), RAPD (Paran et al. 1995), AFLP (Park et al. 2004), CAPS (Yang et al. 2004) and SSR (Smulders et al. 1997). Among different types of DNA marker systems, the use of a $(GATA)_4$ oligonucleotide probe proved to be useful to discriminate among cultivars and accessions that are otherwise difficult to differentiate (Andreakis et al. 2004; Kaemmer et al. 1995; Caramante et al. 2009; Rao et al. 2006). Furthermore, $(GATA)_4$ fingerprinting produced hierarchical classifications consistent with the history of tomato cultivation (Kaemmer et al. 1995).

In some crops, population structure correlated with geographic origin or pedigree (Asfaw et al. 2009; Chao et al. 2010; Lu et al. 2009). In tomato, inferred subpopulations associated to breeding history, market classes and age of the cultivar (Sim et al. 2011; Sim et al. 2009). It has also been reported that selection for market specialization and for geographic adaptation contributes to the population structure of tomato (Sim et al. 2011; Yi et al. 2008). While the majority of the studies have focused on the comparison of contemporary and traditional cultivars, analyses at the regional level are less abundant. It is believed that information about the variation present within tomato landraces is still limited (Mazzucatto et al. 2009; Terzopoulos and Bebeli 2010) also because locally cultivated traditional accessions should not rigorously be considered as homogenous.

In this work, we evaluated the genetic variability in a population of cultivated genotypes that have not been subjected to breeding programmes. Specifically, we analysed a total of 26 accessions of the cultivar types Muchamiel, De la Pera (both originating from the Southeastern area of Spain), Sorrento and San Marzano (both from the Campania region in Southern Italy). To evaluate possible relationships among those Italian and Spanish traditional cultivars, we used the $(GATA)_4$ oligonucleotide probe not only for its discrimination power, but also because it is expected that the accuracy of estimating various parameters in conservation genetics is higher using neutral markers (Vosman and Arens 1997; Allendorf 2010).

## Materials and methods

### Plant material

A collection of 26 tomato accessions were studied. Accessions belong to the Spanish tomato type Muchamiel and De la Pera (8 accessions per type), and the Italian Sorrento and San Marzano (5 accessions per type). Three to five plants per accession were fingerprinted. Names, origin and codes are listed in Table 1. The four tomato types have indeterminate growth habit and were characterised by different fruit shapes. Muchamiel fruits are large (>250 g), flattened and in some accessions strongly ribbed. Fruits of the De la Pera type weigh between 100 and 200 g, varying from rectangular to an elongated-oval shape, without ribs. Both tomatoes have green shoulder. Sorrento fruits have medium size (150–250 g) and are not strongly flattened or ribbed, with green shoulder. San Marzano fruits have an elongated shape and their weight ranges between 100 and 200 g.

**Table 1** List of tomato accessions used in this study

| Country | Type | Accession | Code | Seed source |
|---|---|---|---|---|
| Spain | De la Pera | Pera 1 | Pera1 | Farmer |
| | | Pera 7 | Pera7 | Farmer |
| | | Pera 16 | Pera16 | Farmer |
| | | Pera 19 | Pera19 | Farmer |
| | | Pera 21 | Pera21 | Farmer |
| | | Pera 22 | Pera22 | Farmer |
| | | Pera 25 | Pera25 | Farmer |
| | | Pera 44 | Pera44 | Farmer |
| | Muchamiel | Muchamiel BNY | MuchBNY | Local seed company |
| | | Muchamiel 4 | Much4 | Farmer |
| | | Muchamiel 11 | Much11 | Farmer |
| | | Muchamiel 18 | Much18 | Farmer |
| | | Muchamiel 29 | Much29 | Farmer |
| | | Muchamiel 30 | Much30 | Farmer |
| | | Muchamiel 128 | Much128 | Farmer |
| | | Muchamiel 198 | Much198 | Farmer |
| Italy | San Marzano | San Marzano C | SMARC | Local seed company |
| | | San Marzano 4 | SMAR4 | Local seed company |
| | | San Marzano 5 | SMAR5 | Farmer |
| | | San Marzano 12 | SMAR12 | Farmer |
| | | San Marzano 22 | SMAR22 | Farmer |
| | Sorrento | Campano GGragnano | Gragnano | Farmer |
| | | Semiorto | SemiOrto | Local seed company |
| | | P98-65 | Sorre65 | Local seed company |
| | | P98-62 | Sorre62 | Local seed company |
| | | P98-61 | Sorre61 | Local seed company |

## DNA extraction and $(GATA)_4$ oligonucleotide probe fingerprinting

Leaves were collected from young plants, deep frozen in liquid nitrogen and stored at −80 °C until molecular analysis. DNA isolation was carried out using the GenElute™ Plant Genomic DNA Miniprep kit (Sigma-Aldrich, Madrid, Spain), according to the manufacturer's instructions. For $(GATA)_4$ fingerprinting, 2–4 μg of DNA were digested for 16 h with 45 U of *Taq* I (Promega, Milan, Italy) according to the manufacturer's instructions. DNA molecules were separated by a 0.8 % (w/v) agarose gel electrophoresis and transferred onto Hybond-N + nylon membranes (Amersham Biosciences, Milan, Italy). Hybridization and stringent washes were performed as reported (Caramante et al. 2009). The oligonucleotide $(GATA)_4$ (Sigma-Aldrich, Milan, Italy) was end-labeled using T4 polynucleotide kinase (Promega) and [$\gamma^{-32}$P]-ATP (Perkin Elmer, Milan, Italy) according to standard protocols (Sambrook et al. 1989).

## Hierarchical clustering

Bands were scored as present (1) or absent (0). Only clear and repeatable fragments were introduced in the genetic analysis. Two indexes were calculated: PIC (polymorphic information content) and RP (resolving power). For dominant (presence/absence) markers the PIC is defined as $1-Faa^2-Fan^2$, where $Faa^2$ is the frequency of the amplified allele and $Fan^2$ is the frequency of the non amplified allele. The RP is defined as $\Sigma I_b$, being $I_b = 1-(2|0.5-p|)$, where $p$ is the frequency of the genotypes that contain the band and it represents the ability of a marker to discriminate between samples. Genetic similarity (GS) between units $i$ and $j$ was calculated using the Dice coefficient: $GS_{ij} = 2a/(2a + b + c)$, where $a$ is the number of

matching present bands, $b$ is the number of bands present only in unit $i$ and $c$ is the number of bands present only in unit $j$. Genotypes were clustered by the Unweighted Pair Group Method with Arithmetic averaging (UPGMA) method. A cophenetic value matrix of the UPGMA clustering was used to test for the goodness-of-fit of the clustering to the resemblance matrix on which it was based by computing the product moment correlation ($r$) with 1,000 permutations (Sneath and Sokal 1973). These analyses were conducted using the NTSYS-PC v. 2.1 package (Rohlf 1998).

### Analysis of the population structure

Possible population structure was estimated using a model based Bayesan procedure implemented in the software STRUCTURE v2.3 (Pritchard et al. 2000). The analysis was carried out using a burning period of 10,000 iterations and a run length of 200,000 MCMC replications. We tested a continuous series of $K$, from 1 to 12, in 10 independent runs. We did not introduce prior knowledge about the population of origin, and assumed correlated allele frequencies and admixture (Falush et al. 2003). The most informative $K$ was identified using the *ad hoc* statistic $\Delta K$, which is based on the rate of change in the log probability of data between successive $K$ values (Evanno et al. 2005). Subsequently, population structure was inferred for $K = 6$ with a burring period of 50,000 iterations and a run length of 500,000 MCMC replications. The estimated cluster membership coefficient matrices of these 10 runs, were permuted so that all replicates have as close a match as possible, and then averaged across replicates using the Greedy algorithm of the software CLUMMP (Jakobsson and Rosenberg 2007). To validate the predefined or the estimated population structure, we calculated pairwise Fst (Lynch and Milligan 1994) and Nei's standard genetic distance between populations (Nei and Li 1979). The reference distribution for $P$ value calculation of the Fst analysis was calculated using 10,000 permutations. These analyses were performed with the AFLP-SURV 1.0 software (Vekemans 2002).

## Results

### Genetic diversity and hierarchical classification

A total of 30 clear and repeatable fragments were scored, whose size ranged from 3 to 14 kb. Fragments smaller than 3 kb, with minor intensity and repeatability were not scored. Figure 1 shows an example of DNA fingerprint of the different tomato types under investigation. All fragments were polymorphic among the four tomato types (Table 2). The number of polymorphic bands within each tomato type varied

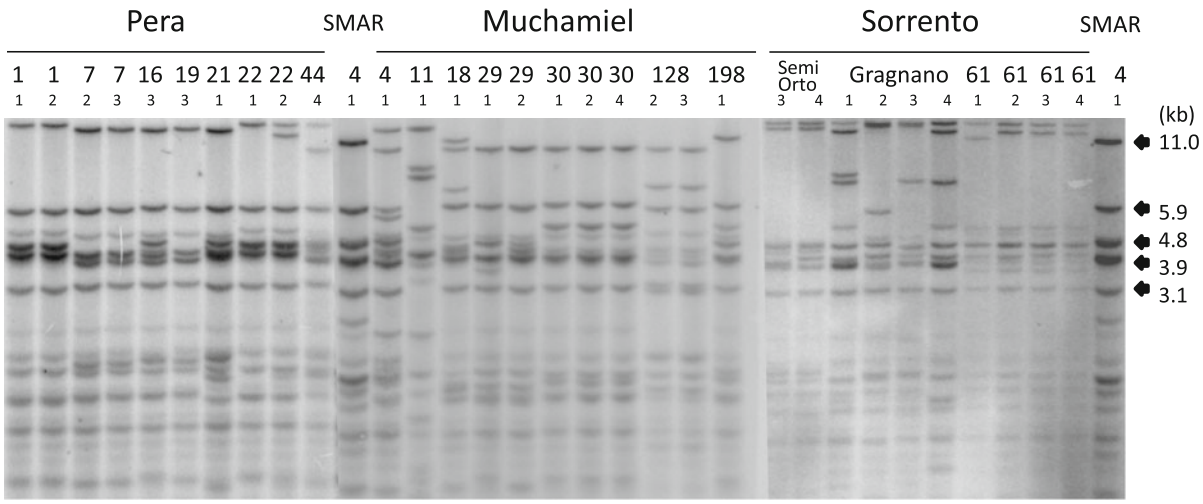


**Fig. 1** $(GATA)_4$ DNA fingerprinting of some plants analyzed. In the top line appears the name of the tomato type, followed by the accession number (Table 1) and the number of the plant analysed. The estimated molecular size (kb) of the San Marzano 4 bands, used as reference, is indicated on the *right hand side*

**Table 2** Genetic variation obtained with the $(GATA)_4$ oligonucleotide probe for the tomato accessions evaluated and grouped according to the fruit type

| Tomato type | Number of plants | PIC | RP | Average similarity ± SDa |
|---|---|---|---|---|
| De la Pera | 34 | 0.124 | 6.06 | $0.69 \pm 0.19^{b}$ |
| Muchamiel | 35 | 0.225 | 9.14 | $0.48 \pm 0.26^{a}$ |
| San Marzano | 20 | 0.029 | 1 | $0.91 \pm 0.09^{c}$ |
| Sorrento | 20 | 0.106 | 4.6 | $0.72 \pm 0.18^{b}$ |
| All types | 109 | 0.235 | 9.30 | $0.64 \pm 0.24$ |

* Different letters represent statistically different group (Tukey; alpha = 0.05)

*RP* resolving power, *PIC* polymorphic information content, *SD* standard deviation

from 4 (in the San Marzano) to 23 (in the Muchamiel) and did not significantly correlate with the number of genotypes per tomato type ($P = 0.051$; Spearman's rho test). PIC and RP values varied considerably in the four tomato types (Table 2). Differences in the $(GATA)_4$ profile among plants of the same accession were present in all accessions but Pera1, Pera7 and Pera25, San Marzano4, San Marzano12 and San Marzano22, Sorrento62, Sorrento65, and Muchamiel11, Muchamiel128, Muchamiel198 and MuchamielBNY. Similarity between genotypes of the same accession, calculated using the Dice coefficient, was highest for the San Marzano type and lowest for the Muchamiel (Table 2).

Genetic similarities were used for hierarchical clustering. The cophenetic correlation between the ultrametric distances of the dendrogram and the similarity matrix revealed a very good degree of fit ($r = 0.90$; $P < 0.01$). The UPGMA tree indicated that the plants under investigation essentially clustered according to the tomato type (Fig. 2a). Specifically, plants of the De la Pera type grouped in two different neighboring clusters. Despite the lower genetic similarity, the Sorrento accessions mostly grouped together, with two Gragnano plants joining in a small cluster together with the accession SemiOrto. The other two Gragnano plants were present in a more genetically diverse cluster. All the plants of the San Marzano accessions grouped in a single cluster that also included Muchamiel198, and, with a lower similarity, with three of the four plants analysed of the accession Muchamiel29. The remaining Muchamiel plants formed another group, with the exclusion of the accession Muchamiel11 and Muchamiel128. These two accessions, however, showed a low level of similarity between them.

## Population structure and differentiation

The identification of genetically homogeneous groups of plants was performed using an admixture model-based clustering analysis implemented in the software STRUCTURE. The Evanno's test indicated that the most informative number of subpopulations ($K$) is 6 (Fig. 3). The inferred population structure is presented in Fig. 2b. Population structure analysis defined clusters that were associated to a priori tomato type-based groups. Specifically, the De la Pera accessions were clearly divided in two equally homogenous groups (P1, dark blue and P2, orange, Fig. 2b). Similarly, the San Marzano plants grouped together and they were all characterised by a high estimated membership coefficient (SM, brown, Fig. 2b). The plants of the Muchamiel198 accession were also assigned to this group. Another group (SO, purple, Fig. 2b) was constituted only by Sorrento accessions, yet the plants of the Gragnano and the SemiOrto accessions were characterised by some degree of possible admixture with one of the De la Pera or the San Marzano group. The Muchamiel subpopulation (M1, light blue, Fig. 2b) was also homogenous and included all but the Muchamiel128 accession. The latter was grouped separately (M2, green, Fig. 2b).

We tested whether the groups inferred by the population structure analysis or those defined by the country of origin, or by the tomato type, represent statistically significant subpopulations by pairwise comparison of two measures of differentiation, Fst and Nei' Standard Genetic Distance (Dst) (Table 3). Results of the two indices were correlated ($P < 0.01$, Spearman rho test) as in other tomato studies (Sim et al. 2011). Significant differences in the Fst were found for all the pairwise comparisons, for either the predefined or the predicted groups. However, the lowest genetic distance and Fst (and $P$ value) were found comparing the Italian and the Spanish groups. These results suggest that the country of origin does not represent a major factor of differentiation for the investigated accessions. Such proposition is also supported by the fact that genetic differentiation and distances were fairly similar between tomato types or

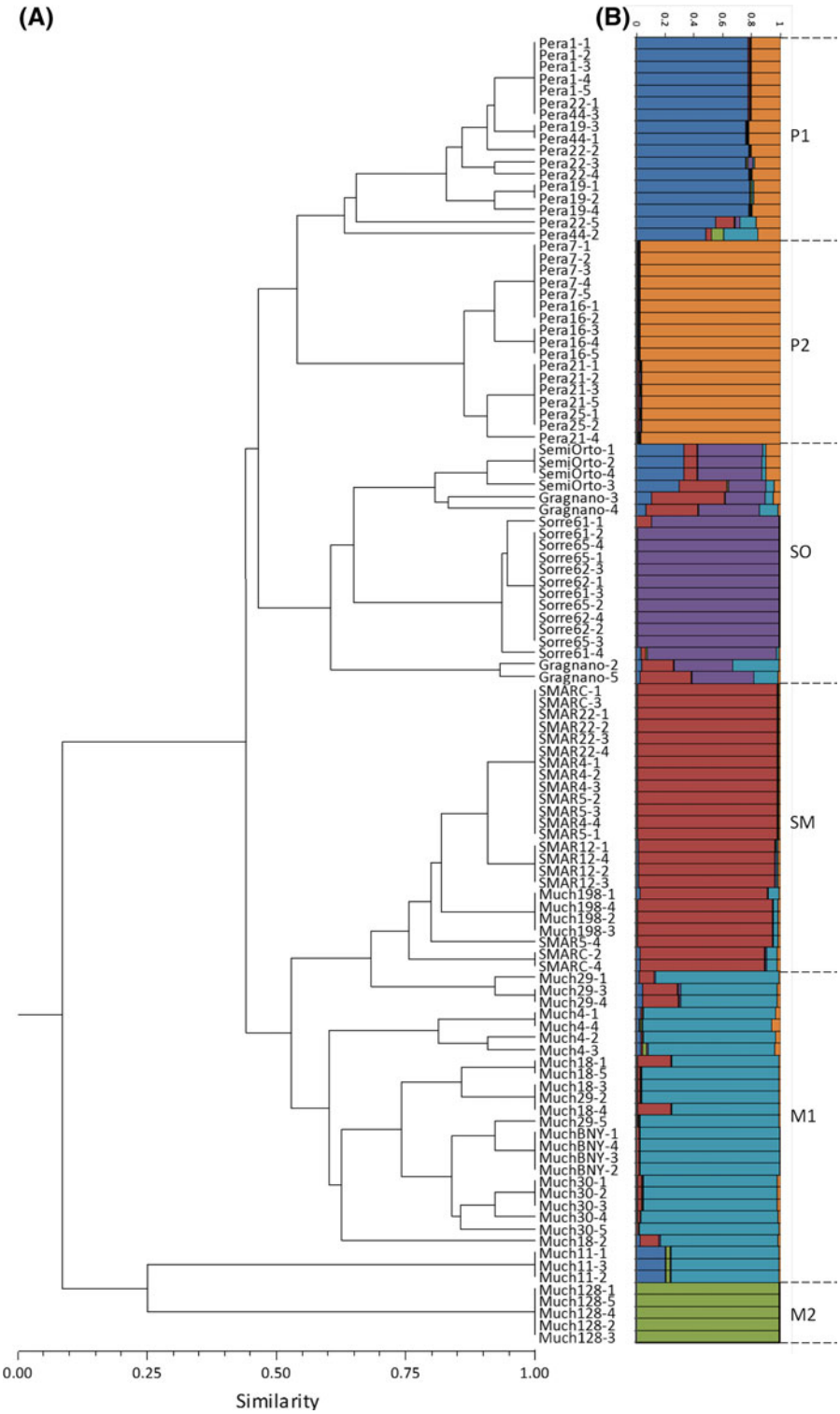


**Fig. 2** Genetic relationship among accessions. **a** Dendrogram (UPGMA algorithm) of the tomato genotypes based on genetic distances calculated with the Dice coefficient. **b** Estimated population structure of the tomato genotypes for $K = 6$ (see also Fig. 3). Each genotype is represented by a *horizontal line*, which is partitioned into *colored segments* that represent the estimated membership fractions in the $K$ clusters. (Color figure online)

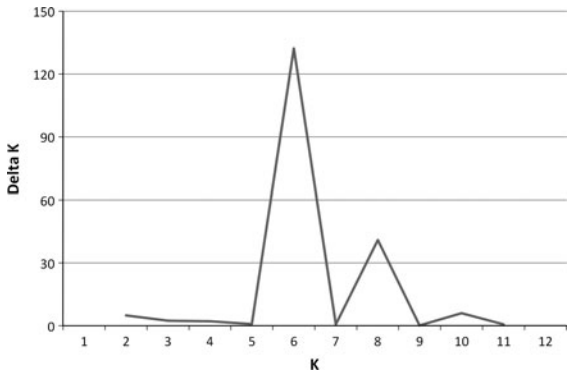


**Fig. 3** Estimation of the optimum number of clusters of tomato accessions according to the Evanno's method. The graph displays the Delta *K* [mean (lL''(*K*)l/SD(L(*K*))] for each *K* value

between groups identified by STRUCTURE. The only exceptions were about the comparisons involving the M2 group. The high Fst and Dst values can be explained considering the low number of (identical) genotypes that are present in that subpopulation. While a well-built division could be not made comparing the two geographic regions, further structure was apparent for the two Spanish accessions. Whereas for the Muchamiel type this was due to the presence of one very different accession, the genetic analyses employed in this study showed a good congruence in indicating the presence of two relatively similar but distinct De la Pera subpopulations.

## Discussion

In a previous study, by using 19 SSRs on a subset of the De la Pera and Muchamiel Spanish cultivars here analysed, we were not able to discriminate all the accessions evaluated, although they had different phenotypes as assessed by morphological characterization (García-Martinez et al. 2006). Distinctive molecular profiles of the most closely related tomato cultivars could be achieved only by using a combination of SSR and AFLP markers (Ruiz et al. 2005a, b, c; García-Martínez et al. 2006). It is therefore remarkable that the use of the $(GATA)_4$ oligonucleotide probe, although labour intensive and not amenable to high-throughput genotyping methods, detected clear and reproducible differences between all the accessions investigated. Furthermore, within-accession polymorphism was found in 14 out of the 26 accessions. This is not surprising, because the lack of uniformity is a characteristic of the plants that are grown and replicated by farmers. García-Martínez

**Table 3** Pairwise estimates of Fst and Nei's standard genetic distance (Dst) between predefined groups or between groups of tomato accessions as inferred by the Bayesian analysis implemented in the STRUCTURE software

| Predefined groups | Italy (40) | S. Marzano (20) | Sorrento (20) | Spain (69) | Muchamiel (35) | Pera (34) |
|---|---|---|---|---|---|---|
| Italy | – | | | 0.258* | | |
| S. Marzano | | – | 0.657** | | 0.368** | 0.563** |
| Sorrento | | 0.173 | – | | 0.511** | 0.530** |
| Spain | 0.088 | | | – | | |
| Muchamiel | | 0.116 | 0.256 | | – | 0.369** |
| Pera | | 0.133 | 0.164 | | 0.142 | – |

| STRUCTURE groups | SM (24) | SO (20) | M1 (26) | M2 (5) | P1 (17) | P2 (17) |
|---|---|---|---|---|---|---|
| SM | – | 0.650** | 0.443** | 0.947** | 0.699** | 0.799** |
| SO | 0.180 | – | 0.574** | 0.884** | 0.617** | 0.705** |
| M1 | 0.119 | 0.258 | – | 0.733** | 0.550** | 0.532** |
| M2 | 0.566 | 0.680 | 0.380 | – | 0.912** | 0.958** |
| P1 | 0.163 | 0.182 | 0.579 | 0.203 | – | 0.707** |
| P2 | 0.178 | 0.223 | 0.167 | 0.534 | 0.157 | – |

The number of genotypes per group is indicated in parenthesis. Above the diagonal is the pairwise estimate of Fst (Lynch and Milligan 1994), while Dst (Nei and Li 1979) appears below the diagonal. Global Fst within the four tomato types was 0.505 ($P < 0.01$)

The *P* value for estimated Fst was calculated using 10,000 permutations (* $P < 0.05$, ** $P < 0.01$)

et al. (2006) reported that the proportion of non-uniform accessions detected by SSR markers was lower (2 out of 16). Hence, these comparisons confirm the power of $(GATA)_4$ fingerprinting to detect intra-cultivar variability (Caramante et al. 2009). Although a more subtle discrimination between and within accessions was achieved with the $(GATA)_4$ marker, it is also necessary to add that we obtained a fairly similar clustering of the De la Pera accessions using SSR or SRAP markers (Ruiz et al. 2005a, b, c; García-Martínez et al. 2006). However, the use of SNPs (García-Gusano et al. 2004), SSRs (Ruiz et al. 2005a, b, c) or AFLPs (García-Martínez et al. 2006), did not reveal the presence of two De la Pera subpopulations.

The most genetically variable type was the Muchamiel, which is in agreement with previous analysis (García-Martínez et al. 2006). De la Pera and Muchamiel are two types of traditional cultivars that include accessions with similar morphological traits. The latter is much more spread in Spain and with time, Muchamiel has become an unspecific denomination for big-sized tomatoes that are cultivated in a wider area in the South-East of Spain (Ruiz et al. 2006). This fact could explain the higher genetic variability found between the accessions of this tomato type. In the Italian group, the Sorrento accessions were characterized by a greater genetic diversity than the San Marzano accessions. However, differently from the De la Pera type, a clear partition of genetic variability among accessions was not evident. While Sorrento is a denomination that includes varieties with similar fruit-shape, San Marzano is a specific tomato type that, despite the numerous improper labelling at a world-wide scale, since the early years of last century was explicitly selected in a discrete agro-environment for industrial transformation (Monti et al. 2004).

The result of the structure analysis implied that the genetic dissimilarities between the tomato types analyzed, rather than depending on an initial difference in the introduced germplasm from the primary centre of origin, are most likely due to other factors, such as spontaneous or planned out-crossing and farmers' selection for adaptation to production niches and uses. For heirloom varieties, the possible strong influence of farmers' selection in shaping genetic diversity is very likely because the tomato produces many seeds per fruits, and fruits of local varieties are traditionally hand-harvested, thus enabling selection from individual plants or fruits (Zeven 2002). Another reason for the lack of a country-specific structure may be the existence of a selective pressure to relatively comparable climates and to culturally similar cooking style and market orientation. As commented before, during the period lasting from the end of the thirteenth century to the beginning of the eighteenth century parts of the South of Italy belonged to the Spanish Crown of Aragon, which also included an important part of the Mediterranean coast of Spain (Muñoz-Falcón et al. 2008). The exchange of materials of tomato among these regions could also have contributed to the lack of a country-specific structure. To corroborate such analysis it will be interesting to investigate also other tomato types.

Tomato is a cultivated species well suited to the analysis of the effect of breeding in shaping diversity (Sim et al. 2009). Our data confirmed that the GATA fragments are a powerful tool to fingerprint closely related tomato accessions because this marker system allowed us to discriminate at the molecular level accessions that previously we were not able to distinguish using either SRAP, SSR or AFLP markers. Using neutral DNA markers, we did not find a clear evidence of specific residual from founder parents. The genetic differentiation, tested on the basis of Fst and Nei's standard genetic distance, indicated that breeding efforts by farmers (which include the need of market specialization), rather than agro-ecological adaptation, is a major driving force for tradition tomato accessions. The results of this work are meaningful not only for germplasm conservation and exploitation (Zeven 2002) but also for breeding programs. These could easily take advantage of the molecular description presented here to identify genotypes that represent the most valuable gene sources based on distinct subpopulations (Pritchard et al. 2000). A further analysis of the phenotypic variation will be necessary to identify the most important plant material for current breeding programs in both regions

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