UNIVERSITÀ DEGLI STUDI DI SASSARI

SCUOLA DI DOTTORATO DI RICERCA
Scienze e Biotecnologie
dei Sistemi Agrari e Forestali
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Indirizzo Produttività delle Piante Coltivate

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Characterization of a wide collection of tomato (*Solanum lycopersicum* L.) for morpho-phenological, quality and resistance traits

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Abstract

Tomato (*Solanum lycopersicum* L.) is considered one of the leading vegetable crops worldwide and it is an important model species for plant biology, in particular for the genetic control of quantitative variation, fruit ripening processes and resistance to biotic stress. The availability of the tomato genome has enhanced the chances to unravel the genetic control of simple and complex traits that can be achieved by genome-wide association studies which exploit natural variation.

Accordingly, in the present study it has been investigated a wide collection of tomato mainly including landraces (71 from Italy of which 64 from Sardinia, and 44 from all over the world) which were compared to ten cultivars and five wild-related tomato species. Three experimental trials in two years and two locations were performed and data were collected for a) phenotypic traits by both classic and precision phenotyping, b) genetic diversity by means of 19 micro-satellite markers, c) carotenoid content and d) antixenotic resistance to *Tuta absoluta* (Meyrick).

Results revealed high levels of phenotypic and genetic diversity pointing to these landraces as a valuable model to identify QTLs and genes of relevant interest. Finally, the associations detected between molecular markers and phenotypic traits indicate that our collection is suitable for future association mapping and transcriptome correlation studies in addition to breeding purposes.

Contents

CHAPTER 1

The tomato: a brief introduction

1.1 Economic and nutritional importance

Tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Miller) is one of the most important vegetable crops in the world (FAOSTAT, 2012), consumed not only as fresh fruit but also as processed product such as paste, whole peeled tomatoes, diced products, juice, sauces and soups (Foolad, 2007). In 2012, the worldwide production of tomato exceeded 160 millions tons becoming the ninth most important crop species and the second most important vegetable after potato (Figure 1.1).

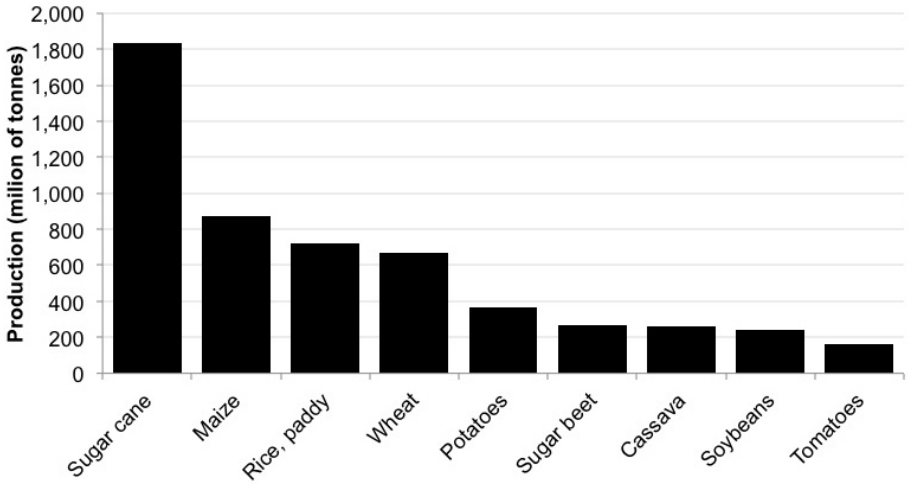

Figure 1.1: Worldwide crop production ranking in 2012 (FAOSTAT, 2012).

Considering a 20 years interval from 1992 to 2012, tomato production has more than doubled (Figure 1.2), and this increase is mainly attributed to the growing importance of Asia in the global economy. In fact, its tomato production moved from 39.4% in 1992 to 60.5% in 2012 (Figure 1.3). In particular, China is the leading producer, followed by India, USA, Turkey, Egypt, Iran, Italy and Spain (Figure

Tomato consumption is due to its high nutritional value and for its importance for a balanced diet. In fact, tomato is a rich source of lycopene, β-carotene, potassium, vitamin A, vitamin C, vitamin E and flavonoids (Willcox et al., 2003). Some of these nutrients, in particular carotenoids, are antioxidants so that the regular consumption of tomatoes has been correlated to protect from the risk of contracting various types of cancer and heart diseases (Borguini and Ferraz Da Silva Torres, 2009).

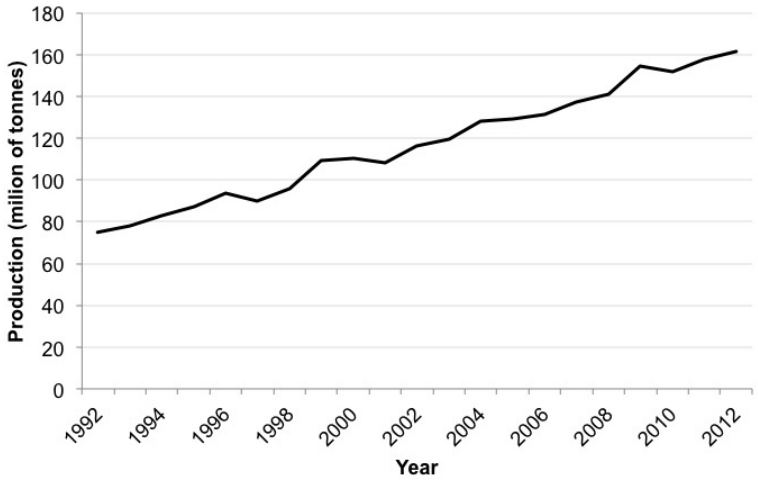

Figure 1.2: Worldwide tomato production for the period 1992-2012 (FAOSTAT, 2012).

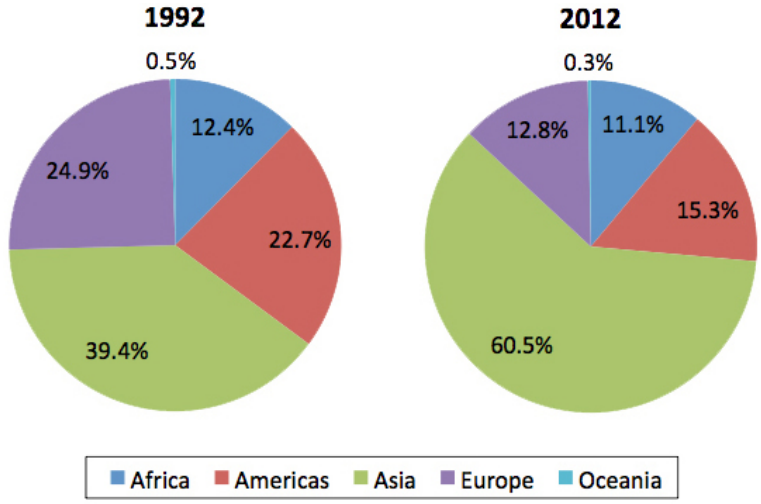

Figure 1.3: Weight in percentage of the different continents in tomato production in 1992 and 2012 (FAOSTAT, 2012).

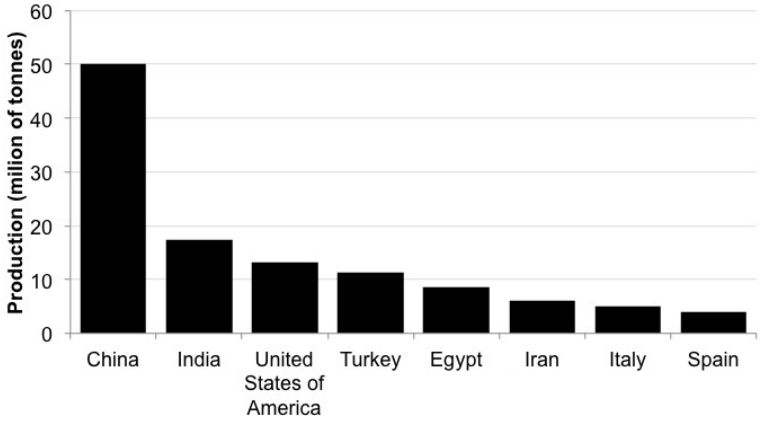

Figure 1.4: Ranking of the eight leading tomato producers (FAOSTAT, 2012).

1.2 Taxonomy and botanical description

The tomato is a fruit berry that is often treated as a vegetable (Foolad, 2007). It belongs to the family of Solanaceae with approximately 90 genera and 3,000-4,000 species, some of which economically important such as potatoes, eggplants, peppers, tobacco and mandrake (Knapp et al., 2004; Weese and Bohs, 2007; Peralta et al., 2008).

The largest genus in Solanaceae is *Solanum*, that includes 1,250 to 1,700 species. The genus and designation of tomato were for a long time subject of debate, as reported by several authors (Foolad, 2007; Peralta et al., 2007). The use of molecular data allowed a final revision of the phylogenetic classification of the Solanaceae and the genus *Lycopersicon* was re-introduced in the *Solanum* genus into the section *Lycopersicon* (Peralta et al., 2008; Spooner et al., 1993; Bohs and Olmstead, 1997; Olmstead and Palmer, 1997; Olmstead et al., 1999; Peralta and Spooner, 2001; Bohs, 2005; Peralta et al., 2005; Spooner et al., 2005).

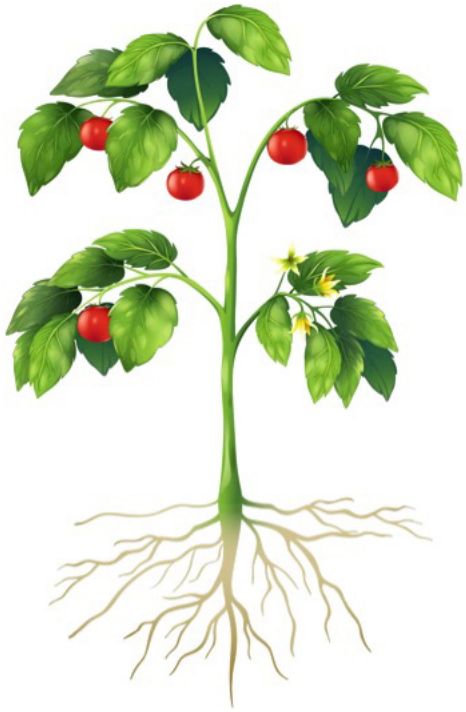

Figure 1.5: Standard representation of a tomato plant.

The tomato (2n = 2x = 24) is an autogamous species with a high degree of homozigosity (Rick, 1979). It is a perennial plant, although it is usually cultivated as an annual plant. Wild tomatoes most probably behave as annuals in their natural environment in the Andean mountains and deserts, because frost or drought kills the plants after the first growing season (Müller, 1940). When the climatic conditions are favorable, wild tomatoes can behave as biennials and perennials depending on the plant capacity for developing secondary growth in basal stems and roots (Liedl et al., 2013).

Tomato plants are dicots, and grow as a series of branching stems, with a terminal bud at the tip that does the actual growing. When that tip eventually stops growing, whether because of pruning or flowering, lateral buds take over and grow into other, fully functional, stems. Tomato stems are typically pubescent,

meaning covered with fine short hairs. The shoots are initially erect, but later, due to the weight of the branches, the plants become decumbent or prostrate and in some cases an develop adventitious roots from basal nodes. In the cultivated species (S. lycopersicum) there are cultivars with semi-determinate or determinate growth habit, with short branches and more compact development (Peralta et al., 2007).

The leaves are 10-25 cm long, bipinnate. Leaflets are quite variable in size and shape from narrowly elliptic, elliptic to broadly elliptic, ovate or orbicular.

This crop present a variety of trichome types and density patterns that are also taxonomically useful. Trichome length range between 10-2,500 μm and four types of glandular trichomes and also four types of non-glandular trichomes have been described in wild tomatoes (Peralta et al., 2008). Glandular trichomes accumulate essential oils that produce the characteristic smell of tomato leaves that varies considerably among species (Darwin et al., 2003).

The basic inflorescence in wild tomatoes is a cyme with different branching patterns. Flowers usually have five petals, although sometimes seven or more flowers can also be found, and its style are usually inserted. The flowers have the anthers fused along the edges, forming a column surrounding the pistil's style.

Tomato fruit is classified as a berry and, as a true fruit, it develops from the ovary of the plant after fertilization, its flesh comprising the pericarp walls. The fruit contains hollow spaces full of seeds and moisture, called locular cavities, that can vary among cultivated species. The fruit color is produced by a combination of pigments in the epicarp and subepidermical tissues. Some species have green fruits due to the presence of chlorophyll in the pericarp. In the cultivated tomato, a wide range of varieties with fruits of different colors, shapes and sizes are currently commercialized (Diez, 1995).

1.3 Tomato origin and domestication

Wild tomato species putatively originated in western South America along coasts and high Andes from central Ecuador, through Peru, to northern Chile, and in the Galapagos Islands (Peralta et al., 2008; Blanca et al., 2012). This wild species grow in a variety of habitats ranging from sea level on the Pacific coast up to 3300 m above sea level in the Andean highlands, and from arid to rainy climates (Warnock, 1988). It is likely that the Andean geography, the diverse ecological habitats and the different climates together contributed to wild tomato diversity (Nakazato and Housworth, 2011) that is expressed through morphological, physiological and sexual characteristics (Peralta et al., 2005; Spooner et al., 2005).

Recently, Peralta et al. (2008), in the monograph of wild tomatoes and their relatives, recognized 13 species of wild tomatoes (Table 1.1).

Although the natural distribution of the wild species is restricted to the Andean region, the site of domestication remain uncertain (Labate et al., 2007). Two alternative hypothesis have been proposed, one supporting Peru, another southern Mexico. According to the Mexican hypothesis, the feral populations of tomatoes migrated from Peru into Central America and were domesticated in Mexico (Jenkins, 1948; Rick and Fobes, 1975; Rick et al., 1974). Following the philology, Jenkins (1948) also argued that the name "tomato" comes from the Mexican Nahua word "tomatl" which refers to "plants bearing globous and juicy

Table 1.1: List of wild tomatoes. From Liedl et al. (2013).

Names in *Solanum* (Peralta et al., 2008)	*Lycopersicon* equivalent
Solanum pennellii Correl	*Lycopersicon pennellii* (Correll) D'Arcy
Solanum habroichates S. Knapp and D. M. Spooner	*Lycopersicon hirsutum* Dunal
Solanum chilense (Dunal) Reiche	*Lycopersycon chilense* Dunal
Solanum huaylasense Peralta	Part of *Lycopersycon peruvianum* (L.) Miller
Solanum peruvianum L.	*Lycopersicon peruvianum* (L.) Miller
Solanum corneliomuelleri J. F. Macbr. (1 geographic race: Misti near Arequipa)	Part of *Lycopersicon peruvianum* (L.) Miller, also known as *L. glandulosum*
Solanum Arcanum Peralta (4 geographic races: 'humifusum', lomas, Marañon, Chotano-Yamaluc)	Part of *Lycopersicon peruvianum* (L.) Miller
Solanum chmielewskii (C.M. Rick, Kesicki, Fobes and M. Holle) D.M. Spooner, G.J. Anderson and R.K. Jansen	*Lycopersicon chmeilewskii* C.M. Rick, Kesicki, Fobes and M. Holle
Solanum neorickii D.M. Spooner, G.J. Anderson and R.K. Jansen	*Lycopersicon parviflorum* C.M. Rick, Kesicki, Fobes and M. Holle
Solanum pimpinellifolium L.	*Lycopersicon pimpinellifolium* (L.) Miller
Solanum lyciopersicum L.	*Lycopersicon esculentum* Miller
Solanum cheesmaniae (L. Riley) Fosberg	*Lycopersicon cheesmaniae* L. Riley
Solanum galapense S.C. Darwin and Peralta	Part of *Lycopersicon cheesmaniae* L. Riley

fruit" (Bauchet and Causse, 2012). De Candolle (1886) supported for the first time the Peruvian origin of tomato domestication, based on the linguistic evidence that "mala peruviana" and "pomi del Peru" were used to refer to the tomato, suggesting its initial domestication and transport from Peru to Europe. Arguments supporting were latter maintained by Moore (1935), Muller (1940) and Luckwill (1943). However, none of the evidence is conclusive regarding an initial site of domestication, and tomatoes may have been domesticated independently in both areas (Peralta et al., 2007).

The most likely ancestor of cultivated tomatoes is thought to be the wild cherry tomato, usually identified as *S. lycopersicum* var. *cerasiforme* because of its wide diffusion in Central America. Nevertheless the genetic investigations made by Nesbitt and Tanksley (2002) demonstrated that the plants known as *cerasiforme* are a mixture of wild and cultivated tomatoes. A very recent study based on the analysis of single nucleotide polymorphisms not only confirms that *S. lycopersicum* var. *cerasiforme* is not the ancestor of the cultivated tomato but also reinforces the model that a pre-domestication of the tomato occurred in the Andean region (Peruvian hypothesis), with the domestication being completed in Mesoamerica (Mexican hypothesis), followed by its introduction to Europe by Spaniards and then spread all over the world (Blanca et al., 2012; Lin et al., 2014).

Tomatoes were introduced into Europe at the beginning of the 16^{th} century by Spaniards (Peralta et al., 2006). It was probably the Spanish conquistador Cortes who first introduced the small yellow tomato to Spain (McCue, 1952). From Spain, the tomato reached Italy where this species was first recorded in 1544 by the botanist Matthiolus. Introduced tomatoes were cultivated first as an ornamental plant and thought by many to be poisonous, than it was incorporated into the local cuisine only in the late17^{th} or early 18^{th} century (McCue, 1952).

Afterwards, tomato consumption expanded to the north. From England, tomatoes were "exported" to the Middle East/Asia and North America due to English colonization (McCue, 1952)). The real domestication of the tomato as an edible vegetable started during the 19^{th} century. The first european cultivars had yellow to red flattened fruits with deep furrows. Development of new cultivars happened by

spontaneous mutation, natural outcrossing or recombination of pre-existing genetic variation (Bauchet and Causse, 2012). Because tomatoes are mostly autogamous, crosses between two different individuals were quite rare and the plants developing from the seeds had a parental phenotype. This allowed obtaining and maintaining fixed populations called "heirlooms" which were unique in their size, color and shape. With expansion of tomato's use, the 20^{th} century was marked by the development of private seeds industries which developed the principle of the F1 hybrid (Bai and Lindhout, 2007).

1.4 Genetic diversity in wild and cultivated tomato

The high diversity of the wild tomato species makes them highly valuable for tomato breeding. The levels of genetic variation can vary among species and within species among populations. Moreover, variation in the levels of the genetic diversity can be due to mating system, historical events, selection or adaptation to local environmental conditions (Arunyawat et al., 2007; Städler et al., 2008). For this reason, many efforts have been devoted to genetically characterize these species that can be further used for classical and association mapping studies (Arunyawat et al., 2007), and for the identification of traits useful for crop improvement.

On the contrary, the cultivated tomato is characterized by a limited variability, largely because of bottleneck events and natural and artificial selection that occurred during the domestication and evolution of the modern cultivars (Rick, 1976). It is reported that tomatoes that were first introduced to Europe by Spanish explorers, furnished the entire genetic base for the modern cultivars and consequently the modern European and U.S. cultivars are highly similar (Miller and Tanksley, 1990; Rick and Fobes, 1975). It is estimated that only the 5% of the total *Solanum* genetic variation can be found within *S. lycopersicum* (Miller and Tanksley, 1990; Rick and Fobes, 1975) and genes for many desirable agricultural characteristics do not exist in this species. The related wild tomato species, however, are a rich source of desirable genes and characteristics for crop improvement, such as high fruit quality and tolerance to abiotic stresses. Indeed, during the past 70 years, wild species of tomato have been utilized in breeding programs to improve the cultivated tomato (Rick, 1979, 1982, 1973). For example, much of the disease resistance in most commercial cultivars has been derived from the related wild species.

Curiously, despite the low levels of genetic diversity, domestication and selection has led to a greater variety of morphological shapes and colors in domesticated tomato fruits than in wild species. Size ranges from small to very large fruits and shape ranges from round to pear-shaped, torpedo, oval and even bell-pepper, whereas ripe fruit color includes various shades of red, pink, orange, yellow, gold green (Male et al., 1999; Paran and van der Knaap, 2007). Wild tomato relatives, on the other hand, produce small round fruits that are often green in color.

1.5 Challenges of tomato breeding

The role of genetic diversity is crucial for future crop improvements especially under the predicted climate change scenario and it is expected that the demand for tomato production will increase, also based on the

current trends in population growth. Accordingly, as breeding is based on the most compelling demands of the consumers yield and improved agronomical traits represent among the most important objectives also in the breeding of tomato. Among these, other important issues are resistance to pests and diseases, adaptability to the environment, followed by fruit quality and nutritional value, fruit shelf life and taste (Bai and Lindhout, 2007; Bauchet and Causse, 2012; Causse et al., 2007; Foolad, 2007; Bergougnoux, 2014).

The resistance to biotic stresses that always cause significant economic losses is a first concern in both processing and fresh market industries as it is estimated that tomato is the target of more than 200 pests and diseases (Lukyanenko, 1991; Schoonhoven et al., 2005; Bai and Lindhout, 2007). The control of these pest and diseases usually rely on repeated chemical treatments that can often lead to the resistance of these pests or pathogens to a number of chemical active ingredients. The heavy reliance of agriculture on pesticides to manage arthropod pests has led to well-documented negative effects on producers and the environment (Hond et al., 2003). Host plant resistance is one of the most effective forms of insect control and offers a very good alternative to the use of insecticides. To be able to develop insect-resistance varieties, it is essential therefore to identify, characterize and categorize effective sources of resistance (Broekgaarden et al., 2011). Within plant species, there is considerable variation in defence mechanisms that has been shaped by differences in selection pressure (Thompson, 2005; Tumlinson et al., 1993). However, by now only very little of this natural variation has been exploited in agriculture (Broekgaarden et al., 2011). Therefore natural variation among wild relatives of crop plants, or even cultivated accessions, needs to be explored to identify sources of resistance specific for a plant species and to introduce these into modern crop plants (Broekgaarden et al., 2011).

Another important breeding objective is to increase the level of carotenoid content in modern cultivars. The strategy mainly rely on the necessity to improve the overall antioxidant activity that can be obtained by selecting for genotypes with high concentrations of these compounds (Kochian and Garvin, 1999; Tucker, 2003). In terms of human health, tomato fruit provides significant quantities of lycopene and β-carotene (Hanson et al., 2004). Lycopene, the major carotenoid in tomato fruit, is a natural antioxidant that is increasing in demand because numerous studies have demonstrated its positive effects on human health such as a decrease of heart diseases, age-related diseases or an association with a lower risk for certain cancers (Bramley, 2000; Heber and Lu, 2002; Kun et al., 2006; Omoni and Aluko, 2005). Also, β-carotene, or vitamin-A precursor, have been shown to be an effective antioxidant, to help in the prevention of free radical chain reactions and diminish risk for eye diseases (Clevidence et al., 2000; Omaye and Zhang, 1998).

1.6 Genetic resources for tomato breeding

Tomato plant breeding over the past century has been associated with a narrowing of the available genetic diversity within elite germplasm. Cultivars (domesticated varieties) have been selected by humans in the last 10,000 years and inevitably represent a subset of the variation found in their wild ancestors (McCouch, 2004). For this reason, the use of genetic resources plays an important role in breeding programs. New

sources of variation useful for crop improvement include landraces and wild relatives of crop species (McCouch et al., 2013).

Multiple tomato resources are available for both research and crop improvement purposes. Many of these resources are publicly available, contributing greatly to a thriving cooperative worldwide tomato research community. Wild and cultivated germplasm of tomato, as well as various genetic stocks are maintained and made available through various gene banks within the US and around the world (Tanksley and McCouch, 1997; Gonçalves et al., 2009; Zamir, 2001), and this germplasm has been continuously used for a wide variety of basic and applied researches.

As aforementioned, the wild germplasm represents a key resource for tomato improvement. A major objective in modern breeding is to return to the wild ancestors of crop plants and employ the diversity that was lost during domestication for the improvement of yields and other agricultural traits under optimal as well as stress field conditions (Bessey, 1906; Tanksley and McCouch, 1997; Zamir, 2001; Gur and Zamir, 2004). The key role of wild relatives in tomato is demonstrated by the ongoing identification of agronomically useful traits in exotic germplasm (Fulton et al., 2002; Frary et al., 2004; Labate et al., 2007). A major drawback to the use of wild genetic resources in breeding is the linkage drag. This phenomenon is due to the fact that the introgressed gene is often in linkage with other genes, carrying a potential negative impact on other elite varieties (Tester and Langridge, 2010).

Despite the significant loss of genetic diversity, the cultivated tomato shows a large diversity that is particularly evident in the fruit morphology (Figure 1.6). Therefore the diversity within cultivated tomato germplasm might also represent a resource for a variety improvement. In particular, tomato landraces (also called regional varieties) are the earliest form of cultivars and represent the first step in the domestication process (Harlan, 1975; Villa et al., 2005). They constitute the main source of variation in the cultivated species, justifying the increasing interest for their utilization in scientific studies (Chable et al., 2009). Landraces are highly heterogeneous, having been selected for subsistence agricultural environments where low, but stable yields were important and natural environmental fluctuation required a broad genetic base. Landraces are closely related to the wild ancestors and embody a great deal more genetic variation than modern, high-yielding varieties that are selected for optimal performance within a narrow range of highly managed environmental conditions. The value of both the wild species and the early landrace varieties in the context of modern plant breeding is that they provide a broad representation of the natural variation that is present in the species as a whole (Brown, 2000; Brush, 2000; Feuillet et al., 2008). Wild relatives and early landrace varieties have long been recognized as the essential pool of genetic variation that will drive the future of plant improvement (Bessey, 1906; Burbank, 1921). The tomatoes classified as landraces are farmer or gardener-selected and are adapted to the local environment, typically in areas of local subsistence. For these reasons, the information obtained with the analysis of wide collections of landraces would be of great interest in the management of the *ex-situ* collections, for their utilization in breeding programs or for their direct use in quality markets (Brown, 2000; Brush, 2000).

Other tomato resources interesting for their diversity level and made available through various gene banks, are heirloom tomatoes (Gonçalves et al., 2009), mutant stocks (Emmanuel and Levy, 2002) and mapping populations (Eshed and Zamir, 1995).

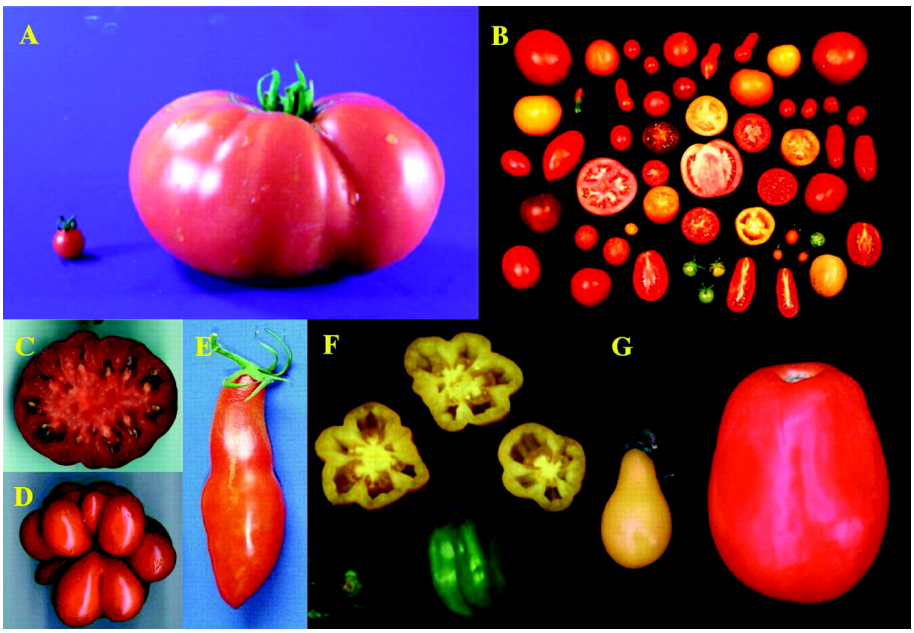

Figure 1.6: Tomato fruits are characterized by different sizes and shapes. Source: Tanksley (2004).

Different monogenic mutants stocks, accumulated through years, are available and they represent one of the most direct ways to determine gene function by analyzing the variant phenotype in the organism that is mutated for a particular gene (Emmanuel and Levy, 2002). However, extensive screening of tomato mutants have been limited by the number of plants that can be managed in a field trial. As an example, the most of characterized ripening mutants have reflected mostly spontaneous mutations or wild allele variants (Giovannoni, 2007).

Mapping populations have been widely used to determining the molecular basis of quantitative and qualitative phenotypic variation in tomatoes. One of the first molecular linkage maps of tomato was published in 1992 (Tanksley et al., 1992) and was based on 62 F2 plants from a cross between *S. lycopersicum* (cv. VF36-Tm2a) and *S. pennellii* (LA716) locating, among the others, 100 genes of known function of phenotype and was subsequently extensively enriched until the highly saturated tomato maps now available (http://www.sgn.cornell.edu/cview/map.pl?map_id=9). Among the publicly available mapping populations there are lines that derive from crosses between wild and cultivated germplasm. In particular, the interspecific crosses from which most linkage maps of tomato have been developed are between the cultivated species and *S. pennellii* or *L. pimpinellifolium*); these have contributed to the identification of thousands of QTLs in tomato (Foolad, 2007; Lippman et al., 2007).

1.7 Importance of plant phenotyping

In order to harness the phenotypic variation of cultivated tomato, and to employ this diversity in basic and applied research projects, it is important to measure and quantify these traits accurately and objectively.

Phenotypic characterization is based upon a set of methodologies and protocols used to measure plant growth, architecture, and composition at different scales of plant organization, from organs to canopies (Fiorani and Schurr, 2013).

Analysis of phenotypes of germplasm resources is fundamental to subsequently evaluate the molecular basis underlying their traits and their overall performances (McCouch et al., 2013). However, evaluating phenotypic traits such as fruit morphology, color intensity, nutritional quality, firmness, flavour and aroma are challenging and time-consuming because of the quantitative nature of these traits (Fiorani and Schurr, 2013). Moreover, the objective and accurate quantification of these traits can be difficult. Therefore, cost reductions and time gains are among the objectives most desirable of the phenotyping of wide collections (Bilder et al., 2009; Fiorani and Schurr, 2013; McCouch et al., 2013).

Many of the ongoing developments in plant phenotyping are driven by the increasingly available technologies, such as imaging sensors (e.g., high-resolution imaging spectrometers) and advanced software for image analysis and feature extraction for 2D and 3D analyses of shoot and root growth and architecture (Biskup et al., 2007; Mühlich et al., 2008; Paproki et al., 2012). Accordingly, in the last few years, conventional phenotyping have been supported by phenomics, defined as the acquisition of high-dimensional phenotypic data on an organism-wide scale (Houle et al., 2010). Phenomics improves the identification of the genetic basis of complex traits and overtakes our limited ability to understand many important biological phenomena by measuring different important variables (Houle et al., 2010). Two-dimensional images derived from photography or scanning is just an example of how to apply phenomics in a cheap and quick manner.

Using these phenotypic data in combination with molecular data, geographic and ecological information will enable researchers to strategically target field experiments and to develop models that can predict plant performances. This will make plant breeding faster, more efficient and cheaper (McCouch et al., 2013).

1.8 Association mapping in tomato

Collection of high quality phenotypic data is essential for genetic mapping research (Zhu et al., 2008). The phenotypic variation of many complex traits of agricultural or evolutionary importance is influenced by multiple quantitative trait loci (QTLs), their interaction, the environment, and the interaction between QTL and environment. Aside to classical QTL mapping, association mapping is one of the tools that is increasingly been adopted for dissecting complex traits (Zhu et al., 2008; Huang et al., 2010; Atwell et al., 2010).

Association mapping, also known as linkage disequilibrium (LD) mapping, has emerged as a tool to dissect complex traits that is based on the establishment of causal relationships between genotypes and phenotypes in natural or breeding populations (Oraguzie et al., 2007; Nordborg and Tavaré, 2002; Flint-Garcia et al., 2003)). Association mapping offers three different advantages, (i) increased mapping resolution, (ii) reduced research time, (iii) greater allele number (Yu and Buckler, 2006). Moreover, in association mapping studies, phenotypic data collected over years in multiple locations are needed (Flint-Garcia et al., 2005). In this framework, newly discovered candidate gene polymorphism can be tested for association with existing phenotypic data.

To date, several marker systems can be used for association studies in tomato. Since the sequencing of

the tomato genome (Tomato-Genome-Consortium, 2012), an increasingly of higher numbers of sequences and SNPs are being used (Lin et al., 2014). Nonetheless, micro-satellite or simple sequence repeat (SSR) markers based on short tandem repeats are still of interest in genetic analyses since they are reliable, less costly and show a high level of polymorphism even in closely related material such as the cultivate tomato (Liedl et al., 2013). They are multi-allelic, which is very useful for association studies (Bredemeijer et al., 2002). Approximately 2000 well characterized and mapped micro-satellite markers have been described and characterized in tomato lines (Smulders et al., 1997; Areshchenkova and Ganal, 2002; Shirasawa et al., 2010a; Geethanjali et al., 2010).

CHAPTER 2

Phenotypic and genetic characterization

2.1 Introduction

The tomato (*Solanum lycopersicum* L.) is an autogamous species having its primary center of diversity in a narrow belt along the Andean region of Ecuador and Peru (Blanca et al., 2012). During its evolution and domestication in Mexico *S. lycopersicum* has undergone various genetic 'bottlenecks' determining the current narrow genetic base (Rick, 1991; Saavedra et al., 2001; Barrero and Tanksley, 2004; Bai and Lindhout, 2007; Foolad, 2007). Moreover, the introduction of the species into Europe at the beginning of the 16th century represented a genetic bottleneck for the cultivated tomato germplasm in Europe (Rick, 1976; Tanksley and McCouch, 1997; Foolad, 2007). The genetic heritage of the tomato was further eroded by the development of vintage and modern cultivars with a high degree of genetic uniformity, also considering that only a limited number of genotypes were used for breeding (Saavedra et al., 2001; Williams and Clair, 1993; Barrero and Tanksley, 2004; Miller and Tanksley, 1990). The decrease in the intraspecific genetic diversity of cultivated species is a consequence of the continuous selection by breeders of more homogenous genotypes with more specific adaptability (Haussmann et al., 2004). Moreover, due to the replacement or disappearance of wild species and local varieties, countless genomic forms with genes that could have been of interest for breeders were lost (Bai and Lindhout, 2007). For these reasons, genetic variation in modern cultivars or hybrids is limited (Archak et al., 2002; Wang et al., 2005; Sharma et al., 2006; Benor et al., 2008; Yi et al., 2008; Chen et al., 2009), as it has been estimated that cultivated tomato genome contains less than 5% of the genetic variation of the wild relatives (Miller and Tanksley, 1990).

This narrowing of the genetic diversity increases the probability of pest occurrence and vulnerability, as well as diseases. Also considering that, in order to develop new cultivars, genetic variation is necessary for breeding programs. For this reason, it is necessary to promote the decrease of the genetic erosion by introducing common and rare alleles locally distributed, from wild species, or from local and traditional varieties. Moreover, with the increasing consumers request for both quality and diversity of tomato products, it is increasing the need to extensively collect, exploit and evaluate unknown tomato germplasm as well as the collections preserved in germplasm banks with the consequent deployment of new alleles (Agong et al., 2000; Hammer et al., 2003; De Castro et al., 2010). Germplasm banks provide information about the preserved accessions, identifying significant characteristics for genetic breeding programs that can facilitates breeding for wider geographic adaptability and traits of interest, especially with respect to biotic and abiotic stresses (Carvalho and Quesenberry, 2009; Nass and Paterniani, 2000; Agong et al., 2000; Saavedra et al., 2001; Saha et al., 2010).

The demand for improvement drove collection expeditions to geographic centers of diversity beginning

in the 1930s and allowed the subsequent exploitation of wild species' alleles (Boswell, 1933; Porte et al., 1941). Spain played a major role in the spread of tomato from the countries of origin and considering that Spain and Italy were the first countries cultivating this crop in Europe, both countries have been recognized as secondary centre of diversification for tomato (Cebolla-Cornejo et al., 2013; Bauchet and Causse, 2012; García-Martínez et al., 2006; Mazzucato et al., 2008). Over five centuries of cultivation, numerous ecotypes adapted to different agroclimatic conditions have been developed (Hammer, 1999; Terzopoulos and Bebeli, 2008; Mazzucato et al., 2008, 2010; García-Martínez et al., 2013). Since the early days of cultivation, a vast number of tomatoes with different fruit shapes have been documented in these countries (Grandillo et al., 2004; De Cillis, 1961). It was the farmers themselves who contributed to the diversification of this crop, by carrying out distinct selections in different cultivation areas (Cebolla-Cornejo et al., 2013). All these types gave rise to landraces, that have been adopted for centuries and are still common in the local markets (Soressi, 1969; Ruiz et al., 2005).

Tomato landraces have been grown and selected by farmers under specific conditions of a limited geographic area (Figàs et al., 2014; Villa et al., 2005). They are usually associated with traditional farming systems and have evolved under natural and farmers' selection often in low-input agricultural systems (Terzopoulos and Bebeli, 2008). A strong decline in the cultivation of tomato landraces has been recorded during the last decades, because of the introduction of pure lines and hybrids, and the evolution of highly mechanized farming systems (Grandillo et al., 2004; Cebolla-Cornejo et al., 2012; Casals et al., 2011; Ruiz et al., 2005; Terzopoulos and Bebeli, 2008). However, this genetic material is typically characterized by a good stress tolerance and local adaptability (Newton et al., 2010; Hawtin et al., 1996).

Tomato landraces represent a wealth of interesting traits such as biotic stress-resistance and high quality fruits as well (Acciarri et al., 2010; Digilio et al., 2010; Andreakis et al., 2004). In fact, they usually present great variability for agronomic traits and contain higher genetic diversity than modern cultivars or hybrids (Williams and Clair, 1993; Zeven, 1998; Zhu et al., 2003; García-Martínez et al., 2006; Terzopoulos and Bebeli, 2008; Yi et al., 2008; Terzopoulos et al., 2009). Therefore they are among the most important sources of genetic variation within the cultivated tomato and to date, a large number of local varieties have been collected (Robertson and Labate, 2007), which provide a potential for increasing the genetic variation in modern breeding (Hawtin et al., 1996; Hoisington et al., 1999; Huang et al., 2010). Moreover, landraces are gaining increasing attention because of their value for niche markets, their yield stability in low input agricultural systems and their growing popularity for sustainable farming (Andreakis et al., 2004; Fernie et al., 2006; Berg, 2009; García-Martínez et al., 2013; Caramante et al., 2011), thus determining an increasing demand by consumers (Brugarolas et al., 2009; Causse et al., 2010). However, in spite of their great potential as a source of variability, the lack of information about their origin, genealogy, agronomic traits and genetic background has limited the use of these varieties in breeding programs (Carelli et al., 2006; Cebolla-Cornejo et al., 2013; García-Martínez et al., 2013). Nowadays, it is still difficult to differentiate in some cases between real landraces, selected by farmers, and old obsolete commercial varieties selected by breeders, as only their designations and not their origins are conserved in the spoken tradition (Cebolla-Cornejo et al., 2013).

In this context, the characterization of landraces is imperative for their efficient use in plant breeding and to improve crop production (Fischbeck, 1989) as an efficient conservation and exploitation of landraces also require the study of their genetic diversity structure (Van Hintum and Elings, 1991; Labate et al., 2011; Corrado et al., 2013; García-Martínez et al., 2013). In fact, genetic profiles of tomato landraces are clearly different from those of modern tomato hybrids and their initial diversity may have been conserved in a range of landraces that have been cultivated for centuries (Carelli et al., 2006; García-Martínez et al., 2006; Terzopoulos and Bebeli, 2008). The evaluation of the diversity of a given collection can be based on phenotypic traits (Yan et al., 2007; Terzopoulos and Bebeli, 2010; Cebolla-Cornejo et al., 2013), genetic markers (Li et al., 2005; Rodríguez et al., 2011; Rao et al., 2012) or their combination (Terzopoulos and Bebeli, 2008; Labate et al., 2011; Hu et al., 2012; Xu et al., 2013). The classification of individuals and quantification of genetic diversity in gene banks is usually aimed at the identification of similarity groups based on separate analyses of continuous (e.g., plant height, fruit weight, days to flowering) and discrete variables (such as fruit color and shape, the presence or absence of a trait, or a molecular marker) (Mohammadi and Prasanna, 2003; Crossa and Franco, 2004; Sudré et al., 2007).

Despite the low variability of *S. lycopersicum*, several molecular markers based on isozymes (Rick and Fobes, 1974), seed proteins (Van den Berg, 1991; Chakraborti et al., 1992; Wang et al., 2000), RFLP (Miller and Tanksley, 1990), AFLP (Park et al., 2004), CAPS (Yang et al., 2004) and SSR (Smulders et al., 1997; Bredemeijer et al., 2002) have been used to address variety identification and relationships among tomato landrace and cultivar collections, as well as in wild species (McClean and Hanson, 1986; Rick et al., 1990; Miller and Tanksley, 1990; Egashira et al., 2000; Zhu et al., 2003). The current genomic era is characterized by new powerful genome sequencing platforms, such as next-generation sequencing (NGS), providing a better way to develop DNA molecular markers (Davey et al., 2011). Single nucleotide polymorphisms (SNPs) have been identified as powerful markers for use in genome-wide studies and in crop breeding programs for genetic diversity analysis, cultivar identification, characterization of genetic resources, and association with agronomic traits (Edwards and Batley, 2010). As tomato genome has been sequenced and assembled (Tomato-Genome-Consortium, 2012), a large amount of tomato NGS data is available for understanding the genetic variations in the tomato genome (Holton, 2001; Shirasawa et al., 2010b; Sim et al., 2012; Kim et al., 2014; Lin et al., 2014). However, in order to perform a rapid and cost-saving genetic diversity study in tomato, the use of SSR markers may be adequate because of their co-dominance, high reproducibility, easy detection, and multiallelic variation (Smulders et al., 1997; Areshchenkova, 2000; He et al., 2003; Frary et al., 2005; Mazzucato et al., 2008).

Although molecular markers are of great utility for studying the relationships among local tomato varieties (Terzopoulos and Bebeli, 2008; Mazzucato et al., 2010; Cebolla-Cornejo et al., 2013; García-Martínez et al., 2013), phenotypic traits remain indispensable descriptors for evaluating genetic variation and try to link quantitative trait loci (QTLs) responsible for this variation to functional genes. Despite phenotypic traits can be affected by environmental factors (Van Berloo et al., 2008), morphological characterization is essential to define the characteristics of local varieties for their protection and registration as recognized conservation varieties (Spataro and Negri, 2013; Hurtado et al., 2014). In this respect, tomato characterization has usually been performed with conventional highly heritable morphological

descriptors based on seedling, plant, inflorescence, flower, fruit, and agronomic traits (Institute, 1996; Scott, 2010). Morphological traits are intuitive and practical, but as they are subject to environmental influences and selection pressure during domestication and breeding, the interpretation of the results of diversity studies based on such traits can be difficult (Van Berloo et al., 2008).

These conventional descriptors are very useful for characterization of varieties but have some limitations, especially when characteristics used for establishing cultivar groups in local varieties correspond to subtle differences in fruit morphology (Scott, 2010). In these cases, conventional descriptors may need to be complemented with more precise characterization tools. Recently, a free high-throughput phenomics software tool (Tomato Analyzer) for the analysis of fruit shape and flesh color of tomato has been developed (Brewer et al., 2006; Gonzalo and Van Der Knaap, 2008; Rodríguez et al., 2010b,a). Tomato Analyzer allows precision phenotyping by the scoring of a large number of fruit traits (e.g. shape and flesh color) from scanned images of fruit sections. Several studies have been performed with Tomato Analyzer to characterize local tomato varieties (Mazzucato et al., 2010; Scott, 2010; Rodríguez et al., 2011; Panthee et al., 2013; Bota et al., 2014; Figàs et al., 2014) as well as to study the genetics of fruit shape in this crop (Brewer et al., 2007; Gonzalo and Van Der Knaap, 2008; Gonzalo et al., 2009; Rodríguez et al., 2011; Rodriguez et al., 2013). These studies reveal that Tomato Analyzer is a powerful tool for precisely describing tomato fruit morphology and it may be a precise complementary tool to conventional characterization of tomato varieties and to distinguish closely related materials (Figàs et al., 2014).

In Sardinia, tomato landraces were widely cultivated until the introduction of modern cultivars. Today, the vast majority of landraces are cultivated in horticultural gardens for personal consumption. Many crop landraces of different species have been collected during 2006 and 2007 and stored at the "Centro Interdipartimentale per la Conservazione e Valorizzazione della Biodiversità Vegetale" (CBV), University of Sassari, Italy (Attene and Rodriguez, 2008). However, only a subsample of this collection has been previously evaluated (Attene and Rodriguez, 2008), and a complete and depth knowledge is indispensable for their efficient use to improve crop production and quality, for promoting them in quality markets and for future plant breeding studies.

In this context, the aim of this work is to evaluate a wide collection of cultivated tomato, that includes the current complete collection of Sardinian landraces, a collection of landraces from around the world, landraces from different Italian regions, vintage cultivars and wild tomato species. These accessions were characterized in two different locations and cropping seasons trough the evaluation of several morpho-phenological traits of interest, using both conventional descriptors and precision phenotyping. Genetic diversity and structure of the collection was also evaluated by using 19 SSR markers. The main objective of the present study was to assess the morphological and genetic variation of this wide tomato collection and evaluate its suitability for association mapping studies.

2.2 Materials and methods

2.2.1 Plant material and experimental design

A collection of 127 cultivated tomato (*Solanum lycopersicum* L.) and wild-related tomato species was investigated. The cultivated tomato accessions included 64 tomato landraces from Sardinia, 7 landraces from other regions of Italy, 44 landraces from different countries around the world, and 10 vintage cultivars. The Sardinian landraces were mainly collected during 2006 and 2007 (Attene and Rodriguez, 2008) when they had been cultivated locally in the same farm (according to the information given by farmers) no less than 30 years (Louette, 2000). Seeds of Italian landraces, cultivars and wild species *Solanum lycopersicum* var. *cerasiforme* and *Solanum pimpinellifolium* were kindly provided by Prof. Andrea Mazzucato, University of Viterbo, Italy. Seeds of accessions from other countries of the world were obtained from the Centre for Genetic Resources, Wageningen University, The Netherlands. The entire collection is now stored at the "Centro Interdipartimentale per la Conservazione e Valorizzazione della Biodiversità Vegetale" (CBV), University of Sassari, Italy. For each group of accessions we use an acronym (L-SAR, Sardinian landrace; L-IT, Italian landrace; L-EXOT, exotic landrace; C, vintage cultivar; W, wild species). The seed stock also includes an accession (Cocktail) that is close to the wild-species *S. pimpinellifolium* (data not shown) and, for this reason, it is classified as wild. The complete list of the accessions is reported in Table A.1.

The accessions have been studied throughout two experimental trials, one in 2012 and one in 2013. In 2012, the experimental trial was carried out in an open-field in Oristano, Sardinia, following a randomized complete block design with five replicates, 124 treatments (accessions) and 4 plants per plot. The field was characterized by eight mulched double rows, with 0.9 m between each double row, 0.6 m between the rows of the same double row and plants spaced 0.4 m apart in-the-row. Transplantation was done by hand at the beginning of June, 2012. Plants of a commercial tomato variety were transplanted all around the field as borders of the trial. All plants were staked by reeds and pruned to one stem, excepting genotypes with a determined growth type. When the plants with undetermined growth type reached the height of about 1.8 m, the apex was trimmed. Standard agronomic practices were used. The trial ended in September when all fruits were harvested.

The experimental trial in 2013 was carried out in a greenhouses at the "M. Deidda" experimental farm of Ottava, University of Sassari, Sardinia. The trial followed a randomized complete block design with three replicates, 127 treatments (accessions) and 1 plant per plot. The field was characterized by three mulched double rows, one for each replicate, with 1.2 m between each double row, 0.4 m between the rows of the same double row and 0.4 m among plants on the same row. Transplantation was done by hand at the end of January 2013. Plants of a commercial tomato variety were transplanted all around the field as borders of the trial. All plants were staked by cords and pruned to one stem, excepting genotypes with a determined growth type. When the plants with undetermined growth type reached the height of about 1.8 m, the apex was trimmed. Standard agronomic practices were used. The trials ended in July when all fruits were harvested. Most of the accessions were shared between the two trials from 2012 and 2013,

except for few lines analyzed only in 2013.

The list of the genotypes and the number of accessions studied during each experimental trial is reported in Table 2.1. Details about accession name, group and origin are given in Table A.1.

Table 2.1: Number of accessions analyzed for each group in 2012 and 2013.

Collection	Collection code	2012	2013
Sardinian landraces	L-SAR	61	64
Italian landraces	L-IT	6	7
Exotic landraces	L-EXOT	44	43
Cultivars	C	10	10
Wild-related species	W	3	3
Total accessions		124	127

2.2.2 Phenotypic analysis

Individual plants were characterized using twenty-six agronomic conventional traits, both phenological and morphological traits. The registered descriptors were sowing date (SD, date), transplanting date (TD, date), flowering date (FLD, date), ripening date (RD), days to flowering from sowing date (DTFs, (FLD-SD), days), days to flowering from transplanting date (DTFt, (FLD-TD), days), flowering-ripening interval (FRI, [FLD-RD], days), plant growth type (PGT, score), number of flowers per inflorescence (NFI), inflorescence type (ITP, score), stigma exertion (SE, score), leaf attitude (LAT, score), leaf length (LLE, cm), leaf width (LWI, cm), leaf length/width (LL/W, [LLE/LWI]), foliage density (FD, score), number of harvested fruits (NHF), weight of harvested fruits (WHF, g), mean fruit weight (FW, [WHF/NHF], g), fruit length (FLE, cm), fruit width (FWI, cm), fruit length/width (FL/W, [FLE/FWI]), fruit color (FCO, score), fruit shape (FSH, score), green shoulder (GS, score), shape of pistil scar (SPS, score), fruit blossom end shape (SBE, score) fruit cross-sectional shape (FSS, score), number of locules (NOL), puffiness appearance (PUF, score), pericarp thickness (PTK, cm) and degrees Brix (BRIX, °Bx). Most of the descriptors were taken from the guidelines of the Bioversity International, formerly IPGRI (`http://tinyurl.com/n7k75m6`). All variables used to calculate other parameters (i.e. SD, TD, FLD, RD, NHF, WHF) were not used for further analysis.

The parameters scored in each trial are listed in Table 2.2.

In addition to these conventional descriptors, 38 fruit-related traits were analyzed by using the software Tomato Analyzer (Brewer et al., 2006; Gonzalo and Van Der Knaap, 2008). This analysis was done on all the accessions cultivated in 2013 (Table 2.1). For each accession, six fruits were harvested at the ripe stage. Three of them were cut longitudinally and the others transversally, then scanned with an Mustek Must A3 600S scanner at a resolution of 300 dpi, saved as JPEG images and subjected to morphometric analysis with Tomato Analyzer version 3 software (Rodríguez et al., 2010a), setting 'centimeters' as units, 0.9 as upper position and 0.1 as lower position for blockiness position settings, and 20 degrees as macro distance and three degrees as micro distance for proximal and distal angles settings. The following trait groups were selected from attributes list: basic measurements (seven), fruit shape index (three), blockiness (three), homogeneity (three), proximal fruit end shape (four), distal fruit end shape (four), asymmetry (six), internal eccentricity (five), and latitudinal section (three), for a total of 38 traits. Thirty-five traits

Table 2.2: List of conventional traits evaluated for each trial in 2012 and 2013.

Trait	Code	Type[a]	2012	2013
PHENOLOGY DESCRIPTORS				
Days to flowering from sowing (days)	DTFs	QNT	✓	✓
Days to flowering from transplanting (days)	DTFt	QNT	✓	✓
Flowering-ripening interval	FRI	QNT	✓	✓
HABITUS DESCRIPTORS				
Plant growth type	PGT	QLT	✓	✓
INFLORESCENCE DESCRIPTORS				
Number of flowers per inflorescence	NFI	QNT	✓	✓
Inflorescence type	ITP	QLT	✓	✓
Stigma exertion	SE	QLT	✓	✓
LEAF DESCRIPTORS				
Leaf attitude	LAT	QLT	✓	✓
Leaf length (cm)	LLE	QNT	✓	✓
Leaf width (cm)	LWI	QNT	✓	✓
Leaf length/width	LL/W	QNT	✓	✓
Foliage density	FD	QLT	✓	✓
FRUIT DESCRIPTORS				
Mean fruit weight (g)	FWG	QNT	✓	✓
Fruit length (cm)	FLE	QNT	✓	✓
Fruit width (cm)	FWI	QNT	✓	✓
Fruit length/width	FL/W	QNT	✓	✓
Fruit color	FCO	QLT	✓	✓
Fruit shape	FSH	QLT	✓	✓
Green shoulder	GRS	QLT	✓	✓
Shape of pistil scare	SPS	QLT	✓	✓
Fruit blossom end shape	SBE	QLT	✓	✓
Fruit cross-sectional shape	FSS	QLT	✓	✓
Number of locules	NOL	QNT	✓	✓
Puffiness appearance	PUF	QLT	✓	✓
Pericarp thickness (cm)	PTK	QNT	✓	✓
Degrees Brix (°Bx)	BRIX	QNT	✓	✓

[a] QLT = qualitative trait, QNT = quantitative trait.

were assigned to the longitudinal section, and three to the transversal section.

The parameters scored through Tomato Analyzer are listed in Table 2.3. Full details about the description of each of the measured traits can be found in Rodríguez et al. (2010a).

2.2.3 Molecular analysis

A molecular analysis was also performed on all the accessions cultivated during 2013 (Table 2.1).

The genomic DNA was extracted from young leaves by taking approximately 100-300 mg of tissue from one plant per accession, for a total of 127 samples. The frozen leaf tissue was grinded with a TissueLyser II (Qiagen s.r.l., Milano, Italy) and DNA extracted using the Qiagen DNeasy Plant Mini Kit (Qiagen s.r.l., Milano, Italy). All samples of extracted DNA were stored at -20 °C.

The molecular analyses were conducted with 19 simple sequence repeat (SSR) markers that were selected from the literature to obtain a good coverage of the tomato genome. Ten SSRs were selected to include a group of loci in regions harboring reported QTLs that affect several fruit features (Q-SSRs), whereas the remaining SSRs do not have a known linkage with genes of interest (NQ-SSRs). The complete

Table 2.3: List of conventional traits evaluated using Tomato Analyzer in 2013.

Trait	Section[a]	Attribute group	Trait	Section	Attribute group
Perimeter (cm)	L	Basic measurements	Proximal indentation area	L	Proximal fruit end shape
Area (cm^2)	L	Basic measurements	Distal angle micro (°)	L	Distal fruit end shape
Width mid-height (cm)	L	Basic measurements	Distal angle macro (°)	L	Distal fruit end shape
Maximum width (cm)	L	Basic measurements	Distal indentation area	L	Distal fruit end shape
Height mid-width (cm)	L	Basic measurements	Distal end protrusion	L	Distal fruit end shape
Maximum height (cm)	L	Basic measurements	Obovoid	L	Asymmetry
Curved height (cm)	L	Basic measurements	Ovoid	L	Asymmetry
Fruit shape index external I	L	Fruit shape index	V.Asymmetry	L	Asymmetry
Fruit shape index external II	L	Fruit shape index	H.Asymmetry.Ob	L	Asymmetry
Curved fruit shape index	L	Fruit shape index	H.Asymmetry.Ov	L	Asymmetry
Proximal fruit blockiness	L	Blockiness	Width widest pos	L	Asymmetry
Distal fruit blockiness	L	Blockiness	Eccentricity	L	Internal eccentricity
Fruit shape triangle	L	Blockiness	Proximal eccentricity	L	Internal eccentricity
Ellipsoid	L	Homogeneity	Distal eccentricity	L	Internal eccentricity
Circular	L	Homogeneity	Fruit shape index internal	L	Internal eccentricity
Rectangular	L	Homogeneity	Eccentricity area index	L	Internal eccentricity
Shoulder height	L	Proximal fruit end shape	Lobedness degree	T	Latitudinal section
Proximal angle micro (°)	L	Proximal fruit end shape	Pericarp area	T	Latitudinal section
Proximal angle macro (°)	L	Proximal fruit end shape	Pericarp thickness	T	Latitudinal section

[a] L = longitudinal section, T = transversal section.

list of the markers is shown in Table 2.4. All details are available in Table B.1.

All PCR reactions were performed using the EconoTaq® DNA Polymerase Kit (Tema Ricerca s.r.l., Bologna, Italy) on a Perkin-Elmer PCR machine (Applied Biosystems, Foster City, CA, USA), with a standard program: an initial cycle of 5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at X °C and 30 s at 72 °C, plus 15 min at 72 °C. X °C refers to the annealing temperature variable for the different primers used (Table B.1).

PCR products were finally separated on 8M denaturing polyacrylamide gels and displayed through a silver staining method (Baudoin et al., 2007). All individuals were genotyped by scoring the bands using a ladder with a known molecular weight as a reference.

Table 2.4: List of SSR markers used for genetic analysis.

Marker	Chromosome	Type[a]	Marker	Chromosome	Type
TMS42	11	Q-SSR	EST245053	1	NQ-SSR
TMS52	12	Q-SSR	LE20592	11	NQ-SSR
TMS59	8	Q-SSR	LE21085	4	NQ-SSR
TMS63	1	Q-SSR	LELE25	10	NQ-SSR
EST253712	6	Q-SSR	LELEUZIP	8	NQ-SSR
EST258529	5	Q-SSR	LEMDDNa	5	NQ-SSR
Tom 59-60	3	Q-SSR	Tom 47-48	3	NQ-SSR
Tom 236-237	9	Q-SSR	Tom 162-163	1	NQ-SSR
SLM6-14	6	Q-SSR	SLM12-29	12	NQ-SSR
			SLM6-35	6	NQ-SSR

[a] Q-SSR = marker associated with known QTLs; NQ-SSR = marker without a known linkage with QTLs of interest.

2.2.4 Statistical analysis

All the statistical analyses were done by using JMP 10.0.0 (SAS Institute, Inc. 2012) and when necessary accessions with missing data were excluded from the analysis.

Ranges and mean values were calculated for each accession. For conventional traits, the analysis of variance (ANOVA) was performed over all the accessions to test the presence of significant differences among genotypes. The interaction between the two experimental trials was performed considering only the 120 accessions shared among the two. For Tomato Analyzer descriptors, ANOVA was performed on individual fruits values to detect differences among accessions.

Nei's diversity index (Nei, 1978) was used to evaluate the diversity among qualitative traits. Broad sense heritability was calculated by fitting model with random effects through the restricted maximum likelihood method (REML) (Patterson and Thompson, 1971; Patterson and Nabugoomu, 1992; Lynch et al., 1998).

Pearson's correlations were estimated to verify associations among different traits.

Multivariate analyses were done using cluster analysis by the hierarchical method and by principal components analysis (PCA).

Descriptive genetic statistics were calculated for the overall collection and within each group of genotypes by using PopGen 1.32 (Yeh Francis et al., 1999) and Arlequin 3.5.1 (Excoffier and Lischer, 2010). The genetic distances among the different groups were determined using the F_{ST} statistics (Wright, 1949) and their significance was tested using 10^5 permutations (Arlequin 3.5.1.2; Excoffier and Lischer, 2010).

To investigate the population structure, the model-based clustering method as implemented in Structure 2.3.4 was used (Pritchard et al., 2000). This method assigns each individual to different groups according to a membership coefficient (qi). The admixture model was run using the options 'correlated allele frequencies among populations' and 'infer the degree of admixture (α) by the data'. For each K (number of hypothetical populations), 20 runs (burn-in length, 100,000; iterations, 200,000) were carried out, and the most likely number of K was determined using the ΔK statistic (Evanno et al., 2005), using the online program STRUCTURE Harvester (Earl et al., 2012).

To detect possible marker-trait associations, the GLM (General Linear Model) test (TASSEL 2.1) that accounts for the genetic structure of the collection, was performed using 10^4 permutations.

2.3 Results

2.3.1 Phenotypic analysis

The ANOVA was performed using the year, the genotype and the interaction year by genotype as effects of the model (Table 2.5). The year is highly statistically significant for all the quantitative traits, with the exception of the number of flowers per inflorescence (NFI), the leaf width (LWI) and the fruit weight (FWG). Strong significant differences ($P < 0.0001$) among genotypes and for the interaction year $\times$ genotype were detected for all the parameters (Table 2.5).

Mean, maximum and minimum values of the 14 conventional quantitative traits showed wide variation

Table 2.5: ANOVA analysis for all the conventional quantitative traits evaluated in 2012 and 2013 among cultivated tomato accessions (landraces and cultivars). Year, genotype and the interaction year × genotype have been considered as effects of the model.

	Year				Genotype				Year × Genotype			
Trait[a]	DF	SS	F		DF	SS	F		DF	SS	F	
DTFs	1	503160.11	26202.61	****	119	39345.61	17.22	****	119	14265.53	6.24	****
DTFt	1	125396.97	6813.21	****	119	37496.46	17.12	****	119	13092.69	5.98	****
FRI	1	242044.26	6896.66	****	119	31310.59	7.50	****	119	22560.47	5.40	****
NFI	1	48.83	2.77		119	10688.18	5.10	****	119	4761.10	2.27	****
LLE	1	560.23	35.29	****	119	11519.14	6.10	****	119	3726.68	1.97	****
LWI	1	3.15	0.13		119	17795.51	6.33	****	119	6537.37	2.32	****
LL/W	1	0.55	27.65	****	119	9.23	3.93	****	119	3.82	1.62	****
FWG	1	398.00	0.12		119	12820541.00	31.87	****	119	1192201.00	2.96	****
FLE	1	27.22	55.04	****	119	3835.74	65.18	****	119	216.29	3.68	****
FWI	1	11.70	12.08	***	119	7515.26	65.17	****	119	343.80	2.98	****
FL/W	1	1.78	122.68	****	119	208.94	120.73	****	119	4.37	2.52	****
NOL	1	57.09	16.02	****	119	30125.08	71.02	****	119	1008.77	2.38	****
PTK	1	2.69	350.76	****	119	23.26	25.44	****	119	2.42	2.65	****
BRIX	1	242.60	628.87	****	119	572.35	12.47	****	119	123.87	2.70	****

DF = degrees of freedom, SS = sum of squares, F = F ratio

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

[a] DTFs, days to flowering from sowing; DTFt, days to flowering from transplanting; FRI, flowering-ripening interval; NFI, number of flowers per inflorescence; LLE, leaf length; LWI, leaf width; LL/W, leaf length/width; FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness; BRIX, degrees Brix.

both in 2012 and in 2013 (Table 2.6). This variation is highly significant ($P < 0.0001$) for all the traits in both experiments. This wide variation was particularly evident for some traits, such as the number of flowers per inflorescence (NFI), the fruit weight (FWG) and the number of locules (NOL), showing a high coefficient of variation (CV) of 36.28, 73.77 and 62.41%, respectively, in 2012 and 38.76, 60.23 and 63.40% in 2013.

As regards to the 38 Tomato Analyzer descriptors analyzed in 2013 (Table 2.7), 36 traits showed high significant differences among genotypes ($P < 0.0001$), whereas lower significant differences ($P < 0.01$) were found only for one of them (Proximal fruit blockiness). No significant differences were found for the trait 'Distal angle micro' (Table 2.7). Some descriptors showed an interesting range of variation, such as the Perimeter (7.64-35.84 cm), Area (3.88-66.39 cm^2), Fruit shape triangle (0.65-2.00), Fruit shape index internal (0.50-2.19), Lobedness degree (0.80-6.85) and Pericarp thickness (0.10-0.38).

The Nei's diversity index (H_e) calculated among qualitative traits ranged from 0.11 for the puffiness appearance (PUF) to 0.78 for the fruit shape (FSH) in 2012, and from 0.03 for the green shoulder (GRS) to 0.79 for FSH in 2013 (Table 2.8). All traits showed the same number of variants in the two years except for the inflorescence type (ITP), the fruit shape (FSH) and the shape of pistil scare (SPS), for which one additional variant was detected in 2013. For some of the descriptors many accessions showed only one variant such as the puffiness appearance (PUF) that in 2012 was mostly slight, and the green shoulder (GRS) that in 2013 was mostly absent.

Broad sense heritability (H^2) for conventional traits showed few differences between the two experiments (Figure 2.1). In 2012, H^2 varied between 26.8% for the leaf length/width ratio (LL/W) and 85.2% for the fruit length/width ratio (FL/W), while in 2013 H^2 ranged from 24.6% for the leaf length (LLE) and 90.5% for FL/W. Moving from 2012 to 2013, the mean H^2 value tended to increase with a value

Table 2.6: Significant differences among cultivated tomato accessions for all the conventional quantitative traits in 2012 and 2013.

2012								
Trait[a]	Mean	Min	Max	SD	DF	SS	F	
DTFs	58.45	48	73	4.19	120	36605.15	19.24	****
DTFt	24.44	14	39	4.19	120	36594.51	19.32	****
FRI	47.09	36	57	4.03	120	30281.81	8.31	****
NFI	11.62	4	33	4.18	120	12137.04	5.16	****
LLE	34.00	20.5	44.7	3.49	120	15288.39	8.88	****
LWI	31.82	13.6	45.2	4.45	120	25380.66	9.75	****
LL/W	1.09	0.9	1.5	0.10	120	12.28	4.97	****
FWG	113.83	6	435	94.17	120	8492981.50	19.21	****
FLE	4.94	2.0	9.6	1.45	120	2328.76	32.73	****
FWI	5.79	2.2	10.4	2.00	120	4325.81	33.11	****
FL/W	0.93	0.49	2.05	0.33	120	120.43	55.26	****
NOL	5.70	2	17	3.87	120	14754.16	36.25	****
PTK	0.45	0.2	0.7	0.11	120	11.04	9.97	****
BRIX	4.06	2.6	6.2	0.58	120	373.43	6.50	****
2013								
Trait[a]	Mean	Min	Max	SD	DF	SS	F	
DTFs	100.10	73	118	8.53	123	25352.74	4.28	****
DTFt	45.26	19	63	8.16	123	23670.96	4.66	****
FRI	77.22	55	105	9.57	123	28083.72	3.18	****
NFI	11.07	5	27	4.30	123	6545.15	4.38	****
LLE	35.44	21.5	45.3	4.15	123	5889.64	2.03	****
LWI	31.98	13.2	41.8	5.26	123	9276.99	2.29	****
LL/W	1.13	0.9	1.6	0.13	123	5.42	2.98	****
FWG	127.57	3.2	336.3	76.29	123	5972561.80	16.30	****
FLE	4.89	1.5	9.1	1.3	123	1852.45	39.14	****
FWI	6.27	1.7	10.2	1.9	123	3846.18	37.46	****
FL/W	0.85	0.46	2.12	0.32	123	105.78	80.49	****
NOL	6.51	2	17	4.14	123	17640.25	38.62	****
PTK	0.53	0.1	0.8	0.13	123	16.66	21.29	****
BRIX	4.71	3.2	7.2	0.65	123	377.98	10.79	****

Min = minimum value, Max = maximum value, SD = standard deviation, DF = degrees of freedom, SS = sum of squares, F = F ratio
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.
[a] DTFs, days to flowering from sowing (days); DTFt, days to flowering from transplanting (days); FRI, flowering-ripening interval (days); NFI, number of flowers per inflorescence; LLE, leaf length (cm); LWI, leaf width (cm); LL/W, leaf length/width; FWG, mean fruit weight (g); FLE, fruit length (cm); FWI, fruit width (cm); FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness (cm); BRIX, degrees Brix (°Bx).

of 55.3% in 2012 and of 59.1% in 2013; the highest variations were mainly seen for FRI (+11.8), NFI (+12.1), LLE (-17.6), LWI (-14.7), LL/W (+13.3), PTK (+17.5) and BRIX (+18.3). Interestingly, in 2013 the heritability for fruit morphology and fruit quality traits showed the highest values among all the analyzed traits.

Broad sense heritability (H^2) for Tomato Analyzer descriptors showed an average value of 63% (Figure 2.2). The highest H^2 values was shown by the trait 'Pericarp thickness' (H^2 = 92.1%), whereas the inheritance level of the trait 'Distal angle micro' was null.

Pearson's correlations were observed among conventional quantitative traits in 2012 and 2013 (Table 2.9). As an example, the mean fruit weight (FWG) was strongly and positively correlated to the fruit size (FLE and FWI) and to the number of locules (NOL), as well as to the pericarp thickness (PTK). Moreover, FWG was negatively correlated to the degrees Brix (BRIX) indicating that small fruits are

Table 2.7: Significant differences among cultivated tomato accessions for all the Tomato Analyzer quantitative traits in 2013.

Trait	Mean	Min	Max	SD	DF	SS	F	
Longitudinal section descriptors								
Perimeter	20.75	7.64	35.84	5.90	114	11444.35	20.07	****
Area	27.84	3.88	66.39	13.67	114	61279.28	14.73	****
Width mid-height	6.08	2.40	11.75	1.98	114	1287.43	17.37	****
Maximum width	6.16	2.42	11.79	1.98	114	1289.39	17.85	****
Height mid-width	4.76	1.90	8.96	1.41	114	674.19	26.43	****
Maximum height	5.38	1.95	9.29	1.53	114	784.76	25.03	****
Curved height	5.56	2.11	9.25	1.51	114	768.56	22.68	****
Fruit shape index external I	0.92	0.47	1.80	0.28	114	25.97	31.54	****
Fruit shape index external II	0.84	0.28	1.83	0.32	114	34.31	28.51	****
Curved fruit shape index	0.97	0.58	1.89	0.28	114	27.17	25.80	****
Proximal fruit blockiness	0.77	0.51	0.91	0.06	114	1.24	1.55	**
Distal fruit blockiness	0.62	0.37	0.81	0.07	114	1.76	5.21	****
Fruit shape triangle	1.27	0.65	2.00	0.22	114	16.12	3.05	****
Ellipsoid	0.05	0.02	0.12	0.02	114	0.09	7.94	****
Circular	0.11	0.03	0.32	0.05	114	0.85	10.63	****
Rectangular	0.55	0.44	0.71	0.03	114	0.35	2.78	****
Shoulder height	0.06	0.00	0.15	0.03	114	0.39	5.54	****
Proximal angle micro	212.34	117.45	262.18	29.16	114	289535.85	1.88	****
Proximal angle macro	141.20	29.90	200.65	32.20	114	357711.48	11.14	****
Proximal indentation area	0.13	0.00	0.46	0.09	114	2.76	4.70	****
Distal angle micro	144.88	57.81	252.42	42.09	114	594182.53	0.84	
Distal angle macro	115.29	50.70	174.15	24.66	114	203500.28	13.27	****
Distal indentation area	0.02	0.00	0.23	0.04	114	0.57	3.19	****
Distal end protrusion	0.01	0.00	0.16	0.02	114	0.17	2.05	****
Obovoid	0.02	0.00	0.31	0.04	114	0.61	4.40	****
Ovoid	0.16	0.00	0.32	0.06	114	1.40	2.93	****
V.Asymmetry	0.12	0.02	0.32	0.07	114	1.52	3.22	****
H.Asymmetry.Ob	0.02	0.00	0.37	0.06	114	1.13	3.03	****
H.Asymmetry.Ov	0.20	0.00	0.49	0.11	114	4.40	5.22	****
Width widest pos	0.45	0.32	0.64	0.05	114	0.85	3.83	****
Eccentricity	0.82	0.64	0.95	0.06	114	1.29	6.02	****
Proximal eccentricity	1.05	0.77	1.26	0.11	114	4.50	6.69	****
Distal eccentricity	0.89	0.80	1.11	0.05	114	0.76	4.95	****
Fruit shape index internal	0.99	0.50	2.19	0.34	114	39.67	21.41	****
Eccentricity area index	0.37	0.18	0.54	0.09	114	2.96	11.43	****
Transversal section descriptors								
Lobedness degree	2.08	0.80	6.85	1.11	122	449.13	2.61	****
Pericarp area	0.64	0.38	0.80	0.11	122	4.24	24.03	****
Pericarp thickness	0.20	0.10	0.38	0.07	122	1.79	36.03	****

Min = minimum value, Max = maximum value, SD = standard deviation, DF = degrees of freedom, SS = sum of squares, F = F ratio
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

usually more sweeter than bigger ones.

Some interesting relations were also observed between qualitative and quantitative conventional traits (Table 2.10). The multiparous inflorescence type (ITP) was generally associated to a higher number of flowers per inflorescence (NFI), a higher fruit weight (FWG) and a lower Brix level (BRIX) than the uniparous inflorescence. Moving from sparse to dense foliage density (FD), both the leaf width (LWI) and the leaf length/width ratio (LL/W) increased. Fruits with a heavy weight are characterized by an irregular shape at pistil scare (SPS), an indented shape at blossom end (SBE) and an irregular sectional shape; medium size fruits showed a stellate or linear SPS, a flat or pointed SBE and a round or

Table 2.8: Nei's diversity among genotypes for all qualitative traits evaluated in 2012 and 2013.

	2012				2013			
Trait[a]	N[b]	N_c[c]	N_e[d]	H_e[e]	N	N_c	N_e	H_e
ITP	109	3	2.1	0.54	123	2	2.0	0.50
SE	114	4	3.9	0.75	113	4	2.5	0.60
LAT	115	3	1.5	0.33	123	3	1.1	0.08
FD	114	3	1.7	0.41	124	3	1.3	0.23
PGT	120	2	1.2	0.19	124	2	1.1	0.11
FCO	117	4	1.8	0.44	124	4	1.4	0.30
FSH	116	8	4.3	0.78	124	9	4.7	0.79
GS	116	2	1.5	0.34	123	2	1.0	0.03
SPS	116	3	2.4	0.58	123	4	2.8	0.65
SBE	117	3	2.7	0.63	123	3	2.6	0.63
FSS	115	3	2.2	0.54	122	3	2.1	0.53
PUF	118	3	1.1	0.11	122	3	1.6	0.38
Mean				0.47				0.40

[a] ITP, inflorescence type; SE, stigma exertion; LAT, leaf attitude; FD, foliage density; PGT, plant growth type; FCO, fruit color; FSH, fruit shape; GRS, green shoulder; SPS, shape of pistil scare; SBE, fruit blossom end shape; FSS, fruit cross-sectional shape; PUF, puffiness appearance.

[b] Number of observations, [c] Number of categories, [d] Number of effective categories, [e] Unbiased Nei's diversity (Nei, 1978).

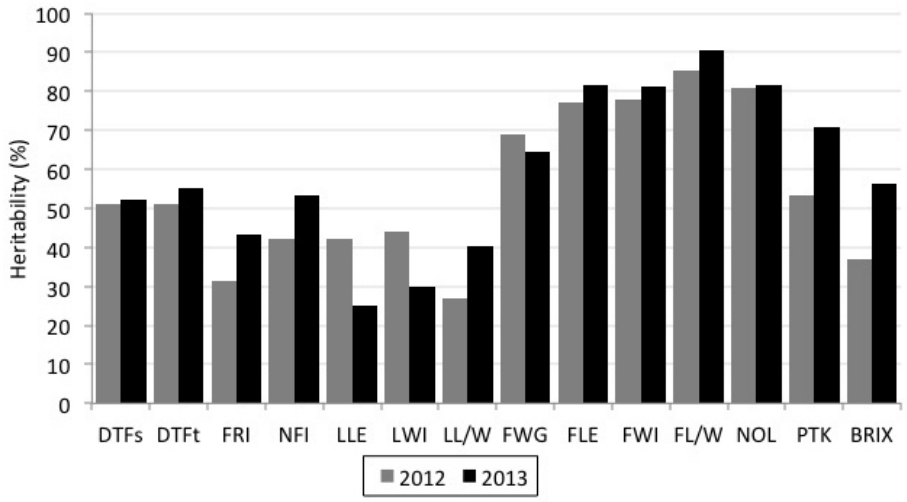

Figure 2.1: Broad sense heritability (H^2) among cultivated tomato for each conventional quantitative trait in 2012 and 2013. Note: DTFs, days to flowering from sowing (days); DTFt, days to flowering from transplanting (days); FRI, flowering-ripening interval (days); NFI, number of flowers per inflorescence; LLE, leaf length (cm); LWI, leaf width (cm); LL/W, leaf length/width; FWG, mean fruit weight (g); FLE, fruit length (cm); FWI, fruit width (cm); FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness (cm); BRIX, degrees Brix (°Bx).

angular FSS, whereas smallest fruits generally showed a dot SPS, a flat SBE and a round FSS. The fruit shape (FSH) showed many significant relations with several quantitative fruit-related traits, such as the the mean fruit weight (FWG), the fruit length/width ratio (FL/W), the number of locules (NOL), the pericarp thickness (PTK) and the degrees Brix (BRIX). As an example, the flat shape showed the highest mean fruit weight and number of locules, a medium pericarp thickness and a low level of degrees Brix, whereas the fruits with a round shape were characterized by the lowest weight, number of locules and pericarp thickness, and by the highest level of degrees Brix. FSH was also related to the fruit-ripening interval (FRI) so that the fruit shapes usually corresponding to a heavy fruit (flat and heart shape) are characterized by a longer interval.

The cluster analysis performed on all the accessions using all conventional quantitative traits split

Table 2.9: Estimates of Pearson's correlations among cultivated tomato accessions for all conventional quantitative traits evaluated in 2012 and 2013.

2012												
Trait[a]	DTFs	DTFt	FRI	NFI	LLE	LWI	LL/W	FWG	FLE	FWI	FL/W	NOL	PTK	BRIX
DTFs	-													
DTFt	1.00 ****	-												
FRI	0.05	0.05	-											
NFI	-0.13	-0.12	-0.05	-										
LLE	0.39 ****	0.39 ****	0.07	0.23 **	-									
LWI	0.49 ****	0.49 ****	0.22 *	0.06	0.82 ****	-								
LL/W	-0.35 ****	-0.35 ****	-0.27 **	0.18 *	-0.20 *	-0.70 ****	-							
FWG	0.10	0.09	0.49 ****	0.06	0.26 **	0.26 **	-0.16	-						
FLE	0.22 *	0.22 *	0.41 ****	0.05	0.25 **	0.38 ****	-0.35 ****	0.56 ****	-					
FWI	0.16	0.16	0.52 ****	0.02	0.27 **	0.28 **	-0.18 *	0.94 ****	0.44 ****	-				
FL/W	0.01	0.01	-0.14	0.08	-0.10	0.00	-0.11	-0.41 ****	0.41 ****	-0.58 ****	-			
NOL	0.00	0.00	0.46 ****	0.06	0.19 *	0.14	-0.03	0.89 ****	0.27 **	0.92 ****	-0.61 ****	-		
PTK	0.35 ****	0.35 ****	0.26 **	-0.22 *	0.11	0.36 ****	-0.46 ****	-0.07	0.54 ****	-0.03	0.44 ****	-0.29 **	-	
BRIX	-0.10	-0.10	-0.44 ****	0.32 ***	0.10	0.04	0.10	-0.34 ***	-0.09	-0.44 ****	0.38 ****	-0.39 ****	-0.05	-
2013														
Trait[a]	DTFs	DTFt	FRI	NFI	LLE	LWI	LL/W	FWG	FLE	FWI	FL/W	NOL	PTK	BRIX
DTFs	-													
DTFt	0.97 ****	-												
FRI	-0.34 ***	-0.32 ***	-											
NFI	-0.18 *	-0.14	-0.14	-										
LLE	-0.06	-0.03	0.12	0.00	-									
LWI	0.08	0.10	0.08	-0.07	0.82 ****	-								
LL/W	-0.21 *	-0.23 **	-0.02	0.11	-0.28 **	-0.76 ****	-							
FWG	0.38 ****	0.38 ****	0.02	0.15	0.09	0.21 *	-0.30 ***	-						
FLE	0.32 ***	0.34 ****	0.16	-0.07	0.14	0.30 ***	-0.37 ****	0.45 ****	-					
FWI	0.37 ****	0.38 ****	-0.02	0.17	0.11	0.22 *	-0.27 **	0.94 ****	0.31 ***	-				
FL/W	-0.05	-0.05	0.16	-0.16	-0.01	0.02	-0.05	-0.46 ****	0.50 ****	-0.63 ****	-			
NOL	0.24 **	0.24 **	-0.03	0.32 ***	0.01	0.12	-0.21 *	0.83 ****	0.08	0.87 ****	-0.67 ****	-		
PTK	0.28 **	0.33 ***	0.15	-0.17	0.11	0.22 *	-0.27 **	0.30 ***	0.69 ****	0.30 ***	0.24 **	-0.07	-	
BRIX	-0.29 **	-0.31 ***	0.00	-0.08	0.00	-0.11	0.23 **	-0.58 ****	-0.32 ***	-0.60 ****	0.27 **	-0.53 ****	-0.31 ***	-

* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

[a] DTFs, days to flowering from sowing; DTFt, days to flowering from transplanting; FRI, flowering-ripening interval; NFI, number of flowers per inflorescence; LLE, leaf length; LWI, leaf width; LL/W, leaf length/width; FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness; BRIX, degrees Brix.

Table 2.10: ANOVA analysis and R^2 values between quantitative and qualitative traits evaluated in 2012 and 2013 among cultivated tomato accessions.

2012															
Trait[a]	DTFs	DTFt	FRI	NFI	LLE	LWI	LL/W	FWG	FLE	FWI	FL/W	NOL	PTK	BRIX	
ITP	0.04	0.04	0.01	0.29 ****	0.04	0.01	0.07 *	0.32 ****	0.03	0.40 ****	0.25 ****	0.40 ****	0.21 ****	0.07 *	
SE	0.02	0.02	0.07 *	0.00	0.01	0.02	0.05	0.04	0.05	0.02	0.05	0.05	0.03	0.00	
LAT	0.01	0.01	0.04	0.03	0.12 ***	0.06 *	0.00	0.09 **	0.13 ***	0.04	0.01	0.07 *	0.02	0.01	
FD	0.10 **	0.10 **	0.01	0.01	0.05 *	0.09 **	0.09 **	0.02	0.04	0.04	0.17 ****	0.05	0.10 **	0.04	
PGT	0.00	0.00	0.01	0.04 *	0.03 *	0.00	0.04 *	0.04 *	0.01	0.05 *	0.08 **	0.09 ***	0.12 ****	0.00	
FCO	0.02	0.02	0.04	0.00	0.05	0.05	0.04	0.05	0.07 *	0.04	0.01	0.03	0.04	0.03	
FSH	0.08	0.08	0.22 ***	0.04	0.09	0.08	0.06	0.39 ****	0.53 ****	0.45 ****	0.79 ****	0.61 ****	0.33 ****	0.25 ****	
GS	0.03	0.03	0.04 *	0.00	0.01	0.01	0.01	0.08 **	0.01	0.07 **	0.03	0.12 ***	0.02	0.06 **	
SPS	0.06 *	0.06 *	0.10 **	0.04	0.11 **	0.07 *	0.01	0.47 ****	0.05 *	0.66 ****	0.43 ****	0.66 ****	0.04	0.10 **	
SBE	0.00	0.00	0.08 **	0.01	0.00	0.02	0.05	0.18 ****	0.30 ****	0.28 ****	0.51 ****	0.34 ****	0.13 ***	0.13 ***	
FSS	0.00	0.00	0.26 ****	0.03	0.01	0.00	0.01	0.28 ****	0.07 *	0.37 ****	0.30 ****	0.44 ****	0.09 **	0.15 ****	
PUF	0.07 *	0.07 *	0.01	0.04	0.00	0.01	0.04	0.02	0.01	0.01	0.01	0.01	0.03	0.03	
2013															
Trait[a]	DTFs	DTFt	FRI	NFI	LLE	LWI	LL/W	FWG	FLE	FWI	FL/W	NOL	PTK	BRIX	
ITP	0.02	0.02	0.03	0.30 ****	0.00	0.00	0.01	0.38 ****	0.01	0.43 ****	0.23 ****	0.51 ****	0.00	0.12 ****	
SE	0.03	0.03	0.04	0.04	0.02	0.02	0.03	0.06	0.00	0.08 *	0.06	0.12 **	0.06	0.03	
LAT	0.01	0.01	0.03	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	
FD	0.01	0.01	0.08 **	0.06 *	0.03	0.07 *	0.09 **	0.03	0.02	0.04	0.07 *	0.03	0.02	0.07 *	
PGT	0.00	0.00	0.01	0.03	0.00	0.00	0.00	0.01	0.03 *	0.01	0.06 **	0.03	0.07 **	0.01	
FCO	0.07 *	0.07 *	0.03	0.03	0.02	0.03	0.03	0.05	0.04	0.05	0.01	0.03	0.10 **	0.03	
FSH	0.07	0.08	0.13 *	0.12	0.12 *	0.15 *	0.16 **	0.47 ****	0.55 ****	0.65 ****	0.81 ****	0.73 ****	0.40 ****	0.24 ****	
GS	0.00	0.00	0.01	0.02	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.01	
SPS	0.05	0.05	0.05	0.04	0.05	0.06	0.04	0.50 ****	0.03	0.66 ****	0.49 ****	0.70 ****	0.06 *	0.15 ***	
SBE	0.10 **	0.10 **	0.02	0.03	0.00	0.02	0.06 *	0.22 ****	0.34 ****	0.29 ****	0.46 ****	0.37 ****	0.04	0.14 ***	
FSS	0.02	0.03	0.00	0.05 *	0.01	0.00	0.02	0.23 ****	0.06 *	0.34 ****	0.32 ****	0.43 ****	0.02	0.14 ***	
PUF	0.03	0.03	0.04 *	0.04	0.02	0.02	0.03	0.06	0.00 ***	0.08	0.06 ***	0.12	0.06 ***	0.03	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

[a] DTFs, days to flowering from sowing; DTFt, days to flowering from transplanting; FRI, flowering-ripening interval; NFI, number of flowers per inflorescence; LLE, leaf length; LWI, leaf width; LL/W, leaf length/width; FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness; BRIX, degrees Brix; ITP, inflorescence type; SE, stigma exertion; LAT, leaf attitude; FD, foliage density; PGT, plant growth type; FCO, fruit color; FSH, fruit shape; GRS, green shoulder; SPS, shape of pistil scare; SBE, fruit blossom end shape; FSS, fruit cross-sectional shape; PUF, puffiness appearance.

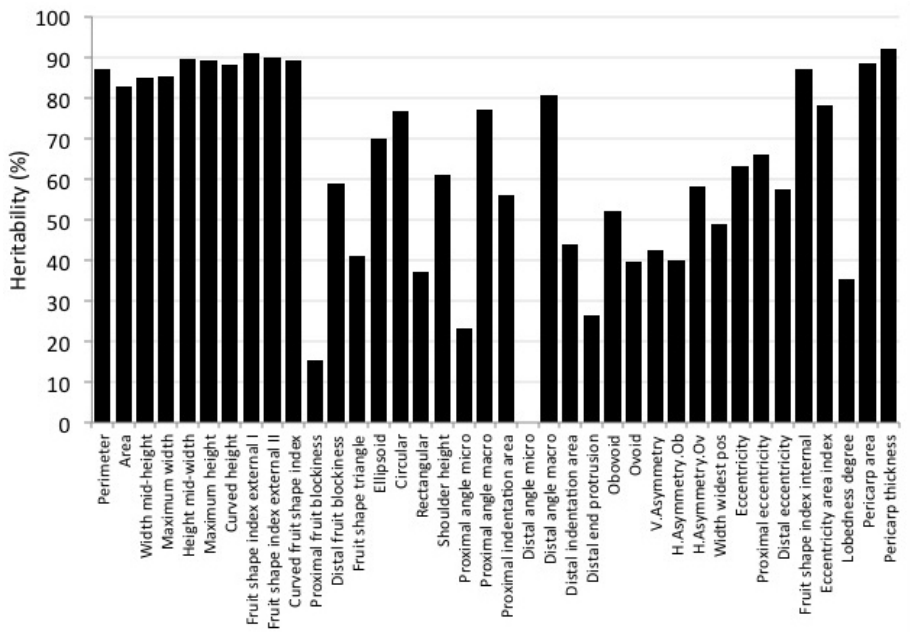

Figure 2.2: Broad sense heritability (H^2) among cultivated tomato for each compound Tomato Analyzer quantitative trait in 2013.

all accessions into four main groups, both in 2012 and 2013. Table 2.11 shows the mean values of each conventional quantitative trait within each cluster. Considering these data and the results of contingency analysis (Table 2.12), it is possible to outline the main characteristics of each cluster (Figure 2.3).

Table 2.11: ANOVA analysis and R^2 values between quantitative and qualitative traits evaluated in 2012 and 2013 among all the accessions.

2012															
Cluster	Accessions	DTFs	DTFt	FRI	NFI	LLE	LWI	LL/W	FWG	FLE	FWI	FL/W	NOL	PTK	BRIX
1	21	59.8 a	25.8 a	48.2 ab	9.9 b	32.2 b	32.5 a	1.0 c	68.1 c	6.1 a	4.5 c	1.4 a	2.5 c	0.6 a	4.1 b
2	60	59.4 a	25.4 a	47.1 b	11.4 b	34.8 a	32.4 a	1.1 b	98.8 b	4.5 b	5.9 b	0.8 c	5.5 b	0.4 b	3.9 b
3	29	57.9 a	23.9 a	50.3 a	12.2 ab	34.9 a	33.0 a	1.1 b	268.4 a	6.3 a	8.7 a	0.7 c	11.6 a	0.4 b	3.8 b
4	14	52.3 b	18.3 b	42.2 c	17.6 a	30.0 b	25.2 b	1.2 a	22.1 d	3.0 c	3.0 d	1.1 b	2.7 c	0.3 c	5.2 a
2013															
Cluster	Accessions	DTFs	DTFt	FRI	NFI	LLE	LWI	LL/W	FWG	FLE	FWI	FL/W	NOL	PTK	BRIX
1	18	101.2 a	46.5 a	84.7 a	8.4 b	33.9 ab	32.0 a	1.1 b	85.7 b	6.5 a	4.8 c	1.4 a	2.3 c	0.7 a	4.8 b
2	37	104.4 a	49.4 a	72.1 c	9.1 b	36.8 a	33.5 a	1.1 b	108.3 b	4.7 b	6.0 b	0.8 b	4.9 b	0.6 b	4.9 b
3	51	100.3 a	45.4 a	79.2 ab	12.8 a	35.7 a	32.4 a	1.1 b	190.4 a	5.1 b	7.9 a	0.7 c	10.4 a	0.5 b	4.3 c
4	21	92.1 b	36.6 b	75.8 bc	13.5 a	32.2 b	26.4 b	1.3 a	21.3 c	2.7 c	3.1 d	0.9 b	2.9 c	0.3 c	5.7 a

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Different letters indicate statistical differences according to Tukey test ($P < 0.05$).

DTFs, day to flowering from sowing; DTFt, days to flowering from transplanting; FRI, flowering-ripening interval; NFI, number of flowers per inflorescence; LLE, leaf length; LWI, leaf width; LL/W, leaf length/width; FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness; BRIX, degrees Brix.

In particular, accessions belonging to cluster I are usually characterized by fruits with a medium mean weight and a cylindrical or high rounded shape; some accessions have a determined growth type and a dense foliage density; the shape at the blossom end is usually pointed and the cross-sectional shape round or angular; fruit length is higher than fruit width and the pericarp is very thick. Accessions belonging to cluster II produce fruits with a medium size and a flattened or slightly-flattened shape; the inflorescence type can be uniparous or multiparous and the foliage density is intermediate; fruits are usually red with an intermediate pericarp thickness. Cluster III is characterized by flattened or hearth-shaped fruits with

Table 2.12: Contingency analysis and R^2 values between conventional qualitative traits and cluster among all accessions obtained in 2012 and 2013.

2012																							
ITP		SE		LAT		FD		PGT		FCO		FSH		GS		SPS		SBE		FSS		PUF	
0.23	****	0.12	****	0.09		0.20	****	0.22	***	0.09	*	0.25	****	0.09	*	0.39	****	0.19	****	0.27	****	0.12	
2013																							
ITP		SE		LAT		FD		PGT		FCO		FSH		GS		SPS		SBE		FSS		PUF	
0.31	****	0.06		0.06		0.20	***	0.32	***	0.11		0.34	****	0.18		0.32	****	0.27	****	0.28	****	0.20	****

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

ITP, inflorescence type; SE, stigma exertion; LAT, leaf attitude; FD, foliage density; PGT, plant growth type; FCO, fruit color; FSH, fruit shape; GRS, green shoulder; SPS, shape of pistil scare; SBE, fruit blossom end shape; FSS, fruit cross-sectional shape; PUF, puffiness appearance.

the highest mean weight; the color is red and sometimes pink; the section and the shape of pistil scare are irregular and the foliage density is intermediate; these fruits have many locules, a medium pericarp thickness but the lowest value of degrees Brix. Cluster IV is characterized by the smallest fruits, usually red but also pink or yellow; the fruit and the section shapes are rounded; the foliage density can be intermediate or sparse; these fruits have the thinner pericarp and the highest level of degrees Brix.

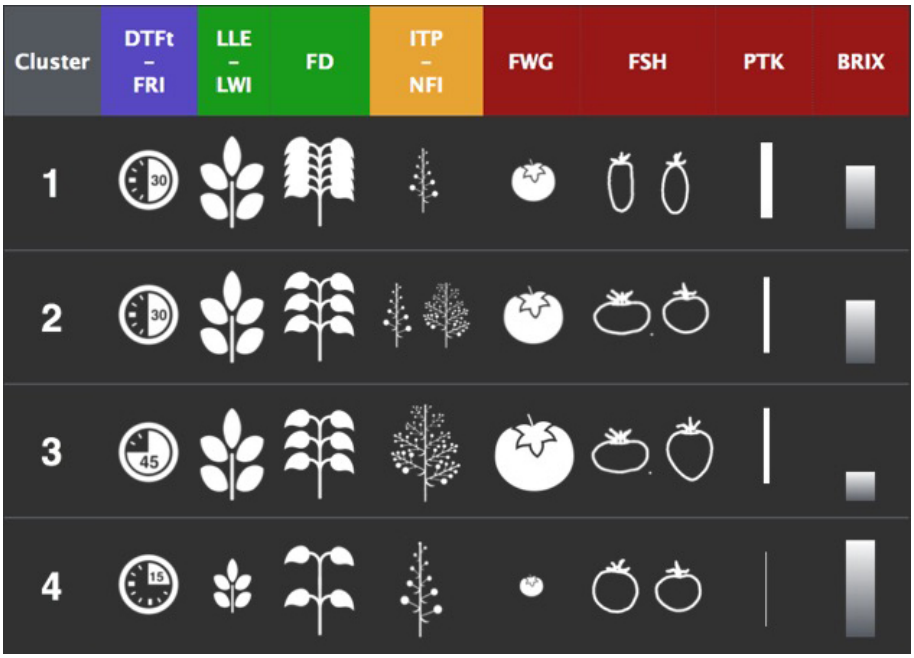

Figure 2.3: Main characteristics of each cluster for some qualitative and quantitative traits among all accessions. Note: DTFt, days to flowering from transplanting; FRI, flowering-ripening interval; LLE, leaf length; LWI, leaf width; FD, foliage density; ITP, inflorescence type; NFI, number of flowers per inflorescence; FWG, mean fruit weight; FSH, fruit shape; PTK, pericarp thickness; BRIX, degrees Brix.

Principal component analysis (PCA) of all conventional quantitative traits identified the first four principal components (PCs) that cumulatively explain nearly the 78% of the total phenotypic variation for both experiments (Table 2.13). In detail, the PC1 captured about the 35% of the total variation and showed high loadings for DTFs, DTFt, LWI, FWG, FLE, FWI and NOL. The PC2 explained around 20% of the total phenotypic variation and was correlated to FL/W. The PC3 and PC4 explained around 12% and 10%, respectively, of the total variation (Table 2.13 and Table 2.14). The first two PCs are plotted in Figure 2.4 and show that all clusters are clearly distinguished for the conventional morpho-phenological traits, in fact accessions of the different groups, are in general plotted in different areas of the graph (Figure 2.4). As an example, accessions of cluster IV, that are usually characterized by smaller fruits,

usually present negative values for both components. The accessions from cluster I, usually characterized by elongated fruits, had usually positive values for the second component. On the other hand, cluster III accessions, that are usually characterized by bigger fruits, had positive values for PC1 and negative values for PC2.

The cluster analysis performed on the TA traits subdivided the whole collection into four groups, the

Table 2.13: Total variance explained by the first four PCs and their eigenvalues in 2012 and 2013 among all the accessions.

2012				2013			
Component	Total	% of variance	Cum %	Component	Total	% of variance	Cum %
1	4.94	35.26	35.26	1	4.83	34.51	34.51
2	3.02	21.58	56.83	2	2.64	18.82	53.33
3	1.57	11.22	68.06	3	2.07	14.80	68.14
4	1.40	9.98	78.04	4	1.35	9.65	77.78

Table 2.14: Associations between the first four PCs and all conventional quantitative traits among all accessions in 2012 and 2013.

2012					2013				
	Component					Component			
Trait[a]	1	2	3	4	Trait	1	2	3	4
DTFs	0.60	0.50	0.41	-0.20	DTFs	0.56	-0.01	-0.75	-0.22
DTFt	0.60	0.50	0.41	-0.20	DTFt	0.58	0.01	-0.73	-0.20
FRI	0.43	-0.27	-0.45	-0.01	FRI	-0.01	0.29	0.46	0.53
NFI	-0.37	-0.13	0.24	0.72	NFI	-0.11	-0.44	0.32	0.10
LLE	0.61	0.22	0.44	0.31	LLE	0.38	0.42	0.52	-0.42
LWI	0.74	0.39	0.23	0.25	LWI	0.56	0.50	0.45	-0.47
LL/W	-0.60	-0.39	0.15	-0.02	LL/W	-0.60	-0.41	-0.20	0.31
FWG	0.74	-0.56	-0.09	0.25	FWG	0.87	-0.32	0.10	0.21
FLE	0.68	0.18	-0.50	0.36	FLE	0.62	0.54	-0.10	0.41
FWI	0.81	-0.55	0.00	0.07	FWI	0.89	-0.37	0.15	0.13
FL/W	-0.25	0.69	-0.48	0.31	FL/W	-0.32	0.77	-0.23	0.25
NOL	0.61	-0.74	0.05	0.11	NOL	0.69	-0.61	0.22	0.05
PTK	0.47	0.61	-0.43	-0.06	PTK	0.57	0.50	-0.12	0.37
BRIX	-0.56	0.21	0.19	0.59	BRIX	-0.72	0.11	-0.01	-0.21

[a] DTFs, days to flowering from sowing; DTFt, days to flowering from transplanting; FRI, flowering-ripening interval; NFI, number of flowers per inflorescence; LLE, leaf length; LWI, leaf width; LL/W, leaf length/width; FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness; BRIX, degrees Brix.

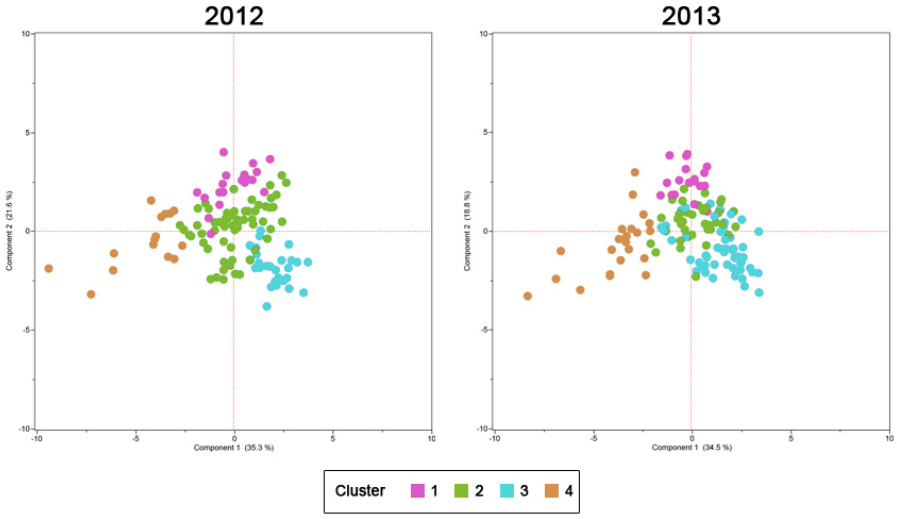

Figure 2.4: Scatter plot of the first and second components obtained by the principal component analysis (PCA) based on 14 conventional quantitative traits in 2012 and 2013. Different colors refers to different groups as obtained by cluster analysis.

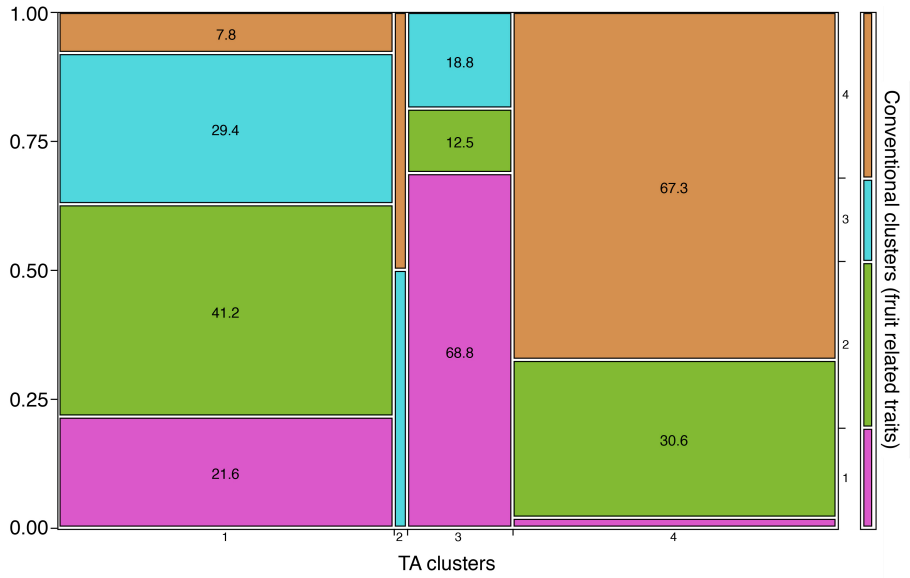

Figure 2.5: Contingency analysis showing the results of the association between clusters obtained with conventional fruit traits and clusters obtained through TA traits.

Table 2.15: R^2 values between Tomato Analyzer principal components and some quantitative and qualitative conventional fruit traits among all the accessions.

Percent	Cum Percent	PC	FWG		FLE		FWI		PTK		FSH		SBE		FSS	
36.4	36.4	PC1	0.60	****	0.00		0.75	****	0.00		0.71	****	0.35	****	0.38	****
20.2	56.6	PC2	0.13	****	0.74	****	0.07	**	0.33	****	0.53	****	0.45	****	0.14	****
12.9	69.5	PC3	0.02		0.09	**	0.00		0.02		0.35	****	0.02		0.03	
5.9	75.4	PC4	0.04	*	0.00		0.01		0.03		0.43	****	0.07	*	0.11	**
4.5	79.9	PC5	0.00		0.01		0.01		0.23	****	0.24		0.11	**	0.03	
3.3	83.2	PC6	0.01		0.00		0.00		0.00		0.17		0.05		0.06	
3.2	86.3	PC7	0.00		0.00		0.00		0.01		0.13		0.00		0.02	
2.5	88.8	PC8	0.01		0.01		0.01		0.06	**	0.12		0.02		0.02	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; PTK, pericarp thickness; FSH, fruit shape; SBE, fruit blossom end shape; FSS, fruit cross-sectional shape.

same number of groups obtained when using only the conventional fruit descriptors (FWG, FWI, FLE, FW/L, NOL, PTK, BRIX). However, the two clusterizations did not show a perfect overlapping (Figure 2.5). In fact, some accessions were attributed to different groups by the two methods. Moreover, the characterization obtained by TA provided about 20% additional information in respect to that obtained by the conventional characterization (Table 2.15).

2.3.2 Molecular analysis

All of the 19 SSR markers used for the genetic analysis were polymorphic for a total of 103 alleles observed. In Table 2.16 is showed the result of the genetic diversity of the entire collection and within groups of genotypes. A high level of polymorphism was observed within the group of Sardinian landraces, exotic landraces, cultivars and wild species, whereas a slightly lower diversity was detected within the Italian landraces. The exotic landraces showed a number of private alleles (15) close to the wild-species (16), followed by the Sardinian landraces (3) and the vintage cultivars (3), whereas the Italian landraces did not show private alleles. The expected heterozygosity level was higher for wild-related species (0.64) and

lower for the Italian landraces, whereas the Sardinian landraces showed diversity values similar to that of the exotic landraces.

Table 2.16: Diversity statistics calculated over SSR data. Analyses have been performed both overall the accessions and within groups.

		Polymorphic loci					
Collection[a]	Sample	no.	%	na[b]	ne[c]	Private alleles	H_e[d]
L-SAR	64	18	94.70%	3.5	1.9	3	0.39
L-IT	7	13	68.40%	2.1	1.8	0	0.36
L-EXOT	43	18	94.74%	3.7	2.0	15	0.41
C	10	17	89.50%	2.7	2.1	3	0.44
W	3	17	89.50%	2.5	2.4	16	0.64
Total	127	19	100%	5.5	2.2		0.43

[a] L-SAR, Sardinian landraces; L-IT, Italian landraces; L-EXOT, exotic landraces; C, cultivars; W, wild species.

[b] Observed number of alleles, [c] Effective number of alleles (Kimura and Crow, 1964), [d] Expected heterozygosity Nei (1978).

Genetic distances (F_{ST}) among these groups of genotypes (Table 2.17) were higher between wild and cultivated tomato, with higher values when compared to the Sardinian landraces ($F_{ST} = 0.377$) in respect to the Italian landraces ($F_{ST} = 0.336$), the exotic landraces ($F_{ST} = 0.320$) and the vintage cultivars ($F_{ST} = 0.298$). The Sardinian landraces were more similar to the exotic landraces ($F_{ST} = 0.045$) than to cultivars ($F_{ST} = 0.079$). The genetic distances among the Italian landraces and the other genetic groups was not evaluated because the comparison was not significant.

Table 2.17: Genetic distances (F_{ST}) among the different groups of genotypes. The analysis among the Italian landraces (L-IT) and the other genetic groups was not significant.

Group[a]	L-EXOT	L-SAR	C	L-IT	W
L-EXOT	-				
L-SAR	0.045	-			
C	0.074	0.079	-		
L-IT	0.085	0.050	0.022	-	
W	0.320	0.377	0.298	0.336	-

[a] L-SAR, Sardinian landraces; L-IT, Italian landraces; L-EXOT, exotic landraces; C, cultivars; W, wild species.

Structure analysis of all the accessions indicated K = 3 as the uppermost hierarchical level of the genetic structure, and secondary peaks were observed at K = 6 and K = 8 (Figure 2.6).

Therefore the subdivision of the collection intro three genetic groups was considered for further analyses. According to this subdivision, 33 accessions (26%) were admixed (qi < 0.7), 43 accessions were attributed to the blue genetic group, 28 to the green group and 22 to the red group. The blue and green groups were mainly constituted by accessions from Sardinia (47% and 89% respectively), wheras the red group was mainly composed by exotic landraces (59%). Among the wild accessions, two (Cocktail and *S. pimpinellifolium*) were attributed to the red group and one (*S. lycopersicum* var. *cerasiformae*) was admixed with $q_{GREEN} = 0.6$ and $q_{RED} = 0.4$.

The worldwide distribution of these groups showed a clear geographical pattern, with the red group showing a more widespread distribution in respect to the remaining two (Figure 2.7). Also at local level we observed a definite geographic structure, with a wider distribution in Sardinia for the blue and green groups and a localized pattern for the red group (Figure 2.8).

All the morphological and molecular data were afterwards used to search for possible associations between the genetic diversity and structure of the population with the phenotypic structure. A weak

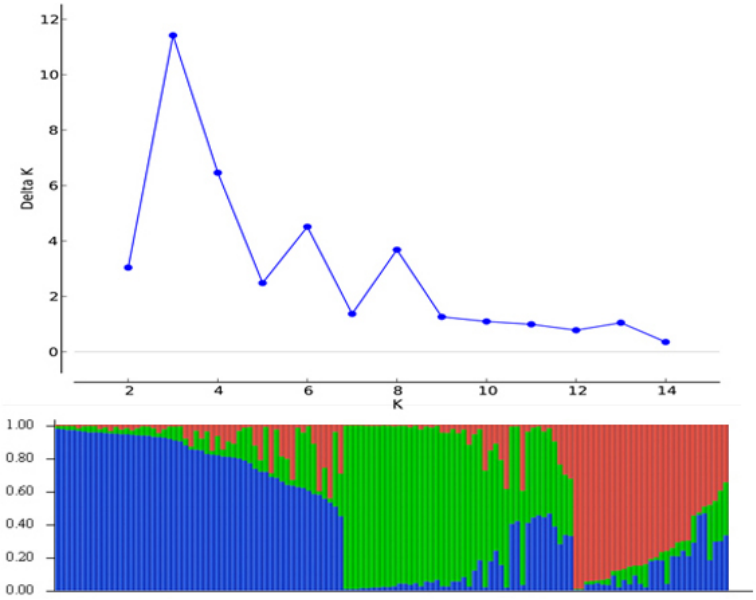

Figure 2.6: Estimation of the optimum number of clusters of tomato accessions according to the Evanno's method. The upper graph displays the Delta K [mean(|L''(K)|)/sd(L(K))] for each K value. The bottom graph shows the estimated population structure of the tomato genotypes for K = 3 were each individual is represented by a vertical bar subdivided into 3 colored segments. The length of each segment indicates the proportion of the genome attributed to each cluster (qi).

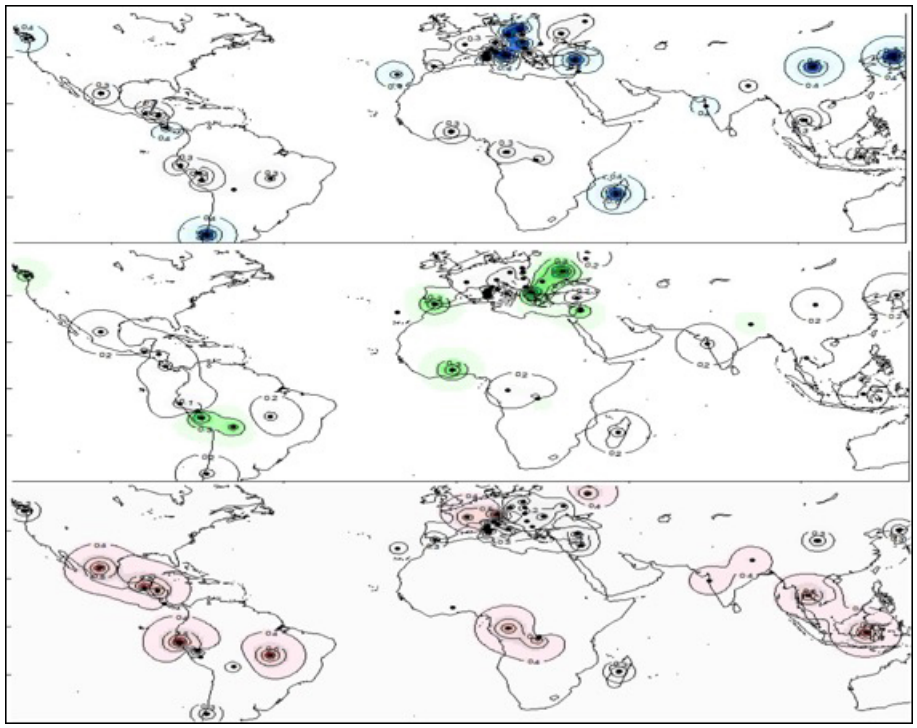

Figure 2.7: Worldwide distributions of the three genetic groups.

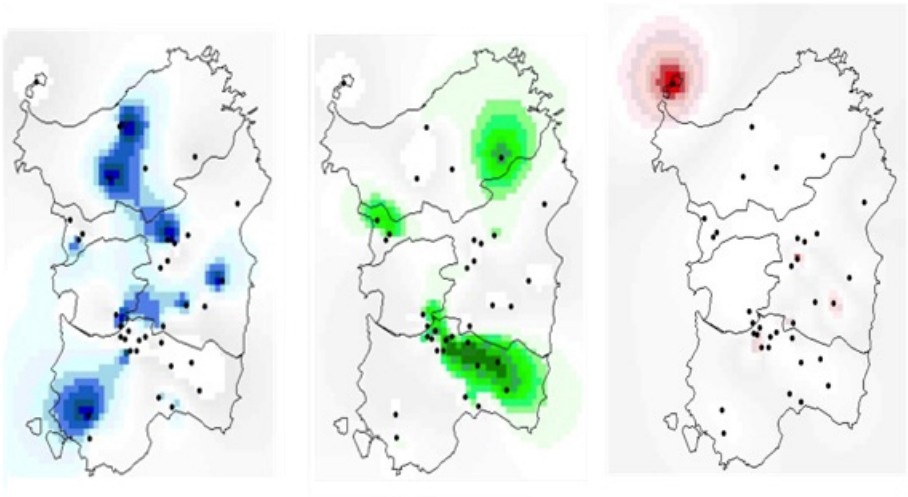

Figure 2.8: Distribution of the three genetic groups in Sardinia.

correlation was detected between the PCA performed with all the phenotypic traits (conventional and TA) and the genetic structure as obtained by Structure, indicating a low level of linkage disequilibrium across the studied collection (Figure 2.9).

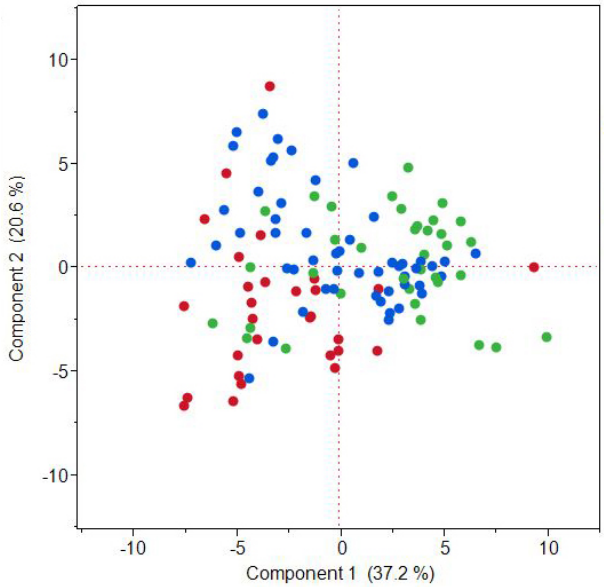

Figure 2.9: Scatter plot of the first and second component by the Principal Component Analysis (PCA) based on all conventional fruit related and TA quantitative fruit traits in 2013. The colors refer to the genetic groups carried out by the Structure analysis (Figure 2.6).

This is confirmed when the clusters obtained with morphological data were directly compared to the genetic groups (Figure 2.10) evidencing a not very high despite significant correlation among structures ($R^2 = 0.18$, $P < 0.001$). Indeed, the most of the accessions from cluster I and cluster II (71% and 59%) were mainly attributed to the blue genetic group while the 57% of the accessions from cluster III were attributed to the green genetic group and the 59% of the accessions from cluster IV were attributed to the red genetic group.

Finally, the possible associations between molecular markers and morphological traits were further investigated by factoring out the genetic structure. Tassel analysis highlighted 47 significant associations

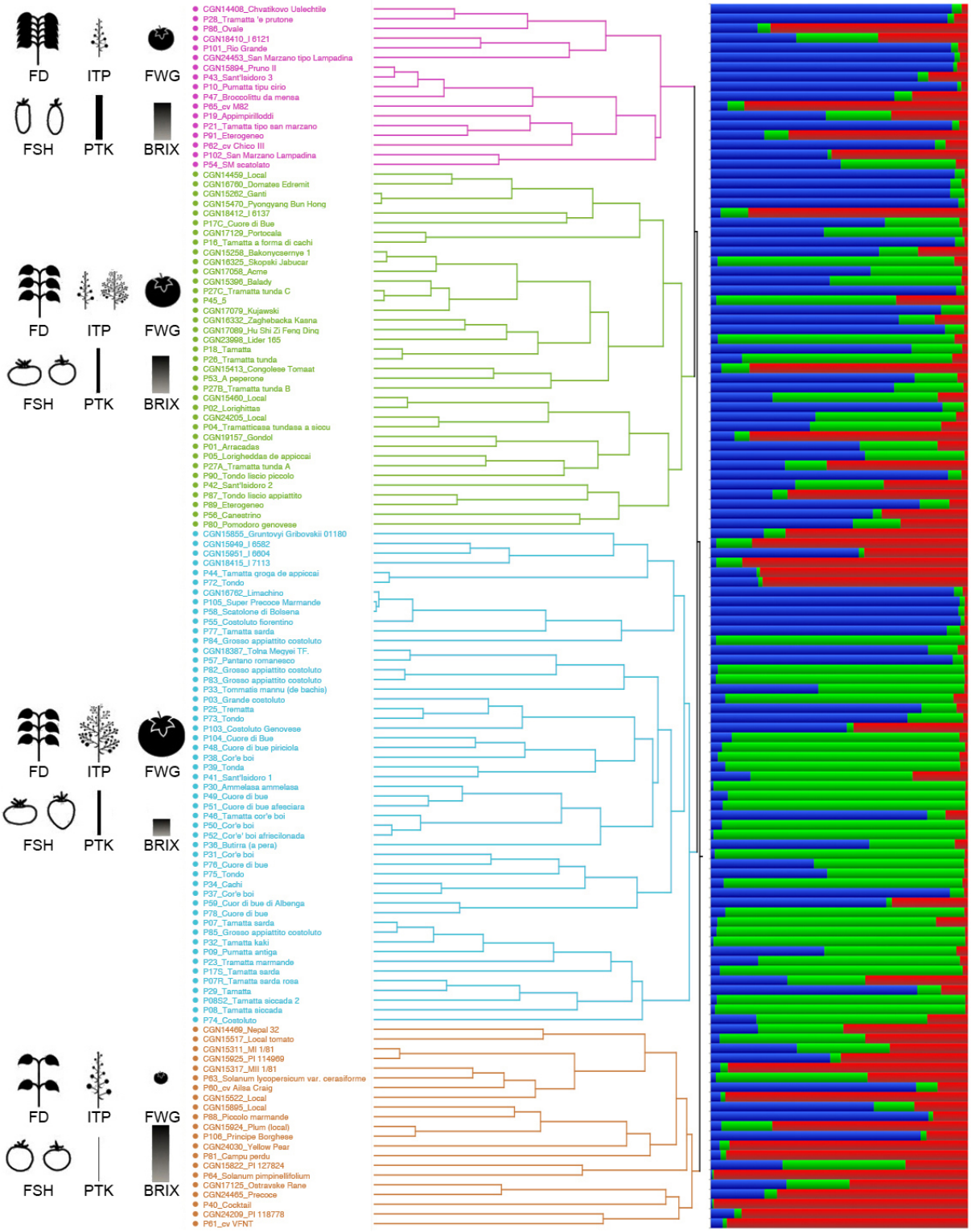

Figure 2.10: Results of the cluster and Structure analyses. On the left, the dendogram shows the 4 morpho-phenological clusters obtained (cluster I = pink; cluster II = light green; cluster III = azure; cluster IV = orange). The icons show the main characteristics of each cluster as shown on Figure 2.3 (FD, foliage density; ITP, inflorescence type; FWG, mean fruit weight; FSH, fruit shape; PTK, pericarp thickness; BRIX, degrees Brix). On the right, each individual is represented by a bar of the histogram in 3 colored segments, one for each genetic group (group I = blue; group II = green; group III = red). The length of each segment is determined by the q value.

between molecular markers and phenotypic traits (conventional and TA descriptors) showing significant associations mainly for the Q-SSR markers (Table 2.18). In particular, the highest number of associations were detected for the locus TMS59, located on chromosome 4, that was associated with fruit shape (FSH) and numerous shape-related fruit traits, both conventional and TA ones. The loci Tom 236-237 and EST2585529, respectively located on chromosome 9 and 5, were significantly associated with the number of flowers per inflorescence (NFI). The locus TMS52, showed significant associations with fruit weight, width and degrees Brix (BRIX), the locus SLM6-14 showed significant associations with the pericarp thickness (PTK) and the degrees Brix (BRIX), while the locus TMS63 was highly significantly associated with the fruit color (FCO) and the 'Obovoid' TA descriptor (related to fruit asymmetry). Some minor associations were also observed with some of the NQ-SSR markers, but overall a significantly higher number of associations were observed for the markers that are indeed associated with known QTLs (Q-SSR) than for the ones that do not show any association to known QTLs (NQ-SSR) (Figure 2.11).

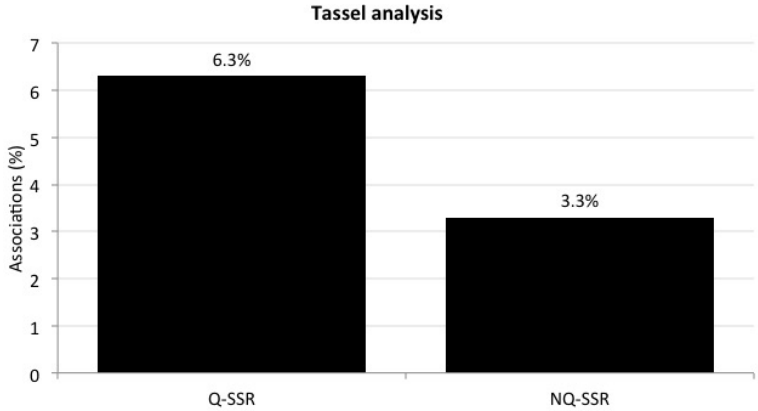

Figure 2.11: Tassel analysis showed a significantly higher number of associations for Q-SSR markers.

2.4 Discussion

The main objective of this work was to evaluate a wide collection of tomato, mainly landraces, through conventional and precision phenotyping. Concurrently, a genetic diversity analysis through micro-satellite markers was performed to describe the population diversity and structure of the population. The comparison of phenotypic and genetic data has then allowed evaluating the potentials of the collection for future association mapping studies.

The value of the present work is primarily represented by the throughput phenotyping of the present collection that nowadays is the most challenging, costly and time-consuming characterization stage (Fiorani and Schurr, 2013; McCouch et al., 2013) when compared to the efficiency so far achieved by plant genomics and molecular genotyping (Tuberosa et al., 2011; Davey et al., 2011; Lin et al., 2014).

The analyses carried out using 52 conventional and TA descriptors highlighted that the present collection, mainly consisting of landraces, is characterized by a valuable level of phenotypic diversity. Additionally, the high levels of heritability observed for most of the registered traits indicate that this diversity is maintained across environmental changes and these landraces represent an interesting model to identify QTLs and genes of relevant interest.

Table 2.18: Significant association between molecular markers and morphological descriptors showed by Tassel analysis.

		Significant associations				
			Present study		Mazzucato et al. (2008)	Previous studies[c]
Locus	Type [a]	Total	Conventional trait[b]	Tomato Analyzer trait		
LE20592	NQ-SSR	5	FLE****, PTK****	Curved height****, Height mid-width****, Maximum height****		
LE21085	NQ-SSR	0				
LELE25	NQ-SSR	0			NOL, FWG, SES	
LELEUZIP	NQ-SSR	0				
LEMDDNa	NQ-SSR	1	PTK**			
Tom 47-48	NQ-SSR	1		Proximal angle micro****		
Tom 162-163	NQ-SSR	2	NFI****	Proximal angle micro**		
SLM12-29	NQ-SSR	0				
SLM6-35	NQ-SSR	2	FCO****,NFI****			
EST245053	NQ-SSR	4	BRIX****, NFI****, PTK*	Proximal angle micro****		
TMS42	Q-SSR	1	PTK**			*ljfs11*
Tom 59-60	Q-SSR	0				*fw3.1*, *ljfs3*, *sblk3.1*, *hrt3.1*, *fw3.2*, *nsf3.1*, *lcn3.1*, *nX3.1*
TMS52	Q-SSR	3	BRIX****, FWG*, FWI*		PTKi	*sblk12.1*, *nsf12.1*, *lcn12.1*
Tom 236-237	Q-SSR	2	NFI*	Proximal angle micro****	GS	*nsf9.1*, *nfl9.3*
SLM6-14	Q-SSR	2	BRIX*, PTK*			*fw6.2*, *fs6.2*
TMS59	Q-SSR	21	FLE****, FSH****, NOL*, FL/W****, PTK****, PUF****, SBE**, SPS****	Curved fruit shape index****, Curved height****, Distal angle macro****, Eccentricity area index****, Fruit shape index external I****, Fruit shape index external II****, Fruit shape index internal****, H.Asymmetry.Ov**, Height mid-width****, Maximum height****, Pericarp area****, Pericarp thickness****, Proximal angle macro***	NOL, FSH, SBE	*fs8.1*, *bell8.1*, *bpi8.1*
TMS63	Q-SSR	2	FCO****	Obovoid****	FSH	*fw1.1*, *sblk1.1*, *hrt1.1*, *nsf1.1*, *nfl1.1*
EST253712	Q-SSR	0			FWG, NOL, ITP	*MF6a*
EST258529	Q-SSR	2	NFI****	Proximal angle micro****	PTKi	*nfl5.1*

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

[a] Q-SSR = marker associated with known QTLs; NQ-SSR = marker without a known linkage with genes of interest.

[b] NFI, number of flowers per inflorescence; FWG, mean fruit weight; FLE, fruit length; FWI, fruit width; FL/W, fruit length/width; NOL, number of locules; PTK, pericarp thickness; BRIX, degrees Brix; FCO, fruit color; FSH, fruit shape; SPS, shape of pistil scare; SBE, fruit blossom end shape; PUF, puffiness appearance.

[c] *bell*, bell shape; *bpi*, bumpiness; *fs*, fruit shape; *fw*, fruit weight; *hrt*, heart shape; *lcn*, locule number; *ljfs*, extremely elongated fruit shape; *MF*, mass per fruit; *nfl*, flowers per inflorescence; *nsf*, seed number per fruit; *sblk*, stem-end blockiness.

In particular, it has been observed that some traits related to the fruit yield and quality, such us the mean fruit weight (FWG) and the degrees Brix (BRIX), showed high heritability and high variation among the accessions during both the experimental trials, evidencing the elevated genetic control for these traits. The slightly higher broad sense heritability observed during 2013 (+3.8%) was probably due to the more stable greenhouse environmental conditions when compared to the field trial conditions in 2012. High diversity was also evidenced for other conventional traits such as fruit shape (FSH), that still represent one of the traits of major interest in fresh market cultivars and in breeding programs (Foolad, 2007).

Other studies on phenotypic diversity on tomato landraces from different countries have shown similar or lower values of diversity (Terzopoulos and Bebeli, 2010; Xu et al., 2013). Xu et al. (2013), in the attempt to perform an association study on a wide tomato collection consisting of 127 *S. lycopersicum* var. *cerasiforme*, 44 cultivated tomato and 17 *S. pimpinellifolium* accessions, found out heritability values for the fruit weight ($H^2 = 0.83$), the number of locules ($H^2 = 0.85$) and the degrees Brix ($H^2 = 0.73$) overall slightly higher than the present study, but more similar when considering the cultivated tomato accessions only ($H^2 = 0.75$, $H^2 = 0.81$, $H^2 = 0.62$, respectively). Other diversity studies have focused on the fruit characteristics: Van Berloo et al. (2008), on a collection of 94 commercial cultivars, calculated the mean, minimum and maximum values for the fruit weight (58.2, 6.8 and 161.4 g, respectively) and for the degrees Brix (5.17, 3.83 and 9.27 °B, respectively) resulting, respectively, lower and similar to the results of the present collection, whereas Bota et al. (2014), analyzing 142 *Ramellet* varieties from Balearic Islands and 29 other local varieties, showed a coefficient of variation (CV) for the fruit weight (CV = 34.04) quite lower than the present study ($CV_{2012} = 73.77$, $CV_{2012} = 60.23$). The diversity of the present collection was interesting also considering the qualitative traits: Terzopoulos and Bebeli (2010) analyzed 34 Greek tomato landraces and found similar level of diversity for several qualitative traits, such as the fruit cross-section shape ($H_e = 0.60$), the presence or abscence of green shoulder ($H_e = 0.55$), the blossom end shape ($H_e = 0.48$), the shape at pistil scare ($H_e = 0.64$), the fruit color ($H_e = 0.53$), the fruit shape ($H_e = 0.50$), the inflorescence type ($H_e = 0.45$), the leaf attitude ($H_e = 0.58$), the plant growth type ($H_e = 0.39$) and the foliage density ($H_e = 0.49$).

In the present study was also performed a precision phenotyping of the collection using the high-throughput Tomato Analyzer software to register different fruit morphology descriptors (Brewer et al., 2006; Gonzalo et al., 2009; Rodríguez et al., 2010b). This phenomics analysis was largely concordant with the conventional phenotyping, although it also provided nearly 20% more information thus evidencing the importance of the precision phenotyping both to automatize some of the phenotyping stages, and to acquire additional information otherwise unavailable. As an example, a collection of 69 Spanish landraces were investigated by using both phenotyping strategies and it was evidenced that although combined conventional and Tomato Analyzer analyses are recommended for characterization of local tomato varieties, some of the investigated morphotypes were not clearly differentiated solely by the conventional method (Figàs et al., 2014).

The cluster and principal component analyses showed that the local varieties are not clearly differentiated from commercial varieties both on the basis of the conventional and TA morpho-phenological

descriptors. In fact, the cultivars were distributed among the different clusters together with landraces. This was also true for the wild species, as the accessions *S. pimpinellifolium*, *S. lycopersicum* var. *cerasiforme* and Cocktail were attributed to the cluster IV together with other cultivated accessions characterized by small fruits, indicating that the phenotypic traits were not able to separate the cultivated tomato from its wild relatives. A thorough investigation of the present collection showed that, by using both phenotyping methods, it was possible to subdivide the investigated accessions into four phenotypic clusters. This allowed to trace out for the first time an exhaustive characterization of the tomato landraces collected in Sardinia, which will be useful to define their distinctive traits, set the basis for their traceability and promote their use in local markets, as well as in a more general contest to use them as a source of useful genes for future breeding programs. Roughly, within the cluster including the accessions with large fruit size, it is found one of the typical Sardinian tomato, generically defined "tamatta sarda" ("Sardinian tomato") that is often characterized by a red-orange fruit, generally with a flattened shape, high weight (up to 700-800 g) and high locules number. Usually, a defect of these fruits from a commercial point of view is the low firmness, that is usually compensated by the high quality traits, according to farmers. Some other interesting local varieties are included in the other clusters, such as the "Lorighittas" (earrings), as well as the "Arraccadas", that in Sardinian language indicate the custom to hang up tomatoes in circles to dry up during the winter and use them to flavor soups or other typical dishes; these are usually, characterized by medium size weight, cylindrical shape and thick pericarp.

The main descriptive statistics based on molecular data have also highlighted an interesting level of genetic diversity in the examined collection. An overall similar diversity level (H_e = 0.44) and mean number of observed alleles per locus (na = 5.2) was observed by Mazzucato et al. (2008) in a collection of 61 accessions including some accessions in common with the present study. Tam et al. (2005) discovered a lower genetic diversity (H_e = 0.39) by using 16 SSRs among 34 tomato accessions of different fruit morphology. Instead, a higher SSR genetic diversity was observed in a collection including 32 landraces of Myanmar (H_e = 0.67) and 40 tomato accessions from different countries (H_e = 0.80), showing on average 13.3 alleles per locus. This result may both indicate higher genetic diversity in respect to our collection or that the SSR markers used targeted high polymorphic regions (Yi et al., 2008).

The interesting level of the genetic diversity of the present collection is confirmed by the presence of an overall high number of polymorphic loci that is also observed within the different groups of genotypes. In the present study, the Sardinian landraces showed a valuable diversity value (H_e = 0.38) when compared to the exotic landraces (H_e = 0.41) if we consider that these were collected from different countries in the world. The highest number of private alleles was found for the exotic landraces (15) similar to that of the wild species (16), while only 3 private alleles were detected among the Sardinian landraces. The higher diversity of the wild species was confirmed at nucleotide level in a recent study were 360 accessions were sequenced evidencing a lower diversity for the group of *S. lycopersicum* var. *cerasiforme* accessions and the lowest for the group of tomato landraces and cultivars Lin et al. (2014). High levels of genetic diversity were also observed for the cultivars included into the collection (H_e = 0.42) whereas a low number of private alleles were detected for this group of accessions. These results are comparable with the previous study of Mazzucato et al. (2008), where the H_e values for a group of Italian landraces, cultivars, Latin

American landraces and wild species were, respectively, 0.41, 0.36, 0.42 and 0.33.

Genetic distance analyses have shown that Sardinian landraces are more genetically similar to the exotic landraces ($F_{ST} = 0.045$) than to cultivars ($F_{ST} = 0.074$) while the cultivars are more genetically similar to the wild relatives of tomato than Sardinian and exotic landraces. As tomato is a crop usually harboring a very low genetic diversity when compared to other species, in the present study it has been chosen to collect and investigate mainly landraces, with the aim to maximize the probability to unravel high levels of diversity. Results from both phenotypic and genetic analysis confirmed that these landraces have maintained a wealth of genetic variation likely mediated by human migration, seed exchange, and natural selection (Harlan, 1975; Brush, 2000). Previous studies on the genetic diversity on landrace of different autogamous species have confirmed the distinctiveness and relevance of this type of materials (Angioi et al., 2010; Rodriguez et al., 2012; Bellucci et al., 2013). Considering that Spain and Italy have been recognized as secondary centre of diversification for tomato, the inclusion of the Italian landraces aside to other landraces from different countries, represents a good chance to add diversity to analyze the structure of the variation in tomato and to investigate possible associations (Cebolla-Cornejo et al., 2013; Bauchet and Causse, 2012; García-Martínez et al., 2006; Mazzucato et al., 2008).

Structure analysis showed that the collection is mainly subdivided into three genetic groups with a moderate admixture level. This subdivision is also confirmed at geographic level as the three genetic groups show a clearly different patter both at international and at local level. One of the criticisms often reported about the association studies is the fact that false positives (non-existing associations between phenotypic traits and molecular markers) can be found. This is often observed when the genetic structure of the collection is not taken into account (Flint-Garcia et al., 2005). In order to avoid errors due to the detection of false positives, the analysis of the genetic structure have been included into the association analysis. Moreover, these studies require a thorough understanding of the linkage disequilibrium (LD) of the analyzed species that in tomato remains usually high over genetic distance (Robbins et al., 2011; Xu et al., 2013). The LD level determines the number of markers needed for an appropriate mapping strategy (the higher the LD the lower the number of markers) and is usually faster in allogamous than in autogamous species, in wild relatives more than landraces and more than in modern varieties (Flint-Garcia et al., 2005; Gaut and Long, 2003; Morrell et al., 2005; Caldwell et al., 2006; Rostoks et al., 2006; Mather et al., 2007; Song et al., 2009).

Therefore, to evaluate the suitability of the present collection for linkage disequilibrium mapping studies, the association between molecular markers and phenotypic traits was performed by using some molecular markers with a known association with QTLs for the fruit morphology and some with unknown linkage with any quantitative trait (Mazzucato et al., 2008). The results showed that this approach is highly effective because the number of associations detected by Q-SSR markers was significantly higher than that detected by the NQ-SSR and were mostly associated with the fruit traits. Among the significant associations detected with the Q-SSR markers, one locus (TMS59) showed the most interesting features, as it is associated with the fruit shape and other conventional and TA traits related to the fruit shape, and as also previously found in other studies (Mazzucato et al., 2008, and references therein). Moreover, associations with inflorescence number were detected for the loci Tom 236-237 and EST258529, also

confirming previous associations (Van der Knaap and Tanksley, 2003). Overall four loci were detected as associated with previously known QTLs for the same or similar fruit traits and a few new correlations were detected (such as the locus SLM6-14 and degrees Brix [BRIX]) in regions that are known to be associated with fruit weight. Co-localization of associations for several traits, such as fruit weight and total soluble solid were frequently observed in other studies (Xu et al., 2013) indicating that more and valuable associations may be unraveled with the present collection. The finding of correlations between some morphological and NQ-SSR markers are therefore worthy of further investigations. The fact that in the present study it was possible to detect marker-trait associations previously detected in different mapping populations (Paterson et al., 1991; Grandillo et al., 1999; Van der Knaap et al., 2002; Van der Knaap and Tanksley, 2003; Mazzucato et al., 2008) confirm this collection as valuable for future association mapping studies.

2.5 Conclusions

The main objective of this work was to evaluate the diversity levels of a wide collection of tomato mainly constituted by landrace accessions.

In this regard, a thorough phenotypic characterization was performed by using conventional and precision descriptors as also a genetic characterization with micro-satellite markers. This allowed for the first time obtaining an exhaustive characterization of the Sardinian tomato landraces, which will be useful to define their distinctive characteristics and implement their use in local markets. Additionally, the high levels of phenotypic diversity maintained across different environments point to these landraces as a valuable model to identify QTLs and genes of relevant interest also considering the possibility to automatize some of the phenotyping stages.

Finally, as a proof of concept, the collection has been evaluated for associations between molecular markers and phenotypic traits by using markers in linkage with known QTLs. The results confirmed the previously detected marker-trait associations in different mapping populations thus validating this collection as appropriate for future association mapping studies.

CHAPTER 3

Diversity for carotenoid contents

3.1 Introduction

Fruit organs are unique to plants and their ripening represents the terminal development stage that ends with the releasing of matured seeds (Giovannoni, 2004). In particular, ripening of fleshy fruits as tomatoes represents a summation of physiological and biochemical processes that increase attraction to seed-dispersing animals (Seymour et al., 1993; Fraser et al., 1994). These include, accumulation of colored pigment, textural changes that lead to softening, and metabolic changes related to flavor, aroma and nutrient composition, generally associated with the accumulation of sugars, acids and volatile compounds culminating in a diverse array of tastes and smells varying among species (Bapat et al., 2010; Klee and Giovannoni, 2011; Gapper et al., 2013). Tomato has emerged as a study model of fleshy fruit development and ripening due to its short generation time, a long known history of physiological, biochemical and molecular investigations, as well as because of the availability of advanced genetic and molecular tools (Bapat et al., 2010; Giovannoni, 2007).

Among the other traits, the color of tomatoes is a very important marketing factor that affects the buying decision of the consumer and is also a quality attribute for the tomato industry (Garrett et al., 1960; Gould, 1974; Stevens and Rick, 1986; Radzevičius et al., 2009). Chlorophyll and carotenoids are responsible for the color of tomatoes. In fact, the chlorophyll imparts a green color during the early developmental stages of fruit, and when the ripening process starts the chlorophyll is degraded; afterwards, the chloroplasts of the mature green fruit change into chromoplasts and carotenoid content dramatically increases leading to fruit colors that may vary from yellow, to orange, to red (Harris and Spurr, 1969; Laval-Martin et al., 1975; Arias et al., 2000; Egea et al., 2010).

Carotenoids are the most widespread group of vegetal natural pigments consisting of isoprenoid fat-soluble molecules (Fraser and Bramley, 2004; Frenich et al., 2005). They are present in all the photosynthetic organisms and their yellow, orange, and red colors are due to the presence of a number of conjugated double bonds in a polyene chain that functions as a chromophore (Britton, 1995, 1996; Rodriguez-Concepcion and Stange, 2013).

The hundreds of carotenoid structures known to date can be divided into two major groups: carotenes (non-oxygenated molecules) and xanthophylls (oxygenated molecules). They are produced in plastids from isoprenoid precursors supplied by the MEP (2-C-methyl-D-erythritol 4-phosphate) pathway (Hirschberg, 2001; Fraser and Bramley, 2004; Rodríguez-Concepción, 2010) (Figure 3.1). The carotenoids are important not only because of the color they impart but also because they contribute to protect the photosynthetic apparatus from photo-oxidation, attract pollinators and, have recognized health benefits (Frank and Cogdell, 1996; Grotewold, 2006; Ruiz-Sola and Rodríguez-Concepción, 2012; Arias et al.,

2000).

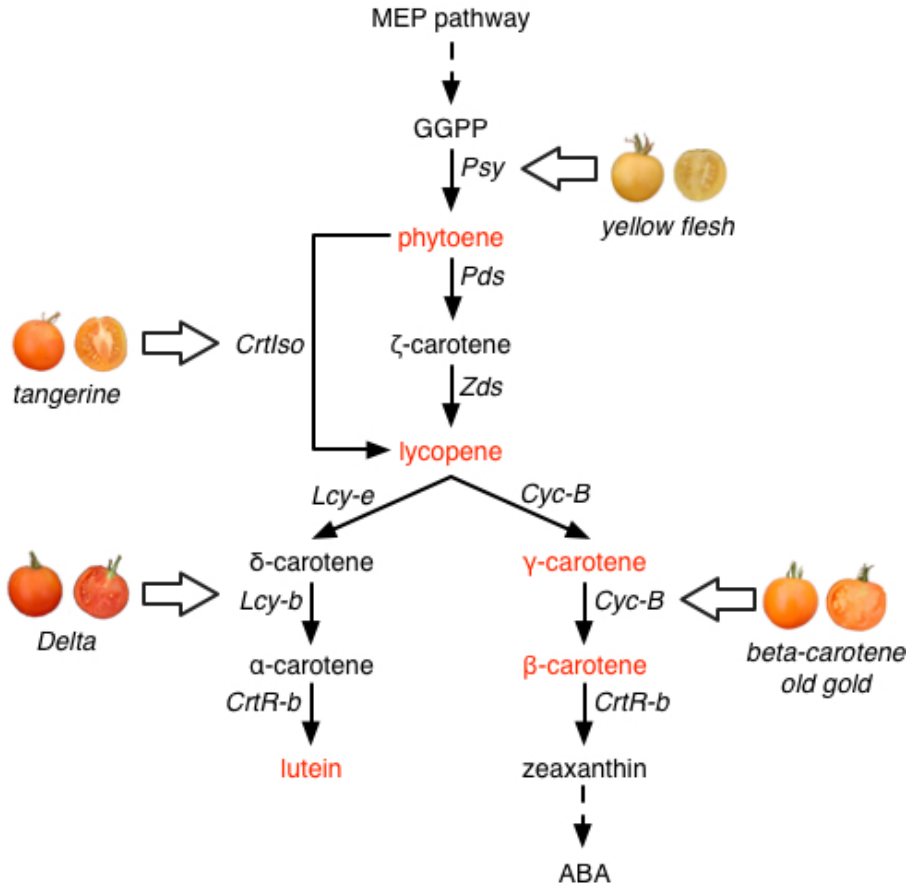

Figure 3.1: Simplified carotenoids biosynthetic pathway and principal tomato mutants. Pictures are from the Tomato Genetic Resource Centre (TGRC). Note: ABA, abscissic acid; CrtR-b: carotene-hydroxylase; Cyc-B, chromoplast specific lycopene synthase; GGPP, geranylgeranyl diphosphate; Lcy-b, lycopene β-cyclase; Lcy-e, lycopene -cyclase; MEP: methylerythritolphosphate; Psy, phytoene synthase; Zds, ζ-carotene desaturase.

Fruits and vegetables are the major sources of carotenoids in the Western-type diet, such as carrots, spinach, broccoli, cantaloupe, watermelon, and tomatoes (Rock et al., 1996; Clinton, 1998; Rao and Rao, 2007). Although tomato does not rank high in nutritional value (Vinson et al., 1998), its high consumption all year round makes it one of the main sources of carotenoids and other nutrients such as polyphenols, vitamins A and C and minerals (Beecher, 1998; Vinson et al., 1998; Khachik et al., 2002; Re et al., 2003; Bugianesi et al., 2004; Dorais et al., 2008; Ashrafi et al., 2012). Therefore, tomatoes are known as healthy fruits because of the antioxidant properties of their main compounds (Radzevičius et al., 2009), and they have been indicated as a functional and nutraceutical food (Jack, 1995; Canene-Adams et al., 2005). A nutraceutical is said any food, or part of a food, that provides medical or health benefits, including disease prevention and treatment (Jack, 1995).

Two carotenoids exhibit the highest nutritional value in tomato: lycopene, which imparts the red color to the fruit and is the most abundant carotenoid in the ripe fruit (approximately 80-90% of the total pigments; Hernández et al. (2007); Helyes et al. (2012)), and β-carotene, which represents around the 7% of the total carotenoid content (Gould, 1974; Adalid et al., 2010; Nguyen and Schwartz, 1999).

Lycopene is transformed into β-carotene by the lycopene β-cyclase (β-Lcy) during the ripening process (Rosati et al., 2000), and begins to accumulate at the 'breaker' stage of fruit ripening when the fruit has

reached the 'mature green' stage (Fraser et al., 1994). This carotenoid is abundantly contained in raw tomato and tomato-based products that contribute approximately for the 85% of our dietary intake of lycopene, whereas the remainder is obtained from watermelon, pink grapefruit, guava and papaya (Rock et al., 1996; Clinton, 1998; Rao and Rao, 2007). Lycopene is known to have antioxidant activity by quenching reactive oxygen species generated by free radicals (Agarwal and Rao, 2000; Shi and Maguer, 2000; Rao and Rao, 2007). Moreover, among the carotenoids, it is the most efficient free radical scavenger with its capacity in inhibiting cell proliferation of various human epithelial cancer cell lines more than twice higher that of β-carotene (Giovannucci, 1999; Violeta et al., 2013). In fact, numerous epidemiological and intervention studies have demonstrated that dietary intake of lycopene is correlated with a decreased incidence of several cancer types (e.g. prostate, lung, mouth, and colon), coronary heart diseases, and cataracts (Gerster, 1997; Gann et al., 1999; Giovannucci, 1999; Rao and Agarwal, 2000; Gupta et al., 2003; Wu et al., 2004; Omoni and Aluko, 2005; Kun et al., 2006; Jatoi et al., 2007). With prostate cancer, it appears that appropriate food choices can slow the onset of the disease and can play a role in prevention (Wilkinson and Chodak, 2003; Cohen, 2002).

Aside to lycopene, tomatoes also contain other carotenoids such as β-carotene, α-, γ-, δ-carotene, zeaxanthin and lutein and also neurosporene, phytoene, and phytofluene (Capanoglu et al., 2010; Ray et al., 2011; Tonucci et al., 1995). These tomato phytochemicals also contribute in reducing cardiovascular diseases and lung cancer (Canene-Adams et al., 2005; Sies, 1997). In particular, β-carotene is the second most abundant carotenoid in tomato and is a precursor of vitamin A, therefore making fruits and vegetables essential contributors to vitamin A in humans (Frenich et al., 2005). Its relevance has been widely demonstrated as extreme deficiency of vitamin A can causes depressed immunological disorders and eye diseases in 40 million children every year (Mayne, 1996). Among the approaches to face dietary deficiencies is the biofortification, a process by which staple crops are enriched of higher nutritional content such as vitamin A (Harjes et al., 2008).

All of the carotenoid benefits to human health demonstrate the importance of tomato and, as a result, this vegetable is in increasing demand (Adalid et al., 2010; Dar and Sharma, 2011). Further, since humans can only acquire lycopene through the diet and it is estimated that the 85% of the lycopene is acquired from tomato-based foods, the availability of tomatoes with higher lycopene content would be highly beneficial to consumers as well as to the tomato industry (Rao and Rao, 2007).

Accordingly, the development of high-lycopene tomato cultivars is one of the major breeding objectives for both fresh-market and processing tomato industries, as well as pharmaceutical and nutraceutical companies (Ashrafi et al., 2012; Causse et al., 2002, 2007). Beyond the functions listed above, carotenoids are also precursors for some of the most important volatile chemicals (apocarotenoids) essential to tomato flavor (Mathieu et al., 2009; Vogel et al., 2010). Aside to the reduced accumulation of sugars and acids, the inferior flavor of commercially produced tomatoes with respect to landraces, is associated with the reduced production of these volatiles (Mathieu et al., 2009; Vogel et al., 2010). All these data underline the importance of the studies on carotenoids.

To date, conventional breeding and genetic engineering are the two main strategies for the enhancement of carotenoids in plants (Fraser and Bramley, 2004; Römer et al., 2000; Zhang and Stommel, 2000).

In particular, conventional breeding programs have improved the overall lycopene content of tomato cultivars by exploiting natural genetic variation available within the cultivated species, including the use of the mutant gene *old-gold crimson* (og^c) (Ashrafi et al., 2012). Genetic improvement requires a wide genotypic variability for the desired characters (Zamir, 2001; Kuti and Konuru, 2005; Willits et al., 2005; Bai and Lindhout, 2007). However, agricultural industrialization has led to a reduction of the type of cultivars commonly cultivated, which has led to a decrease in the genetic diversity of the tomato cultigen with a consequent higher homogeneity in organoleptic and nutritional quality characteristics (Adalid et al., 2010; Ashrafi et al., 2012). Additionally, traditional varieties are disappearing worldwide and they are gradually replaced by modern cultivars (Adalid et al., 2010; Attene and Rodriguez, 2008). For this reason, the wild tomato related species and the landraces still represent an important source of genetic diversity for crop improvement programs with respect to antioxidant and other quality parameters (Kavitha et al., 2014). In fact, several studies have shown that aside to environmental factors, agricultural techniques, and post-harvest storage conditions, the chemical composition of the fruit strongly depends on genetics (Audisio et al., 1993; Buta and Spaulding, 1997; Giovanelli et al., 1999; Dumas et al., 2003; López Camelo and Gómez, 2004; Borguini and Ferraz Da Silva Torres, 2009; Maršić et al., 2011; Vrcek et al., 2011). The bioavailability of lycopene is influenced also by isomerisation as it is naturally present in *trans* configuration in raw tomato and converted to *cis* isomers during processing and storage of foods (Zechmeister and Tuzson, 1938; Lee and Chen, 2001; Frenich et al., 2005; Kuti and Konuru, 2005). However, this carotenoid seems to be more stable than other carotenoids to changes occurring during peeling and juicing (Capanoglu et al., 2010).

The variability of carotenoids contents and other quality parameters observed in tomato genotypes underlines the importance in the choice of the cultivated variety either for direct use or breeding purposes (Arias et al., 2000; Abushita et al., 2000; George et al., 2004; Kotíková et al., 2011). Accordingly, different studies have been performed to compare carotenoid contents in different tomato accessions. Significantly variable levels of carotenoids have been observed in different tomato varieties, with cherry tomato types having high lycopene content and high lipophilic and hydrophilic anti-oxidative activities (Leonardi et al., 2000; Kuti and Konuru, 2005). Moreover, several accessions of the red-fruited *Solanum pimpinellifolium* species, in particular the accession LA2093, have shown a higher lycopene content than that normally found in commercial tomato cultivars (Hyman et al., 2004; Ashrafi et al., 2012; Kinkade and Foolad, 2013). Accessions of *S. pimpinellifolium* can easily hybridize with the cultivated tomato, facilitating their use in tomato breeding when exotic traits are needed for crop improvements; aside, local varieties can provide useful and promising traits for crop improvements without the drawbacks of the linkage drag effects.

In fact, while new varieties of tomato improved for quality traits are being developed, heirloom, local or traditional varieties continue to enjoy prestige among consumers in terms of taste and nutritional value (Attene and Rodriguez, 2008; Casals et al., 2011). Moreover, among the cultivated tomato genotypes, tomato landraces constitute the main source of variation for the cultivated species, justifying the increasing interest for their utilization in scientific studies (Chable et al., 2009).

In accordance with this premise, in this study, we have analyzed a collection of 125 tomato accessions

(114 landraces, 10 vintage cultivars and 1 wild species as reference) for their variation in carotenoid contents at two fruit ripening stage (breaker and ripe stage). In detail, we searched for accessions with intriguing characteristics to be used a) in future breeding programs for improving tomato quality traits related to carotenoid contents and b) in metabolic studies to better understand the genetic basis of the fruit ripening process.

3.2 Materials and methods

3.2.1 Plant material and experimental trials

A collection of cultivated tomato (*Solanum lycopersicum* L.) and wild tomato species was investigated through an experimental trial held in 2013. All details about materials and trial are described in section 2.2.1 on page 21.

3.2.2 Measurements and molecular analysis

We have extracted the carotenoids from three replicates of each accession (one per block) both at breaker stage, when the fruit is rich in chloroplasts, and ripe stage, when the fruit accumulates large quantities of carotenoids (Figure 3.2), for a total of 762 samples. Fruit harvesting was done without the use of any sensorial instrument. In fact, the human perception of color has previously been reported to have a good correlation with the color readings of Hunter and Munsell color disks (Mavis and Gould, 1954) and with the light transmittance and reflectance of tomatoes during ripening (Jahn, 1975). All harvested fruits at breaker and ripe stage were uniformly colored and healthy. At ripe stage, accessions with orange, yellow or pink fruits were harvested when the color reached the maximum intensity. Harvested fruits were left for 6 hours on a laboratory bench at room temperature to reduce harvest stress. Then they were cut into halves and cleaned from seeds, frozen with liquid nitrogen and stored at -80 °C.

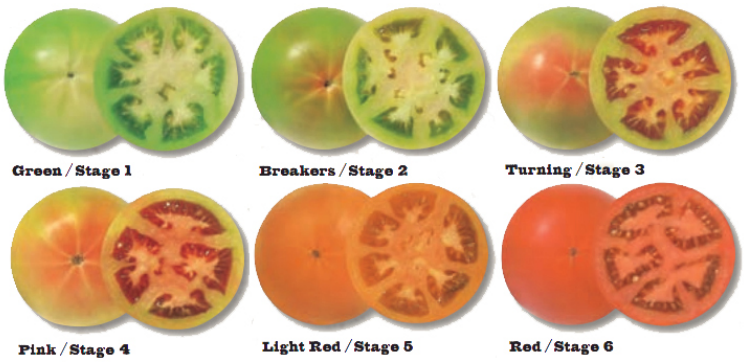

Figure 3.2: Tomato fruit ripening stages. Image courtesy of the California Tomato Commission.

Frozen flesh samples from each fruit stage were rapidly homogenized and carotenoids extraction performed as described in Grassi et al. (2013). Carotenoid detection was performed on a Summit HPLC system and a PDA-100 photodiode array detector (Dionex, Sunnyvale, CA, US). Spectra were collected at 286, 450 and 471 nm and pigments were identified via co-migration with purified standards and/or by

their pigment-specific absorbance spectra. The list of the carotenoids analyzed for each stage is shown in Table 3.1.

Table 3.1: List of carotenoids extracted at the breaker and ripe stages.

Carotenoid	Code	Wavelength	Breaker stage	Ripe stage
Trans-lycopene	T-LYC	471 nm	✓	✓
Cis-lycopene	C-LYC	471 nm	✓	✓
Lutein	LUT	450 nm	✓	✓
β-carotene	β-CAR	450 nm	✓	✓
γ-carotene	γ-CAR	450 nm		✓
Phytoene	PHY	286 nm		✓

Relevant morphological traits were also investigated for possible associations with the carotenoid content of the different varieties. In detail, the mean fruit weight (FW, g), the degrees Brix (BRIX, °B), the fruit color (FCO) and the fruit shape (FSH) were considered. The description of these traits is available in section 2.2.2 on page 22.

3.2.3 Statistical analysis

All the statistical analyses were performed by using JMP 10.0.0 (SAS Institute, Inc. 2012) and when necessary accessions with missing data were excluded from the analysis.

The analysis of variance (ANOVA) was performed over all the accessions to test the presence of significant differences among genotypes.

Broad sense heritability was calculated by fitting model with random effects through the restricted maximum likelihood method (REML) (Patterson and Thompson, 1971; Patterson and Nabugoomu, 1992; Lynch et al., 1998).

Pearson's correlations were estimated to test possible associations among different traits.

Cluster analysis, performed by using the hierarchical method, and principal components analysis (PCA) were also implemented to investigate relations among the different varieties based on carotenoid contents.

3.3 Results

The total amount of carotenoids was higher at the ripe stage than at the breaker stage (Table 3.2). At the breaker stage the total mean content of carotenoids was 6.09 μg/g of fresh weight (fw) and increased of about 20-fold at full ripeness (121.55 μg/g fw). All analyzed compounds showed significant differences among genotypes (Table 3.2) at both breaker and red ripe stages. *Trans*-lycopene, *cis*-lycopene and β-carotene increased their amount during fruit ripening, whereas lutein showed an opposite trend.

Trans-lycopene showed the highest increment, moving from a mean value of 2.72 μg/g fw at the breaker stage to 105.98 μg/g fw at the ripe stage, that means an increase of around 39-fold. The *trans*-lycopene isomer content was also significantly higher than its *cis*-isomer at both ripening stages (data not shown). The second highest compound, the β-carotene, increased of about 2-fold, going from a mean value of 2.03 μg/g fw at the breaker stage to 4.36 μg/g fw at the ripe stage. It accounted for 33% of the

total carotenoid content at the breaker stage and only 3.6% at full ripeness; moreover, its concentration was almost 25-fold lower than *trans*-lycopene at the ripe stage. Lutein decreased its mean concentration from 1.3 μg/g fw at the breaker stage to 0.87 μg/g fw at the ripe stage. At the ripe stage the concentration of γ-carotene and phytoene showed a mean content of 3.58 μg/g fw and 3.78 μg/g fw respectively.

Table 3.2: Significant differences among genotypes of cultivated tomato for all analyzed compounds.

Trait[a]	Stage[b]	Mean (μg/g fw)	Min (μg/g fw)	Max (μg/g fw)	SD	DF	SS	F	
T-LYC	B	2.72	0	16.56	2.05	119	1035.30	1.94	****
C-LYC	B	0.05	0	0.26	0.05	119	0.79	1.88	****
LUT	B	1.30	0.33	2.50	0.29	119	28.36	2.49	****
β-CAR	B	2.03	0.84	3.72	0.69	119	160.57	3.32	****
Total B stage		*6.09*							
T-LYC	R	105.98	0	183.37	34.17	119	375635.92	2.66	****
C-LYC	R	2.99	0	8.97	1.24	119	506.51	2.38	****
LUT	R	0.87	0.15	1.68	0.25	119	20.91	1.61	**
β-CAR	R	4.36	0	10.94	1.95	119	1176.22	4.70	****
γ-CAR	R	3.58	0	6.52	1.07	109	233.14	1.42	*
PHY	R	3.78	0.01	8.32	1.36	109	384.43	2.28	****
Total R stage		*121.55*							

Min = minimum value, Max = maximum value, SD = standard deviation, DF = degrees of freedom, SS = sum of squares, F = F ratio
* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001

[a] T-LYC, *trans*-lycopene; C-LYC, *cis*-lycopene; LUT, lutein; β-CAR, β-carotene; γ-CAR, γ-carotene; PHY, phytoene.

[b] B, breaker stage; R, ripe stage.

The outstanding genotype for *trans*-lycopene content was 'Cocktail', which has traits and molecular characteristics similar to a wild species. The maximum average value of this accession, included as control, was observed at the ripe stage with 245.48 μg/g fw. Among the cultivated tomato group, some accessions showed high *trans*-lycopene content, such as the landraces 'Pomodoro genovese' (183.37 μg/g fw), 'Pruno II' (180.77 μg/g fw), 'Cuore di Bue' (163.71 μg/g fw), 'Tamatta' (162.56 μg/g fw) and 'Ovale' (158.80 μg/g fw). On the other side, the landrace 'Arracadas' showed the lowest content of *trans*-lycopene (maximum value, 13.46 μg/g fw; average value, 10.94 μg/g fw).

The broad sense heritability (H^2) was similar for the two ripening stages with a slightly lower average value at the breaker stage ($\overline{H^2} = 29.3$) than at the ripe stage ($\overline{H^2} = 33.2$) (Figure 3.3). Except for lutein, the heritability increased at the ripe stage for all of the carotenoids (see *trans*-lycopene, *cis*-lycopene and β-carotene, Figure 3.3). At the breaker stage, the β-carotene showed the highest heritability (H^2 = 44.6%), followed by lutein (H^2 = 33.8%), *cis*-lycopene (H^2 = 21.6%), and *trans*-lycopene (H^2 = 17.4%). The β-carotene showed the highest value also at the ripe stage (H^2 = 57.7%), followed by phytoene (H^2 = 38.6%), *trans*-lycopene (H^2 = 36.1%), *cis*-lycopene (H^2 = 31.9%), γ-carotene (H^2 = 18.2%), and lutein (H^2 = 16.8%).

Correlations among the different carotenoid contents at both ripening stages are shown on Table 3.3. At the breaker stage *trans*-lycopene was positively correlated with the content of *cis*-lycopene (r = 0.80) and β-carotene (r = 0.38). The β-carotene was correlated with *cis*-lycopene (r = 0.22) and strongly correlated with lutein (r = 0.38). At the ripe stage more and stronger correlations were detected among the different compounds. The *trans*-lycopene content increased with the increasing of all compounds, in particular *cis*-lycopene (r = 0.65), γ-carotene (r = 0.78) and phytoene (r = 0.69). Also the *cis*-lycopene

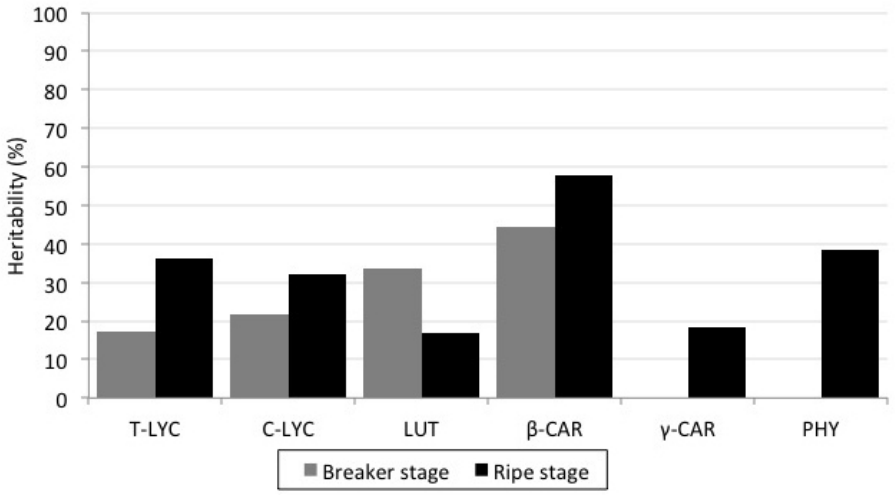

Figure 3.3: Broad sense heritability (H^2) among cultivated tomato for each compound at breaker and ripe stage. The traits γ-CAR and PHY were not scored at the breaker stage. Note: T-LYC, *trans*-lycopene; C-LYC, *cis*-lycopene; LUT, lutein; β-CAR, β-carotene; γ-CAR, γ-carotene; PHY, phytoene.

Table 3.3: Estimates of Pearson's correlations among all the carotenoid contents at both breaker and ripe stage for cultivated tomato accessions.

	Breaker stage								*Ripe stage*											
Trait[a]	T-LYC		C-LYC		LUT		β-CAR		T-LYC		C-LYC		LUT		β-CAR		γ-CAR		PHY	
Breaker stage																				
T-LYC	-																			
C-LYC	0.80	****	-																	
LUT	0.07		0.07		-															
β-CAR	0.38	****	0.22	*	0.38	****	-													
Ripe stage																				
T-LYC	0.23		0.08		0.16		0.14		-											
C-LYC	0.17		0.20		0.15		-0.09		0.65	****	-									
LUT	0.02		0.08		0.52	****	-0.01		0.37	****	0.26	**	-							
β-CAR	-0.02		-0.07		0.16		0.57	****	0.32	****	-0.11		0.27	**	-					
γ-CAR	0.21		0.12		0.13		0.19		0.78	****	0.66	****	0.17		0.27	*	-			
PHY	0.26	*	0.17		0.13		0.18		0.69	****	0.64	****	0.06		0.15		0.75	****	-	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

[a] T-LYC, *trans*-lycopene; C-LYC, *cis*-lycopene; LUT, lutein; β-CAR, β-carotene; γ-CAR, γ-carotene; PHY, phytoene.

was highly correlated with γ-carotene (r = 0.66) and phytoene (r = 0.64), and the γ-carotene was strongly correlated with phytoene (r = 0.75). From the intersection of the two ripening stages, only lutein and β-carotene showed a high significant positive correlation among themselves with a correlation coefficient equal to 0.52 and 0.57 respectively.

Considering the morphological traits, some interesting correlations were detected (Table 3.4). The β-carotene was negatively correlated to the mean fruit weight (FWG) at both breaker stage and ripe stage, and it was positively correlated with the degrees Brix at the breaker and ripe stage. Fruit color (FCO) was correlated with lutein at the breaker stage, and with all compounds at the ripe stage. The β-carotene showed significant differences among the different fruit shapes (FSH) at both ripening stages.

The carotenoid content varied among accessions with different fruit color at both breaker and ripe stages (Figure 3.4). In particular, at the breaker stage, only the lutein showed significant differences among the different colors, whereas at the ripe stage differences among different fruit colors were significant for all the compounds. Moreover, among the varieties with orange fruit color we found a particularly

Table 3.4: Estimates of Pearson's correlations among the carotenoid contents and the quantitative traits, and R^2 values among qualitative traits evaluated for cultivated tomato accessions.

	Breaker stage				*Ripe stage*					
Trait[a]	T-LYC	C-LYC	LUT	β-CAR	T-LYC	C-LYC	LUT	β-CAR	γ-CAR	PHY
Quantitative traits										
FWG	0.05	0.21 *	-0.22 *	-0.51 ****	-0.01	0.31 ***	-0.02	-0.36 ****	0.12	0.16
BRIX	-0.07	-0.16	0.13	0.42 ****	0.06	0.06	0.00	0.18 *	0.05	0.05
Qualitative traits										
FCO	0.05	0.04	0.12 **	0.04	0.25 ****	0.15 ***	0.08 *	0.11 **	0.21 ****	0.16 ****
FSH	0.07	0.08	0.07	0.29 ****	0.11	0.15 *	0.08	0.17 **	0.09	0.13

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

[a] T-LYC, *trans*-lycopene; C-LYC, *cis*-lycopene; LUT, lutein; β-CAR, β-carotene; γ-CAR, γ-carotene; PHY, phytoene; FWG, mean fruit weight; BRIX, degrees Brix; FCO, fruit color; FSH, fruit shape.

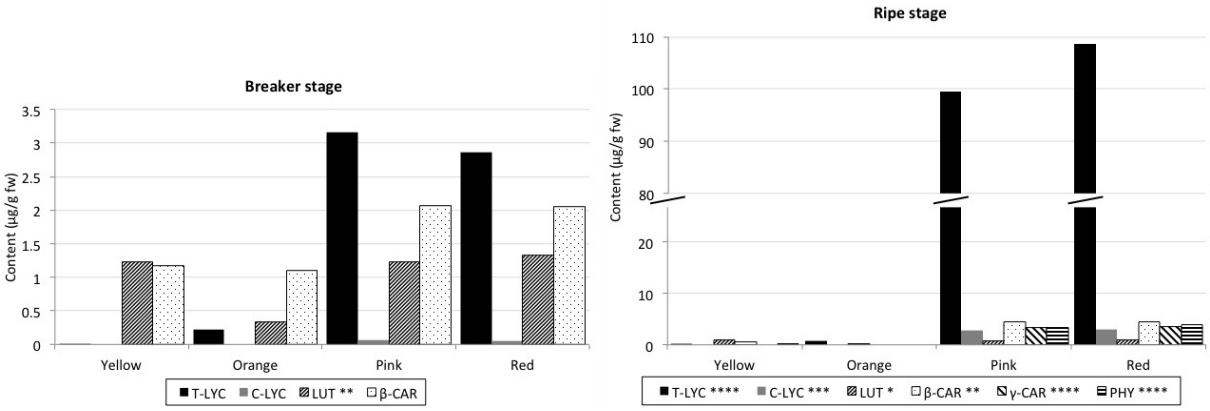

Figure 3.4: Carotenoid contents of the cultivated tomato accessions at the breaker and ripe stage within the different fruit colors. The traits γ-CAR and PHY were not scored at the breaker stage. Significant differences for each compound within the different fruit colors are also indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.). Note: T-LYC, *trans*-lycopene; C-LYC, *cis*-lycopene; LUT, lutein; β-CAR, β-carotene; γ-CAR, γ-carotene; PHY, phytoene.

interesting carotenoid pattern in one of the exotic landraces (Portocala, Romania). In fact, the HPLC chromatograms obtained from two different samples of this line, showed no β-carotene content and several peculiar lycopene-like peaks, but clearly not *cis*- or *trans*-lycopene.

We also investigated the relations between the analyzed carotenoids and the fruit shape, finding an interesting variation of the β-carotene content among the different shapes. In particular, we observed that, at both the breaker and ripe stages, the fruits showing the highest β-carotene content were round (3.61 and 8.18 μg/g fw, respectiely), high-round (2.82 and 6.77 μg/g fw, respectively) and ellipsoid (3.61 and 5.62 μg/g fw, respectively) (Figure 3.5). The round shape is typical of cherry tomatoes, while high round and ellipsoid shape are usually indicated as plum shape.

Finally, in order to maximize the differences for the carotenoid content, a core collection was extracted from the germplasm collection. The accessions were selected by choosing the two accessions with the highest carotenoid content and the two with the lowest carotenoid content. The choice was repeated for each carotenoid at the ripe stage for a total of 19 accessions selected (Table 3.5). For some compounds, the selected accessions were the same. The landraces 'Yellow Pear' (yellow), 'A peperone' (yellow) and 'Portocala' (orange) were included by default because of the fruit color, as well as the accession 'Cocktail' because of its affinity with the wild species and its high lycopene content. As a whole, the subset includes 17 landraces, 7 of which from Sardinia, one vintage cultivar and one wild species (Table 3.5).

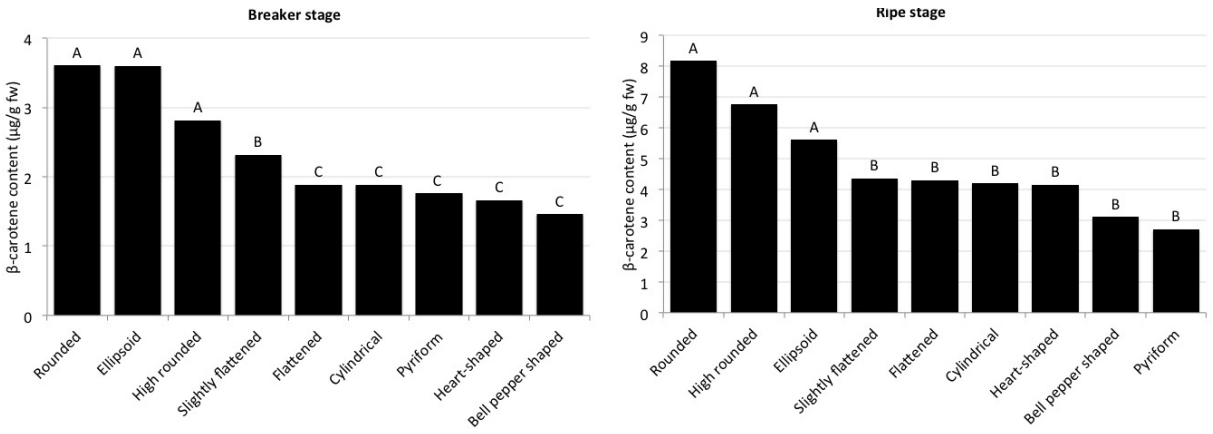

Figure 3.5: β-carotene contents of the cultivated tomato accessions at the breaker and ripe stage within the different fruit shapes.

Table 3.5: Carotenoid contents at the ripe stage of the accessions selected for further analysis.

Code	Name	Population[a]	T-LYC (μg/g fw)	C-LYC (μg/g fw)	LUT (μg/g fw)	β-CAR (μg/g fw)	γ-CAR (μg/g fw)	PHY (μg/g fw)
P01	Arracadas	L-SAR	106.84	1.89	0.67	10.94	4.52	5.22
P16	Tamatta a forma di cachi	L-SAR	31.56	0.89	0.33	1.84		
P18	Tamatta	L-SAR	162.56	5.72	0.99	5.67	4.77	6.59
P44	Tamatta groga de appiccai	L-SAR	34.71	0.74	0.57	6.30	1.10	1.03
P72	Tondo	L-SAR	46.23	0.73	0.76	8.50	1.19	1.25
P80	Pomodoro genovese	L-SAR	183.37	3.27	0.94	7.38	4.56	5.69
P82	Grosso appiattito costoluto	L-SAR	130.73	4.42	0.92	4.72	6.52	8.32
P53	A peperone	L-IT	0.07	0.00	0.57	0.34	0.00	0.01
P59	Cuor di bue di Albenga	L-IT	137.12	4.56	0.89	1.78	4.14	4.56
CGN14469	Nepal 32	L-EXOT	120.82	3.44	1.67	6.40	3.84	4.93
CGN15460	Local	L-EXOT	90.64	1.62	0.98	10.55	3.49	3.32
CGN15522	Local	L-EXOT	53.62	1.20	0.32	3.14		
CGN15894	Pruno II	L-EXOT	180.77	3.42	0.83	4.05	4.62	6.15
CGN17125	Ostravske Rane	L-EXOT	91.15	3.07	1.60	2.91	2.51	1.48
CGN17129	Portocala	L-EXOT	0.73	0.00	0.15	0.00		
CGN24030	Yellow Pear	L-EXOT	0.30	0.00	1.15	0.76	0.00	0.01
P62	cv Chico III	C	50.81	1.38	0.62	1.31	2.68	2.83
P40	Cocktail	W	245.48	4.27	0.81	7.23	4.22	5.72

T-LYC, *trans*-lycopene; C-LYC, *cis*-lycopene; LUT, lutein; β-CAR, β-carotene; γ-CAR, γ-carotene; PHY, phytoene.

[a] L-SAR, Sardinian landraces; L-IT, Italian landraces; L-EXOT, exotic landraces; C, cultivars; W, wild species.

3.4 Discussion

The aim of this work was to evaluate the diversity for the content of different carotenoids of a wide collection of cultivated tomato accessions harvested at two different ripening stages. Most studies compare carotenoid content of fruits, in particular lycopene and β-carotene, at the ripe stage, while a lower number of studies investigate the variation of the carotenoid contents during fruit ripening. We have chosen to analyze the carotenoid concentration at the breaker and ripe stages, to compare the chloroplast rich, photosynthetic fruit with the chromoplast and nutrient rich, non-photosynthetic fruit, as variations among stages might reveal interesting feature useful to gain insight into the fruit ripening mechanisms (Radzevičius et al., 2009).

The high degree of variation and the broad sense heritability (H^2) observed among accessions at both ripening stages for all of the analyzed carotenoids (*trans*-lycopene, *cis*-lycopene, lutein, β-carotene, γ-

carotene, and phytoene) suggests that the genotype is a determinant factor in affecting the carotenoid content, as also shown in previous studies that have investigated tomato collections (Abushita et al., 2000; George et al., 2004; Lenucci et al., 2006; Guil-Guerrero and Rebolloso-Fuentes, 2009).

The present study also revealed that the *trans*-lycopene for cultivated tomato accessions ranged from 0 to 237.09 μg/g fw, corresponding to an average range of 0-180.77 μg/g fw. This is a wide range of variation if compared with other recent studies made on different cultivated tomato collections (Figure 3.6). According to Cortés-Olmos et al. (2014), the lycopene content of 126 Spanish traditional varieties of tomato and four commercial hybrids based on the mean of two trials ranged from 12.4 to 151.93 μg/g fw. Frusciante et al. (2007), in the attempt to address breeding programs in selecting tomato genotypes and to propose an index of antioxidant nutritional quality, analyzed twelve breeding lines and six cultivars for eight quality traits and, among the others, they detected a lycopene content ranging from 23.3 to 169 μg/g fw. Hanson et al. (2004), in order to identify accessions interesting for the improvement of antioxidant content, found a range of 0.4 to 106.4 μg/g fw for the lycopene content among fifty *S. lycopersicum* entries. Other studies (Adalid et al., 2010; Hyman et al., 2004; Kuti and Konuru, 2005; Violeta et al., 2013) found out a smaller lycopene content range despite the inclusion of tomato cultivars and tomato hybrids. This comparison with other studies related to the analysis of accessions interesting for their carotenoid content, places our collection among the most variable, highlighting its value for further metabolic studies in tomato.

In particular, the comparison with the highest reported carotenoid concentration (Frusciante et al., 2007) and with other previous studies, several accessions of our collection, many of which landraces, showed very interesting lycopene contents. Some studies showed that high-lycopene tomato cultivars carrying 'high-lycopene' (*hp*) mutations, reached a lycopene content up to 254 μg/g fw (Ilahy et al., 2011a,b). This attention to lycopene content is justified by the fact that its antioxidant capacity is almost twice that of β-carotene (Di Mascio et al., 1989). These results encourage studies as the present one to unravel valuable genotypes also taking into account further studies on carotenoid content (Dumas et al., 2003; Kuti and Konuru, 2005; Brandt et al., 2006).

When comparing the range for the content of β-carotene at full ripeness, some exotic and Sardinian landraces showed interesting β-carotene and the mean range (0-10.94 μg/g fw) is in line with previous studies (Hanson et al., 2004; Hyman et al., 2004; Frusciante et al., 2007; Adalid et al., 2010) on cultivated tomato accessions (Figure 3.7), except for Cortés-Olmos et al. (2014) that investigated 126 Spanish landraces (10.73-34.72 μg/g fw) and Violeta et al. (2013) that have detected the β-carotene content of ten tomato F1 hybrids (6.4-20.6 μg/g fw).

As also demonstrated in Chapter 2 for relevant morphological traits of the fruits, the diversity of this collection for carotenoid contents and in particular for lycopene and β-carotene, can be deployed for association mapping studies. The successful combination of modern plant breeding tools with nature's biodiversity is demonstrated. As an example, this combination allowed Zheng et al. (2008) to isolate a key gene that can be used to regulate oil content and composition in maize. Similarly, a recent study has reported how the natural variation has been exploited to improve β-carotene content in maize (Harjes et al., 2008). This study combined association analysis with classical linkage mapping, expression and

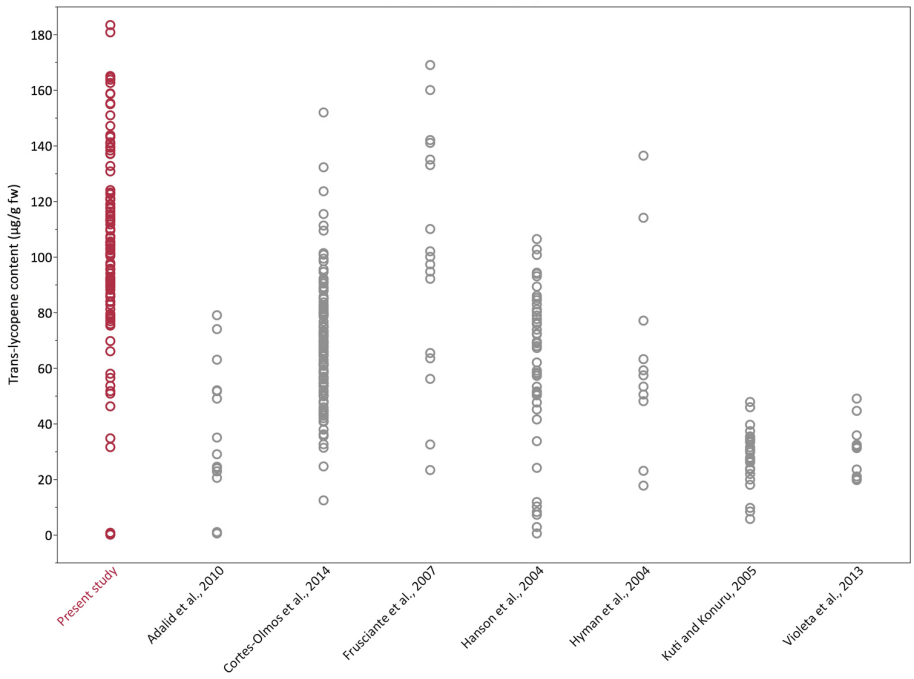

Figure 3.6: Comparison of content-range for *trans*-lycopene at the ripe stage between the present collection and other studies.

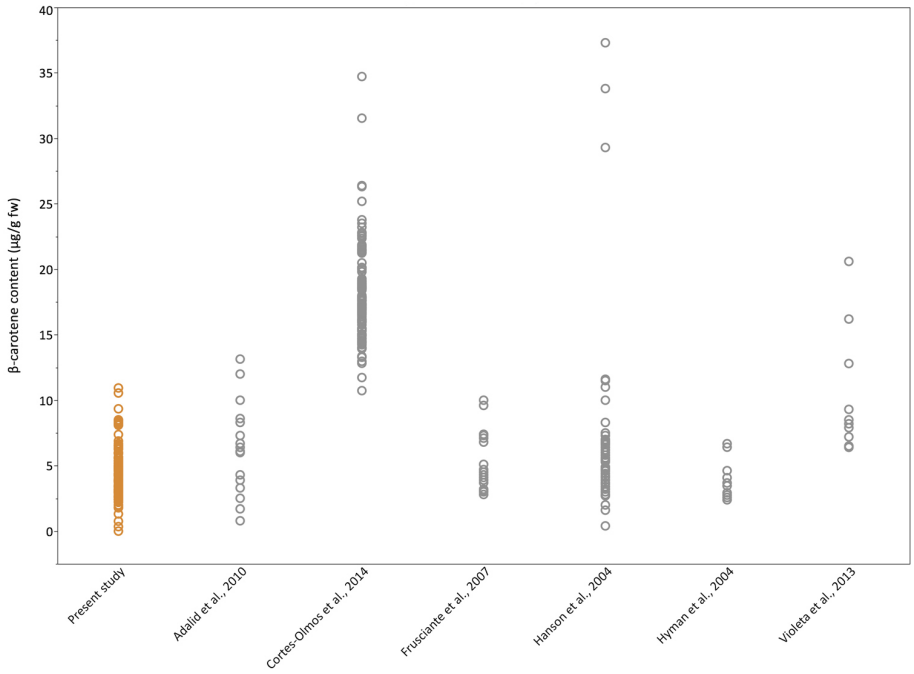

Figure 3.7: Comparison of content-range for β-carotene at the ripe stage between the present collection and other studies.

mutagenesis analyses to show that variation at the lycopene epsilon cyclase (lcyE) locus alters the flux of β-carotene. In particular, new genetic resources enriched for β-carotene or provitamin-A were detected. They also developed a simple PCR-based method that enables developing-country breeders to effectively produce maize grain with higher provitamin A levels (Harjes et al., 2008).

In tomato, a species that harbors much lower genetic diversity than maize, the availability of a highly variable collection for carotenoid contents, provide an unusual chance to identify valuable variants to be exploited in breeding programs. As underlined by previous studies (Mathieu et al., 2009; Vogel et al., 2010), reduced carotenoid negatively impacts flavor acceptability as well as reduced total soluble solids influence the sweetness of the fruits. The strong differences detected among a number of fruit color mutants (Vogel et al., 2010)) and among different genotypes from two different populations of introgression lines (Mathieu et al., 2009) encourages to address fruits with strongly different concentrations of carotenoids as major targets of breeding for improved tomato quality traits.

In addition to lycopene and β-carotene, the present study also presents the results of the variation of some minor carotenoids to better describe the carotenoid pattern of each accessions. To date, few works have analyzed all these compounds and not in wide collections of tomato as the present one. As an example, Abushita et al. (2000) analyzed the content of lutein, lycopene epoxide, lycopene, *cis*-lycopene and β-carotene on ripe fruits of 12 salad tomatoes and 15 processing cultivars, whereas Raffo et al. (2002) evaluated the amount of lycopene, β-carotene, phytoene, phyrofluene, γ-carotene, ζ-carotene, 5,6-dihydroxy-5,6-dihydrolycopene and lycopene 1,2-epoxide on one F1 hybrid.

Aside to the intriguingly high and variable contents of carotenoid, the present collection revealed interesting associations with some other fruit traits. In particular, β-carotene at both ripening stages and *cis*-lycopene at ripe stage have shown significant differences among the different fruit shapes, whereas *trans*-lycopene did not show a significant variation. The highest β-carotene values were shown by the round (cherry), high-round (plum) and ellipsoid (plum) shapes.

Several studies have shown as the carotenoid content may vary with the different fruit shapes. Violeta et al. (2013) found the plum shaped cultivar 'Porto' showing the highest average lycopene content (49 μg/g fw) while other studies found high lycopene content for accessions having the plum shape and cherry tomatoes (Aherne et al., 2009; Muratore et al., 2005). These nutritional characteristics and the high content of lycopene and β-carotene of these genotypes explain why in recent years there has been an increased demand for the plum and cherry tomato varieties (George et al., 2004; Kuti and Konuru, 2005; Aherne et al., 2009; Adalid et al., 2010; Violeta et al., 2013). Kuti and Konuru (2005) found the highest lycopene content in the cherry tomato cultivar 'Sugar Lamp', both in the greenhouse (63.6 μg/g fw) and in the field (116.7 μg/g fw). The cherry tomato 'Tiger' presented the highest content of β-carotene (20.6 μg/g fw) in the study of Violeta et al. (2013). Adalid et al. (2010) found the highest content of β-carotene (13 μg/g fw) in the fruits of a cherry type cultivar as well. It has been suggested that cherry tomatoes may be useful varieties for processing and for improvement of nutritional and health benefits in tomato breeding programs (Tigchelaar, 1986).

The generally negative correlation observed in the present study between fruit weight and carotenoid content, becomes strongly significant for β-carotene. Previous studies detected negative correlations

between fruit weight and lycopene content, suggesting that carotenoid, and consequently color variations could be partly due to pleiotropic effects of fruit weight (Liu et al., 2003; Saliba-Colombani et al., 2001). As well as the associations with fruit weight significant correlations were found between β-carotene content and total soluble solid content (measured in degrees Brix), as also observed previously (Liu et al., 2003), in particular for processing tomatoes (Lin et al., 2014).

The association with different fruit colors is not surprising and numerous studies have widely explained that variable combinations of different carotenoids determine different graduation of colors (from yellow to purple to red) (Fray and Grierson, 1993; Liu et al., 2003; Arias et al., 2000). Aside to the different nutritional values associated to variable carotenoid content, genotypes with diverse colors may also unravel other important utilities. As an example, the accession 'Porto Cala' of the present collection evidenced a peculiar HPLC chromatogram, with no β-carotene content and several peculiar lycopene-like (but clearly not *cis*- or *trans*-lycopene) peaks. This result encourages us to investigate further on the reasons of this pattern and whether this is a natural mutant carrying a mutation similar to the tomato tangerine (Figure 3.8). The carotenoid characterization of this genotype might eventually represent the starting point for the detection of candidate genes associated with the carotenoid biosynthetic pathway (Liu et al., 2003; Giovannoni, 2007).

Figure 3.8: The exotic landrace 'Portocala' with orange fruit color (on the left) does not contain β-carotene. It is probably carrying a mutation similar to the tomato tangerine (on the right).

Genome-wide association mapping, also called linkage disequilibrium (LD) mapping, is an approach that is being increasingly adopted to identify the QTLs in plants that exploits the natural diversity of wild and landraces populations with substantial benefits for conservation genetics and ecology (Vigouroux et al., 2002; Flint-Garcia et al., 2003; Allendorf et al., 2010; Bordes et al., 2011; Galeano et al., 2012). Despite the low genetic diversity of the cultivated tomato, landraces still represent a valuable breeding material showing diversity levels usually higher than modern cultivars that can be exploited to improve tomato features such as fruit nutritional value and quality (Chable et al., 2009; Casals et al., 2011). However, describing the agronomic and quality traits of a large collection is still costly and time-consuming. Therefore, selecting a sub-set of individuals that represents the diversity conserved in the whole collection may be more efficient (Balfourier et al., 2007; Bordes et al., 2011; Corrado et al., 2014).

In here, 19 accessions were extracted from the present tomato collection and selected for their high or low carotenoid content at the ripe stage. These genotypes, present outstanding features in their carotenoid content and therefore represent an interesting sub-sample for pilot studies with molecular and

high-throughput genetic analyses with the aim to gain insight into the fruit ripening mechanism. Sixteen accessions of this sub-set are landraces, so that future information might also provide the basis for a suitable utilization and valorization of tomato landraces.

3.5 Conclusions

The present work has analyzed the variation of the carotenoid content among accessions of a wide tomato collection consisting mainly of landraces. The results have shown a high variability for the carotenoid content, with some outstanding landraces for their high (or conversely very low) content of lycopene and β-carotene. These accessions represent a valuable material to be used for direct valorization or as donor parents in future breeding programs for the improvement of the fruit quality. Likely, the present results will also contribute to promote the *in situ* conservation of these traditional tomato varieties by local farmers.

Moreover, the whole collection and the selected core collection might be the object of future association studies and high-throughput genetic analyses, to search for candidate genes related to fruit quality traits and to deepen the knowledge of the ripening process in tomato.

CHAPTER 4

Characterization for antixenotic resistance to *Tuta absoluta* (Meyrick)

4.1 Introduction

The tomato borer or tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is one of the most devastating pests for tomato (*Solanum lycopersicum* L.) crops both under field and greenhouse conditions (Desneux et al., 2010; Tropea Garzia et al., 2012) and it is currently considered a key agricultural threat to European and North African tomato production. This pest is native to South America and probably arrived in Europe through Spain in late 2006 (Urbaneja et al., 2007). It was subsequently reported in several countries of the Mediterranean Basin and Europe (Potting, 2009; Gharekhani and Salek-Ebrahimi, 2014b). The first report for Sardinia (Nannini et al., 2010) was almost contemporary to the first reports in the Italian peninsula (Sannino and Espinosa, 2009; Viggiani et al., 2009).

Although tomato appear to be the preferred host, *T. absoluta* can feed, develop and reproduce on other species, mainly Solanaceae, as also on wild plants, such as *Solanum nigrum* L., *Datura stramonium* L., *Lycium chinense* Mill., and *Malva* sp. (Vargas, 1970; Campos, 1976; Pereyra and Sánchez, 2006; Tropea Garzia et al., 2009; Desneux et al., 2010). The insect can attack tomato plants at any phenological stage on all epigeal parts (Figure 4.1), and the main damage is caused by larvae that penetrate leaves, stems and fruits, creating mines and galleries (Desneux et al., 2010). In tomato leaves, damages are caused through mine-formation within the mesophyll by feeding larvae, thus affecting the plant's photosynthetic capacity and consequently lowering tomato yield (Bogorni et al., 2003). Furthermore, the insect can damage the plant apex, thereby halting plant development (Desneux et al., 2010). Attacks on both unripe and ripe fruits can cause yield losses of up to 80-100% when no control measures are used (Apablaza, 1992; López, 1991; Gabarra et al., 2014). Moreover, attacked tissues are more vulnerable to secondary diseases (Tropea Garzia et al., 2012). If food is available and climatic conditions are favourable, larvae feed almost continuously and generally do not enter diapause (Tropea Garzia et al., 2012) and under Mediterranean conditions, adults of *T. absoluta* can be detected all around the year (Desneux et al., 2010).

Control of tomato leafminer infestations is difficult, because of the endophytic habit of larvae (Cocco et al., 2013). The primary *T. absoluta* control strategy in most South American countries and in all newly infested countries is chemical control through the use of insecticides (Lietti et al., 2005; Bielza, 2010), harmful to both man and the environment (Picanço et al., 1998). The repeated use of chemicals can also negatively affect beneficial insects which provide biological control to several other tomato pests (Cocco et al., 2013) as well as a decreasing activity of some insecticides used against *T. absoluta* (Lietti et al.,

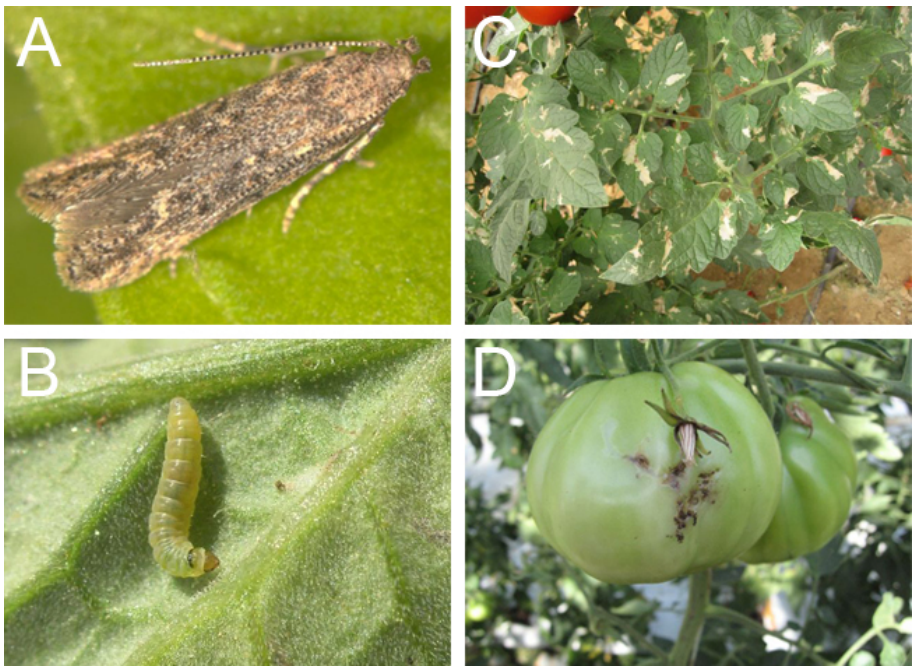

Figure 4.1: Adult (A) and larval (B) stage of *Tuta absoluta*. The larva damages mainly leaves (C) and fruits (D). Source: http://photos.eppo.int.

2005). During the past few years, the cost of tomato production has increased as a consequence of the necessity of developing and applying new pest control and monitoring strategies (Tropea Garzia et al., 2012), such as the use of natural enemies (Urbaneja et al., 2012; Gabarra et al., 2014) and of synthetic pheromone traps (Cocco et al., 2013; Mahmoud et al., 2014).

The use of resistant genotypes would be an alternative to chemical control. The study of the mechanisms and causes of resistance to *T. absoluta* is fundamental for the determination of the resistance factors necessary to establish plant breeding programmes for insect resistance and to provide objective parameters for the crosses (Eigenbrode et al., 1996). The causes associated with resistance may be trichomes, chemical compounds and/or physical morphology (Panda et al., 1995). There are three mechanisms that may be involved in the resistance of *Solanum* spp. to arthropod pests: antixenosis, antibiosis and tolerance. Tolerance is more or less independent from the effect on the insect, because it reflects the degree to which a plant can regrow and reproduce after damage from herbivores (Painter, 1951; Strauss and Agrawal, 1999). Antixenosis determines insects showing less preference for oviposition, food or shelter due to chemical, morphological and/or physical stimuli provided by plants, which are governed by genetic factors that cause different responses in insects (Painter, 1951; Panda et al., 1995; Antônio et al., 2011). Oliveira et al. (2009) observed that the HGB-1497 accession of *S. lycopersicum* presented resistance by antixenosis to *T. absoluta*. One more cause of resistance to insect-pests in tomato is the mechanical action of glandular trichomes (types IV and VI) due to their density, position, length and shape, that may affect feeding, locomotion, oviposition and sheltering of insect-pests (Norris and Kogan, 1980; Channarayappa et al., 1992; Nihoul et al., 1994; Lucatti et al., 2013). Antibiosis, in tomato, is mainly determined by the synthesis and accumulation of acyl sugars that takes place within the glandular head of the trichome (Schilmiller et al., 2012; Resende et al., 2006; Hartman and St Clair, 1999). Acylsugars are non- specific resistance components providing resistance to a broad spectrum of insects of different feeding guilds

(whiteflies, aphids, leaf miners, caterpillars, etc.) (Glas et al., 2012). Some studies suggest that a high level of acylsugars and the presence of glandular trichomes type IV are needed to achieve an effective level of resistance and a fully resistant phenotype (Resende et al., 2006; Lucatti et al., 2013).

Resistant accessions with antibiotic or antixenotic effects have considerable potential for slowing the growth rates of pest populations (Kennedy et al., 1987). Antixenosis would be more desirable because it curtails initial insect colonization and reduces the photosynthetic losses caused by adult feeding (Trumble et al., 1985; Selvanarayanan and Narayanasamy, 2004). However, as *T. absoluta* is polyphagous with several secondary crop hosts, an antibiosis-based resistance for the larvae may provide better regional population reductions than antixenosis approaches, which could favour destructive intercrop movements, especially in open fields (Trumble and Quiros, 1988; Le Roux et al., 2008).

The potential of wild species as a source of genetic variation to bring about crop improvement was recognized early in the twentieth century (Bessey, 1906). Some wild species, such as *Solanum habrochaites* (Leite et al., 2001), *Solanum pennellii* (França et al., 1988; Resende et al., 2006) and *Solanum peruvianum* (Suinaga et al., 1999, 2004) are known to be resistant to this pest. However, undesirable characteristics and/or incompatibility of these species hinder transfer of resistance factors to commercial tomato (Oliveira et al., 2009). In fact, the use of these exotic genetic resources in breeding programs was a time-consuming and laborious process that often ended in failure. The transfer of traits from poorly adapted germplasm that carries many undesirable genes into elite lines required many backcrosses, an efficient selection procedure and much luck (Zamir, 2001). One of the difficulties in obtaining a resistant variety is the low genetic variability that often prevents a breeding program (Fernandes et al., 2012), although genetic variation is the engine that propels breeding (Zamir, 2001). To date, there is no known cultivated tomato variety resistant to *T. absoluta*. This fact could be associated with reduced genetic variability introduced during tomato domestication, leading to the loss of genes that control the production of allelochemicals involved in plant defenses (Oliveira et al., 2009). The genetic variability can be obtained by sources of resistance that are present mainly in germplasm banks (Oliveira et al., 2009). According to Panda et al. (1995) one of the ways to get genetic variability is to get plants from different regions. Landraces still present a unique source of specific traits for diseases and pest resistance (Frankel, 1995; Duvick, 1999).

Very few studies focused on the study of antixenotic resistance in the context of tomato landraces (Gharekhani and Salek-Ebrahimi, 2014a). Moreover, to the best of our knowledge all the available studies were conducted at foliar level (Ecole et al., 2001; Oliveira et al., 2009; Gharekhani and Salek-Ebrahimi, 2014a). Thus, the aim of this study was to screen a collection of 126 cultivated tomato accessions of diverse geographical origins (115 landraces and 11 vintage cultivars) for antixenotic (non-preference) resistance against *T. absoluta*, and compare them to 5 wild genotypes. We tested our collection either under open-field and greenhouse conditions and we afforded this task either at foliar and fruit level.

4.2 Materials and methods

4.2.1 Plant material

The investigated plant material consisted of a collection of 126 cultivated tomato (*Solanum lycopersicum* L.). Moreover some wild accessions were also included for comparison.

The cultivated tomato collection included 64 tomato landraces from Sardinia, 7 from the Italian peninsula and 44 from the rest of the world, as well as 11 cultivars. Seeds of Sardinian landraces are stored at the 'Centro per la Conservazione e Valorizzazione della Biodiversità Vegetale', University of Sassari, Italy (Attene and Rodriguez, 2008). The landraces from the Italian peninsula and the wild species *Solanum lycopersicum* var. *cerasiforme* and *Solanum pimpinellifolium* were provided by prof. Andrea Mazzucato, University of Viterbo, Italy. The sample of accessions of worldwide geographical origin was provided by the Centre for Genetic Resources, Wageningen University, The Netherlands. The accession LA1777 of *Solanum habrochaites* (Monforte and Tanksley, 2000) and an accession of *Solanum pennellii* were provided by the C.M. Rick Tomato Genetics Resource Center (TGRC), University of California, Davis, USA.

Previous molecular analysis conducted using micro-satellite (SSR) markers have shown that the Sardinian collections includes an accession named 'Cocktail' which has a genetic profile that is very close to the wild-species *S. pimpinellifolium* (data not shown). For this reason for all the statistical analyses, we grouped this accession with the wild ones.

4.2.2 Experimental trials

Accessions were studied in two experimental trials, one in 2012 and one in 2013. In 2012, the trial was carried out under open-field conditions at Oristano, Sardinia. The experimental layout was a randomized complete block design (RCBD) with five replicates. One-hundred-twenty four tomato genotypes were investigated and four plants per genotype were considered within each block. The field was arranged with eight mulched double rows, with a distance of 0.9 m between each double row, 0.6 m between the rows of the same double row and with plants spaced 0.4 m apart within the row. Transplantation was done by hand the 5^{th} of June. To reduce environmental effects, a modern tomato variety was transplanted around the border of the field. All plants were staked by reeds and pruned to one stem, excepting genotypes with a determined growth type. The apex of the individuals with undetermined growth type was trimmed when the plants reached the height of ca. 1.8 m. The management of the crop followed standard agronomic practices. The trial ended in September, when all fruits were harvested from all of the accessions.

In 2013, 114 landraces and 11 commercial varieties were analyzed at Oristano, in the same location than in 2012, but under greenhouse conditions. The trial adopted was a RCBD with three replicates, with one plant per replicate. The field was arranged with three mulched double rows, one for each replicate, with a distance of 1.2 m between each double row, 0.4 m between the rows of the same double row and 0.4 m between plants of the same row. Transplanting was done by hand at the 25^{th} of January 2013. As

for the 2012 trial, to reduce environmental effects a modern tomato variety was transplanted around the border of the field. All plants were staked by cords and pruned to one stem, excepting genotypes with a determined growth type. The apex of the individuals with undetermined growth type was trimmed when the plants reached the height of ca. 2.0 m. Standard agronomic practices were used. The trials ended in July when all fruits were harvested. Most of the cultivated accessions (123 out of 130) were shared by the two trials in 2012 and 2013. Moreover, chemical control of pest was not applied, allowing plants to grow under natural infestation of *Tuta absoluta*.

The list of the collections and the number of accessions studied during each experimental trial is reported in Table 4.1. Details about accession name, group and origin are given in Table A.1.

Table 4.1: Number of accessions for each group in 2012 and 2013.

Collection	Collection code	2012	2013
Sardinian landraces	L-SAR	61	64
Italian landraces	L-IT	6	7
Exotic landraces	L-EXOT	44	43
Cultivars	C	10	11
Wild-related species	W	3	5
Total accessions		124	130

4.2.3 Phenotypic characterization

The resistance by antixenosis to *Tuta absoluta* was measured at different stages and on different organs of the plant.

The degree of attack was first determined when plants were about 30 cm high (about 15 days after transplantation, d.a.t.) by counting the number of leaves (PL) and the total number of mines per plant (PM) in order to calculate the mean number of mines per leaf (M/L = PM/PL). At this stage, we also checked if the meristematic apex of each plant was damaged or not (DA).

After that, the attack on the leaves in three different stages was evaluated. First, the number of mines were counted on a basal leaf chosen randomly (ML1) 45 d.a.t. The same scoring was then repeated 60 da.t. considering a randomly chosen middle leaf (ML2) and 80 d.a.t. on an apical leaf (ML3). Furthermore, the mines registered 60 d.a.t. were further classified into big mines (BM; length $>$ 0.5 cm) and small mines (SM; length $<$ 0.5 cm) (Picanço et al., 1995) to calculate the ratio big/small mines (B/S = BM/SM). The synthetic variable 'total number of mines' on the three leaves (TML = ML1+ML2+ML3) was derived as well.

Morphological data were also collected to describe the main features of the foliar architecture of tomato plant: leaf length (LLE), leaf width (LWI), leaf length/width ratio (LL/W) and foliage density (FD). Details about these traits are reported in section 2.2.2 on page 22. A new trait was introduced and called 'Leaf type' (LT) for which nine states are possible (Figure 4.2).

The insect attack was also determined on the fruits. The tomato accessions showed a wide range of fruit sizes (see Chapter 2) and, for a given fruit size, genotypes can differ for the number of fruit produced and, as a consequence, for their yield. Thus, in the absence of variation for antixenosis among genotypes it should be expected that the number of mines on the fruit of a plant will depend by the total fruit surface

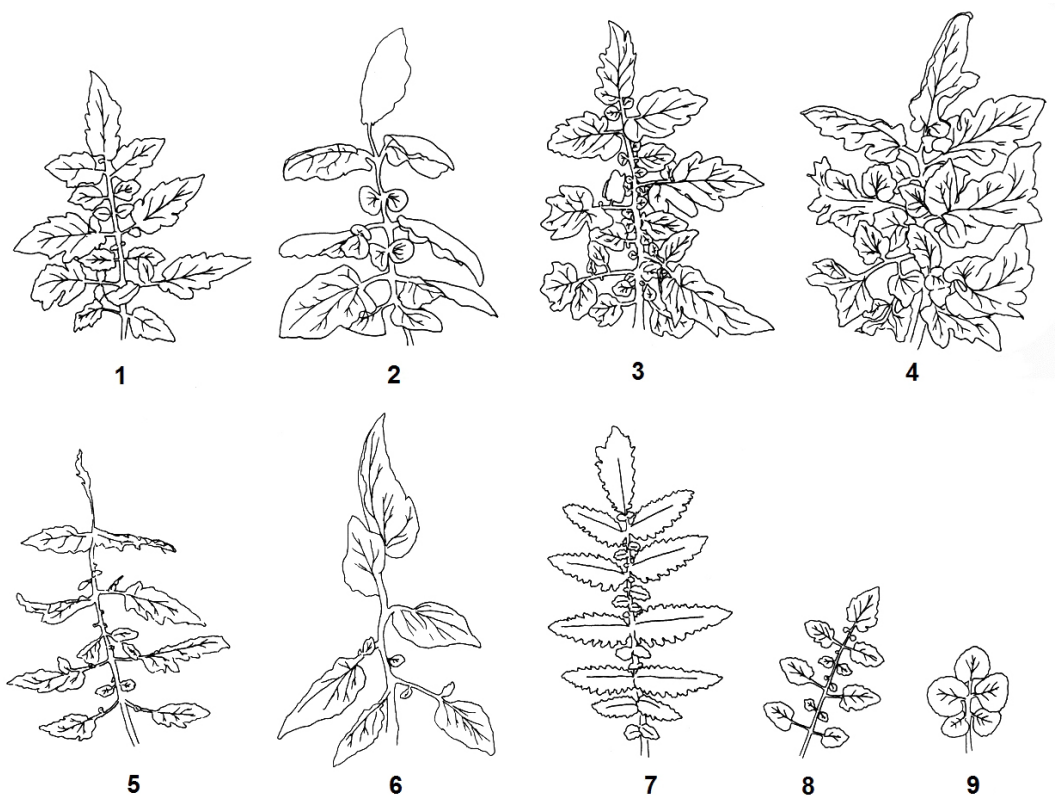

Figure 4.2: Different variants of leaf type trait (LT).

area. For this reason, the insect attack was indexed as mine density on the fruit (mines/cm^2). Thus, the number of fruits produced by each plant (NF) was counted and the average weight of a fruit (FW) was calculated; then, the number of larval holes in the tomato peel or under the calyx were determined. The total number of mines on the fruits of a plant (FM) was calculated. The density of mines on the fruit surface (MDF, mines/cm^2) was estimated as FM/FA, where FA is the total of fruit surface area (cm^2) of a plant. The (approximate) FA was derived from the average fruit weight (FW) and the number of (NF) fruits of a plant, assuming the same density and a spherical shape for all fruits.

The parameters scored in each trial are shown in Table 4.2.

4.2.4 Statistical analysis

For the foliar attack (TML) separated analyses were performed for the 2 years (i.e., for the two different growing conditions, open field in 2012 and greenhouse in 2013), as a standard F test for unequal variance was significant (see results). On the contrary, the same test indicated unequal variance for mine density on the fruit (MDF; see results); for this trait we then performed the analysis also combining the two years. Thus for MDF we first adopted the following model year, genotype, year × genotype and block within year. This analysis was performed considering only the accessions that were shared among the two trials. All factors year, genotype, and block were considered as random factors; indeed we considered the two conditions (open field and greenhouse) representative of the most frequent environmental conditions under which the tomato crop is cultivated; the cultivars utilized were also considered a representative sample of the tomato landraces.

Table 4.2: List of traits evaluated for each trial in 2012 and 2013.

Trait	Code	Type[a]	2012	2013
ANTIXENOSIS				
Mean number of mines per leaf (young plant)	M/L	QNT	✓	
Damaged apex (%)	DA	QNT	✓	
Mines on basal leaf	ML1	QNT	✓	✓
Mines on middle leaf	ML2	QNT	✓	✓
Mines on apical leaf	ML3	QNT	✓	✓
Sum of mines on leaves	TML	QNT	✓	✓
Mines density (mines/cm^2)	MDF	QNT	✓	✓
Big mines/small mines	B/S	QNT	✓	✓
MORPHOLOGICAL				
Leaf length (cm)	LLE	QNT	✓	✓
Leaf widht (cm)	LWI	QNT	✓	✓
Leaf length/width	LL/W	QNT	✓	✓
Foliage density	FD	QLT	✓	✓
Leaf type	LT	QLT	✓	✓
Mean fruit weight (g)	FW	QNT	✓	✓

[a] QLT = qualitative trait, QNT = quantitative trait.

When the analyses were performed each year separately, the model adopted was performed according to the RCBD used, i.e. with the factors genotypes and block. Also in this case the terms were considered random. Variance component estimates associated to each term were obtained through the restricted maximum likelihood method (REML) procedure implemented in JMP 10.0.0 software (SAS Institute, Inc. 2012). Variance components were considered "significant" when their confidence intervals did not assume zero or negative values. Variance components were also used to estimate the broad sense heritability (H^2). In the combined dataset heritability H^2 was estimated as $\sigma^2_G/(\sigma^2_G + \sigma^2_G \times Y + \sigma^2_e)$ where σ^2_G is the genotypic variance; $\sigma^2_G \times Y$ is the variance of the G × Y interaction and σ^2_e is the error variance. In the single-year analysis $H^2 = \sigma^2_G/(\sigma^2_G + \sigma^2_e)$. When nested model of analysis of variance was used, the variance was further partitioned into between groups (σ^2_B), and within groups components. Multiple comparison among cultivar means were conducted adopting the Tukey-Kramer HD test.

Associations among variables were quantified by Pearson's 'r' correlation coefficients or by Spearman's ρ non-parametric correlation. All statistical analyses were performed with JMP 10.0.0.

4.3 Results

As there are only two groups (2012 and 2013), then a standard F-test for unequal variances was performed. This resulted highly significant ($P < 0.0001$). Then the analysis was continued considering the two trials separately.

4.3.1 Year 2012 - Open-field conditions

In 2012, the average TML (7.91) was almost 10 fold lower than in 2013 (77.60) and this difference is highly significant (t-test allowing standard deviation not equal: $P < 0.0001$).

The results of REML analysis for the trials conducted in 2012 is presented in Table 4.3.

The percentage (%) of damaged apex (DA) was significantly different among genotypes and the

heritability (H^2) was 0.26. Successively, significant differences were also detected among young plants where the attack was measured as average number of mines per leaf at whole plant level (M/L). In this latter case, H^2 is strongly reduced ($H^2 = 0.118$); however, the DA was significantly correlated to M/L (Pearson's $r = 0.426$, $P < 0.0001$; Spearman's $\rho = 0.422$, $P < 0.0001$).

When considering adult plants, foliar attack (TML) decreased over time, with attack 40 d.a.t. (ML1) higher than 60 d.a.t. (ML2) and 80 d.a.t. (ML3) (Table 4.3). These differences among sampling times were significant ($P < 0.05$).

In 2012, no significant variation for TML was detected (Table 4.3). REML analysis was not applied for the three sampling times separately.

Table 4.3: Differences among accessions of cultivated tomato for antixenosis at foliar level in 2012 and based on REML procedure. The trait DA was square root transformed prior the REML analysis.

Trait[a]	Mean	SD	Min	Max	C.I. 95%		% of the total			H^2
					Lower	Upper	Genotype	Block	Error	
DA (%)	19.8	19.9	0	75	0.0005	0.0516	26.04	-0.78	74.74	0.260
M/L	1.03	0.29	0.40	1.9	0.0102	0.0547	8.92	21.90	69.17	0.118
TML	7.91	3.59	1	18	-1.6748	5.3741	6.41	2.37	91.22	0.066
ML1	3.66 a	3.38	0	26	nd	nd	nd	nd	nd	nd
ML2	2.50 b	2.53	0	16	nd	nd	nd	nd	nd	nd
ML3	1.50 c	2.07	0	14	nd	nd	nd	nd	nd	nd

SD = standard deviation; Min = minimum value; Max = maximum value; C.I. 95% = confidence interval (95%) for the genotype variance component; % of total = variance components as % of the total variance; H^2 = broad sense heritability.

nd = not determined because of the low of variability and the convergence questionable.

Sampling times not connected by same letter are significantly different ($P < 0.05$) based on Friedman's rank test.

[a] DA, damaged apex; M/L, mean number of mines per leaf (young plant); TML, sum of mines on leaves; ML1, mines on basal leaf; ML2, mines on middle leaf; ML3, mines on apical leaf.

4.3.2 Year 2013 - Greenhouse conditions

In this trial, DA and M/L trait were not collected and the analysis is focused on adult plants. In this case, significant differences among genotypes for the trait TML were found as also together with an interesting heritability value ($H^2 = 0.37$). Moreover, to the opposite than in 2012, the foliar attack increased with time; in fact the number of mines 40 d.a.t. (ML1) was lower than 60 d.a.t. (ML2) and than 80 d.a.t. (ML3) (Table 4.4). On average, the number of mines on the leaves was significantly different among the three sampling times.

The genotypic variance component was not significant for the first sampling time (ML1) but became significant in ML2 and ML3. Broad sense heritability (H^2) increased over time (0.084, 0.269, and 0.300, for ML1, ML2 and ML3, respectively) thus paralleling the increase in the number of mines on the leaves. Also TML had a higher H^2 compared to the single sampling times (Table 4.4).

When considering the top five genotypes for TML (Table 4.5), two were from the Italian peninsula and three from Sardinia. In ascending order for number of mines they are 'Grande costoluto', 'Tamatta', 'Tommatis mannu', 'A peperone', and 'Pantano romanesco'. On the other tail of the distribution, five genotypes showed a much lower number of mines on the leaves and they were in ascending order 'Rybka', 'Kujawski', 'Tramatta 'e prutone', 'Sant'Isidoro 2', and 'cv VFNT'. Among these cultivars, two were from all around the world (Rybka and Kujawski) and two from Sardinia (Tramatta 'e prutone and Sant'Isidoro

Table 4.4: Differences among accessions of cultivated tomato for antixenosis at foliar level in 2013 and based on REML procedure. In this trial, the traits DA and M/L were not scored.

Trait[a]	Mean	SD	Min	Max	C.I. 95%		% of the total			H^2
					Lower	Upper	Genotype	Block	Error	
TML	77.61	27.12	16.33	144.67	268.69	657.38	35.69	3.57	60.74	0.370
ML1	5.33 a	4.02	0	23	-1.26	8.21	8.25	2.29	89.46	0.084
ML2	27.93 b	10.94	6.67	55.33	29.56	94.24	25.49	5.07	69.44	0.269
ML3	44.34 c	18.56	6.33	117	99.37	284.50	29.87	0.55	69.58	0.300

SD = standard deviation; C.I. 95% = confidence interval (95%) for the genotype variance component; % of total = variance components as % of the total variance; H^2 = broad sense heritability.

Sampling times not connected by same letter are significantly different ($P < 0.05$) based on Friedman's rank test.

[a] TML, sum of mines on leaves; ML1, mines on basal leaf; ML2, mines on middle leaf; ML3, mines on apical leaf.

2). Figure 4.3 shows some features of the foliar architecture of all of these 10 genotypes and suggests that these features (particularly leaf size) must be taken into account when comparing diverse tomatoes for antixenotic resistance.

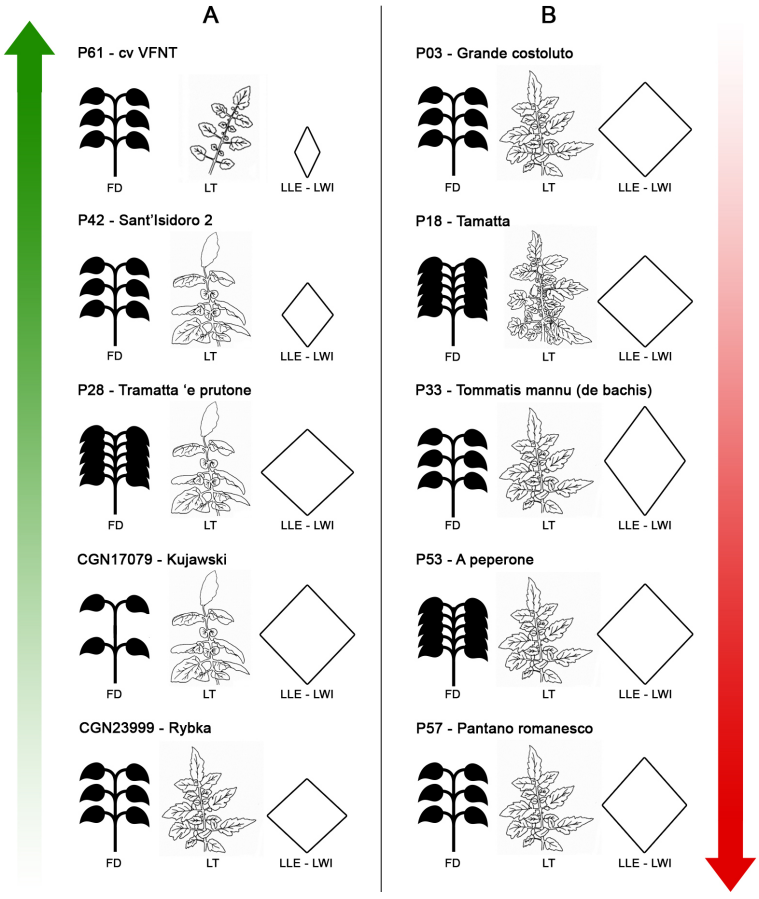

Figure 4.3: Characteristics of the genotypes falling in the opposite tails (A, more resistant; B, more susceptible) of the distribution of the total number of mines on the leaves (TML). Note: FD, foliage density; LT, leaf type; LLE, leaf length; LWI, leaf width.

Consequently, the correlations between TML and leaf size traits was calculated. The best correlation was between TML and the leaf length (LLE), followed by leaf area (LA) and leaf width (LWI). No significant correlation was observed between TML and the leaf length/width ratio (LL/W) (Table 4.6). The variable LLE, LWI and LA were strongly correlated among each other (Table 4.7) and the stepwise multiple regression evidenced that if one of them enter in multiple regression model, no other variables can be further added to explain TML (Table 4.8). This indicates that these three variables are 'redundant'.

Table 4.5: Rank of the tomato cultivars based on the least square means (LSM) for the total number of mines on the leaf (TML). Levels not connected by same letter are significantly different ($P < 0.05$) based on Tukey-Kramer HD test.

Accession		LSM	Accession		LSM
Pantano romanesco	A	144.67	Butirra (a pera)	A B C D E F	77.67
Tommatis mannu (de bachis)	A B	133.33	Gondol	A B C D E F	77.00
A peperone	A B	132.33	Ammelasa ammelasa	A B C D E F	77.00
Tamatta	A B C	125.00	Local	A B C D E F	76.67
Grande costoluto	A B C D	121.33	Tolna Megyei TF.	A B C D E F	76.00
Tramatta tunda B	A B C D	120.33	San Marzano Lampadina	A B C D E F	75.33
Skopski Jabucar	A B C D	119.67	Cuor di bue di Albenga	A B C D E F	74.33
Pomodoro genovese	A B C D	118.67	Tondo	A B C D E F	73.67
Tamatta sarda	A B C D E	118.33	Costoluto fiorentino	A B C D E F	71.00
Pumatta antiga	A B C D E F	115.00	Eterogeneo	A B C D E F	70.67
Zaghebacka Kasna	A B C D E F	114.00	Tramatta tunda A	A B C D E F	70.33
I 6137	A B C D E F	113.33	Tonda	A B C D E F	70.33
Appimpirilloddi	A B C D E F	112.00	Eterogeneo	A B C D E F	70.33
Tramatta tunda C	A B C D E F	109.67	MI 1/81	A B C D E F	69.33
Tamatta a forma di cachi	A B C D E F	107.67	Acme	A B C D E F	69.33
Cuore di bue piriciola	A B C D E F	106.67	Nepal 32	A B C D E F	67.67
Bakonycsernye 1	A B C D E F	105.33	MII 1/81	A B C D E F	66.33
Tamatta	A B C D E F	105.33	Tondo	A B C D E F	65.33
Local	A B C D E F	105.00	Cor'e boi	A B C D E F	63.67
Hu Shi Zi Feng Ding	A B C D E F	104.33	Costoluto	A B C D E F	63.67
Local tomato	A B C D E F	104.00	Grosso appiattito costoluto	A B C D E F	63.67
Plum (local)	A B C D E F	103.67	Limachino	A B C D E F	63.00
I 6582	A B C D E F	102.00	Yellow Pear	A B C D E F	62.33
Arracadas	A B C D E F	102.00	Domates Edremit	A B C D E F	60.67
Tamatta sarda rosa	A B C D E F	101.33	San Marzano tipo Lampadina	A B C D E F	60.67
Lider 165	A B C D E F	100.33	Sant'Isidoro 3	A B C D E F	60.00
Cuore di bue	A B C D E F	99.33	Pruno II	A B C D E F	59.76
PI 114969	A B C D E F	98.67	Tamatta siccada 2	A B C D E F	58.67
Congolese Tomaat	A B C D E F	98.00	PI 127824	A B C D E F	58.67
Cachi	A B C D E F	97.33	Portocala	A B C D E F	57.67
Super Precoce Marmande	A B C D E F	97.00	Broccolittu da mensa	A B C D E F	56.33
Tamatta tipo san marzano	A B C D E F	97.00	Pumatta tipu cirio	A B C D E F	56.33
Balady	A B C D E F	95.76	Rio Grande	A B C D E F	56.00
Cuore di bue	A B C D E F	94.00	Scatolone di Bolsena	A B C D E F	56.00
Tamatta cor'e boi	A B C D E F	93.67	Tamatta kaki	A B C D E F	55.67
Cuore di bue	A B C D E F	93.67	Grosso appiattito costoluto	A B C D E F	54.33
Tramatta marmande	A B C D E F	91.67	Tramatta tunda	A B C D E F	53.33
Grosso appiattito costoluto	A B C D E F	91.67	Tamatta sarda	A B C D E F	51.00
Trematta	A B C D E F	90.33	Lorigheddas de appiccai	A B C D E F	50.67
Tamatta siccada	A B C D E F	90.33	I 6604	A B C D E F	49.00
Cor'e boi	A B C D E F	89.33	Cor'e boi	A B C D E F	49.00
Sant'Isidoro 1	A B C D E F	88.67	Campu perdu	A B C D E F	48.33
Local	A B C D E F	88.00	cv M82	A B C D E F	47.83
Tamatta sarda	A B C D E F	87.67	5	A B C D E F	47.67
Tondo liscio piccolo	A B C D E F	87.33	PI 118778	A B C D E F	47.33
Cor'e' boi afriscilonada	A B C D E F	86.33	Precoce	A B C D E F	44.67
Local	A B C D E F	85.33	Lorighittas	A B C D E F	44.00
Pyongyang Bun Hong	A B C D E F	85.00	Ostravske Rane	B C D E F	42.00
Tramatticasa tundasa a siccu	A B C D E F	85.00	Cor'e boi	B C D E F	40.00
I 7113	A B C D E F	84.33	I 6121	B C D E F	38.33
Ganti	A B C D E F	82.33	Piccolo marmande	C D E F	29.67
Grosso appiattito costoluto	A B C D E F	82.33	cv Chico III	C D E F	29.33
Local	A B C D E F	82.00	Ovale	C D E F	28.67
SM scatolato	A B C D E F	82.00	Rybka	C D E F	27.33
Principe Borghese	A B C D E F	80.67	Kujawski	C D E F	26.67
cv Ailsa Craig	A B C D E F	78.33	Tramatta 'e prutone	D E F	21.00
Chvatikovo Uslechtile	A B C D E F	78.00	Sant'Isidoro 2	E F	16.67
Costoluto Genovese	A B C D E F	78.00	cv VFNT	F	16.33
Cuore di Bue	A B C D E F	77.67			

Table 4.6: Parametric and non-parametric correlations between total number of mines on the leaves of a plant (NTML) and leaf size (LLE, leaf length; LWI, leaf width; LA, leaf area) and shape (LL/W, leaf length/width).

Variable	Count	Pearson's r		Spearman's ρ	
LLE	117	0.317	***	0.274	**
LWI	117	0.274	**	0.253	**
LL/W	117	-0.105		-0.011	
LA	117	0.298	**	0.268	**

Table 4.7: Correlations among leaf size traits. Outside parentheses: Pearson's r coefficient; among parentheses: Spearman's ρ coefficient.

Trait[a]	LLE	LWI	LA	LL/W
LLE	-			
LWI	0.811 (0.770)	-		
LA	0.929 (0.917)	0.959 (0.955)	-	
LL/W	-0.173 (-0.054)	-0.691 (-0.631)	-0.470 (-0.401)	-

[a] LLE, leaf length; LWI, leaf width; LA, leaf area; LL/W, leaf length/width.

Table 4.8: Results of the stepwise multiple regression with TML as dependent variable and when LLE or LA or LWI is entered as independent variable.

Parameter	Estimate	DF	SS	F	
Entering LLE					
Intercept	-6.81086	1	0	0	
LLE	2.480135	1	8557.2	12.82	***
LWI	0	1	73.91	0.11	
LL/W	0	1	221.8313	0.331	
LA	0	1	9.358418	0.014	
Entering LA					
Intercept	41.3399	1	0	0	
LLE	0	1	987.61	1.47	
LWI	0	1	143.82	0.21	
LL/W	0	1	132.73	0.2	
LA	0.032926	1	7579.01	11.21	**
Entering LWI					
Intercept	23.43043	1	0	0	
LLE	0	1	2218.65	3.3	
LWI	1.697736	1	6412.54	9.35	**
LL/W	0	1	1159.28	1.70	
LA	0	1	1310.28	1.93	

[a] LLE, leaf length; LWI, leaf width; LA, leaf area; LL/W, leaf length/width.

Based on these results the number of mines on the leaves (TML) was standardized by dividing for the area of a rhombus calculated as (LLE × LLW)/2. It was assumed that this area was proportional to the real leaf area. This has allowed to roughly estimate the mines density on the leaves (MDL, mines/cm^2). In particular, MDL calculated in this way likely approximates by defect the real density, but it can be useful for comparative purposes. LA was used instead of LLE as mines/cm and it allows the comparison with the mines density on the fruit (see here after). Moreover, the ranks based on the TML/LLE and the TML/LA ratios were very similar as they were determined by the very strong rank correlation between LLE and LA (Spearman's $\rho = 0.917$).

Secondly, it was investigated the relationship between MDL and leaf type (LT) and MDL and foliar density (FD) (Table 4.9). The variance of MDL was significantly explained by LT but not by FD.

Leaf type was significantly associated with MDL with about the 15% of the total variance explained. However, genotypes within LT were also significantly differentiated and they explained about 20% of the total variance. As reported in Table 4.10, MDL varied from a minimum of 0.077 for leaf type 2 up to 0.177 for leaf type 3, i.e., the MDL of the less attacked type is about the half of the most damaged type.

Table 4.9: Association between density of mines on the leaves (MDL) and foliage density (FD) or leaf type (LT). The confidence interval for the random terms were obtained by REML procedure.

Trait	C.I. 95%		% of the total
	Lower	Upper	
Foliage density (FD)			
FD	-0.0002	0.0001	-0.76
Genotypes [FD]	0.001	0.002	34.95
Block	0	0.001	3.24
Residual	0.003	0.004	62.58
Total			100
Leaf type (LT)			
LT	0.0005	0.0022	15.35
Genotypes [LT]	0.0005	0.0018	20.31
Block	-0.0002	0.0006	3.26
Residual	0.0028	0.0041	61.07
Total			100

C.I. 95% = confidence interval (95%) for the genotype variance component; % of total = variance components as % of the total variance.

Table 4.10: Effect of leaf type on leaf mines density.

Leaf type		LSM	SE
3	A	0.177	0.009
1	AB	0.158	0.004
6	ABC	0.130	0.019
5	BC	0.129	0.011
8	BC	0.112	0.034
2	C	0.095	0.009

LSM, least square means; SE, standard error. Levels not connected by same letter are significantly different ($P < 0.05$) based on Tukey-Kramer HD test.

When the components of variance are calculated, LT explains the 15% of the total variance but the variance among genotypes within LT is 20% and it is significant.

Finally, we recalculated broad sense heritability for MDL not correcting (H^2 MDL) or correcting for leaf type effect (H^2 MDL_LT; Figure 4.4). Results evidenced that, similarly to the heritability of TML, the heritability of MDL increases with the increase of the level of infestation (as measured by the number of mines on the laves, ML). Moreover , the effect of leaf type also increases during the period considered.

Figure 4.5 shows the distribution of TML and of DML for cultivated tomato (landraces and commercial varieties) in comparison with the wild species. This allowed some interesting qualitative observations.

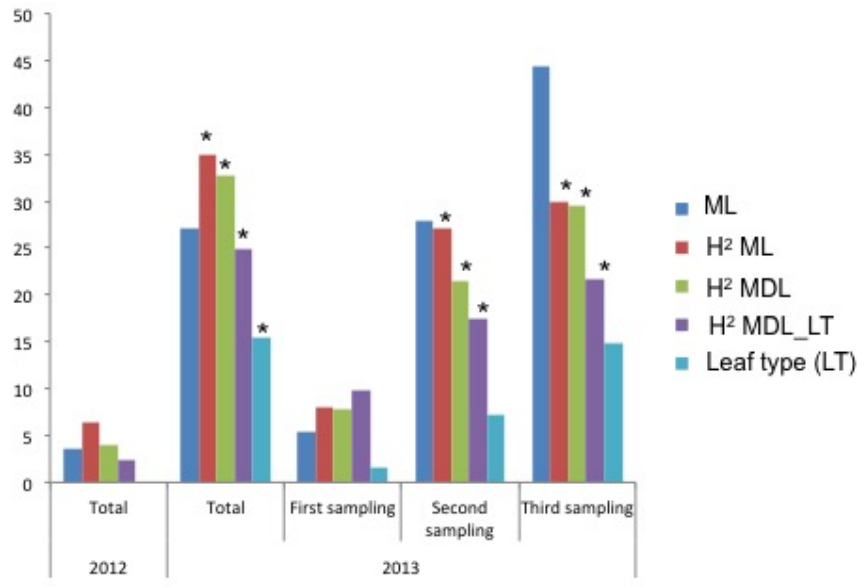

Figure 4.4: Relationship between number of mines on the leaves (ML, in total and for each sampling) and broad sense (H^2) heritability (percentage).

Overall, the wild species tend to be less attractive to *Tuta absoluta* as evidenced by the fact that the scoring for wild species was always below the median observed for cultivated materials. Moreover, differences were also detected among wild species. Indeed, *Solanum lycopersicum* var. *cerasiforme* resulted the wild species with the lowest foliar antixenotic resistance followed by *Solanum pimpinellifolium*, *Solanum habrochaites* and *Solanum pennellii* that is outside the distribution of the cultivated material. The cultivated accession 'Cocktail' that has a molecular profile similar to *S. pimpinellifolium* tends to be similar to *S. lycopersicum* var. *cerasiforme*. The first three accessions for TML were 'cv VFNT', 'Sant'Isidoro 2', and 'Tamatta'e prutone' while the first three for MDL were 'Sant'Isidoro 2', 'Tamatta'e prutone', and 'Kujawski', while 'cv VFNT' ranks thirty-first. Data indicates that some domesticated tomatoes might have interesting levels of antixenotic resistance.

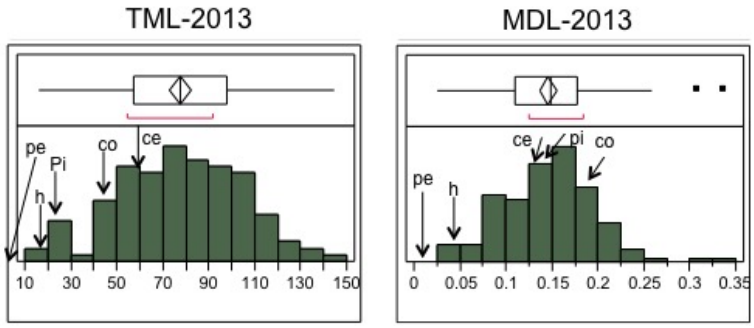

Figure 4.5: Frequency distribution for the sum of mines on leaves (TML) and the density of mines on the leaves (MDL) in 2013 among cultivated accessions. Distributions have been evaluated using accession means for the traits. The arrows indicate the values of the wild species included as references: co, Cocktail; ce, *Solanum lycopersicum* var. *cerasiforme*; pi, *Solanum pimpinellifolium*; pe, *Solanum pennellii*; h, *Solanum habrochaites*.

Either in 2012 and 2013 years, during the second sampling of leaves from adult plants, the number of big (B) and small (S) mines were counted. Significant differences among genotypes for the B/S ratio were detected in 2013 but not in 2012 (REML procedure: $H^2 = 0.160$, $P < 0.05$ and $H^2 = 6.68$, $P > 0.05$,

respectively). If the relative number of big and small mines is measuring a combination of resistance by antixenosis and antibiosis, it should be expected that when the number of mines on the leaf decreases, the percentage of small mines increases. Encouragingly, this was indeed observed even if the effect, that is significant, is quite small (Pearson's r = -0.281; P < 0.0022; Spearman ρ = -0.275, P = 0.0027) (Figure 4.6).

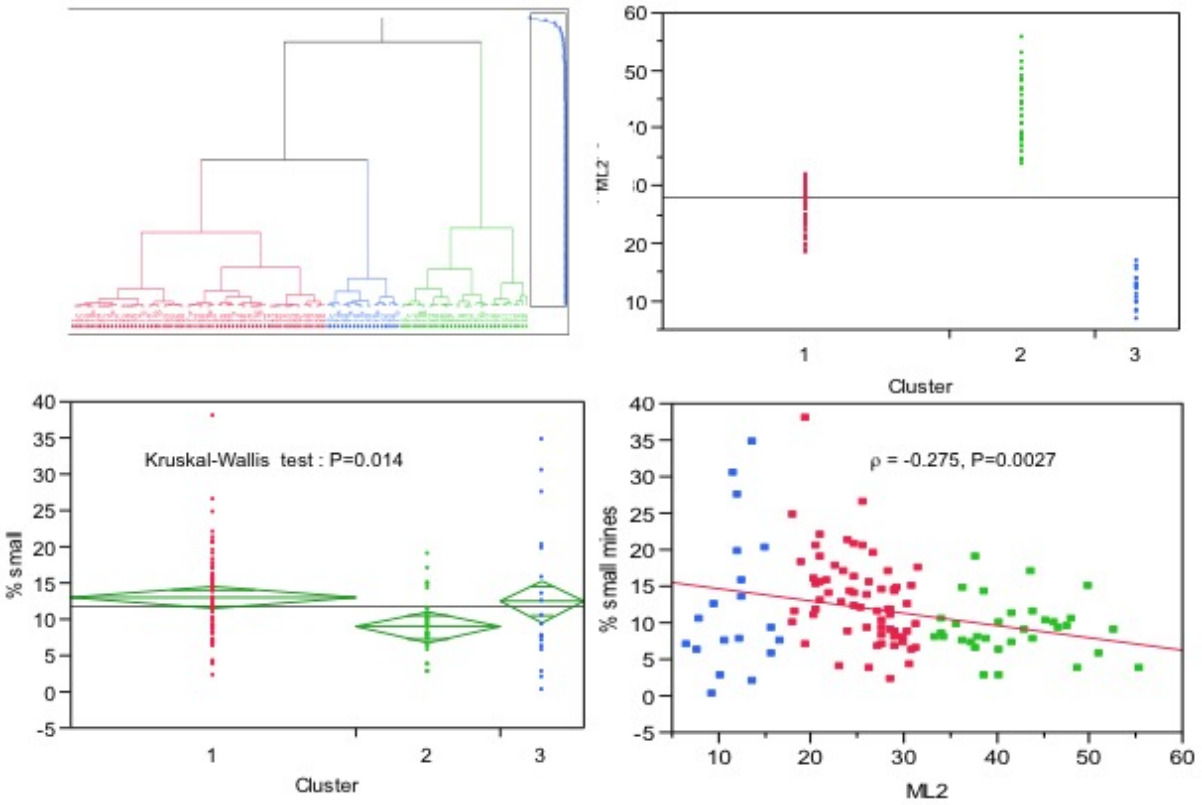

Figure 4.6: Relationship between the number of mines on the leaf (ML2-2013) and the percentage (%) of small mines on the leaves . In clockwise direction: a) dendrogram based on Ward's method to group accessions based on ML2; b) differences among clusters in the number of mines; c) differences among clusters in the % of small mines; d) correlation between % of small mines and ML2.

4.3.3 Antixenotic resistance at fruit level

In 2013, the MDF (0.023) was about 3.5 fold higher than in 2012 (0.0065) and this difference was significant (standard t-test: P < 0.0001). As the standard F test for unequal variance was not significant (P > 0.05) we first performed the statistical analysis by combining the two years. Estimation of the variance components and of their confidence intervals is presented in (Table 4.11).

It is evidenced a significant and strong effect of the growing conditions (open field versus greenhouse) on the number of mines/cm^2 with the 55% of the total variance explained. The analysis showed that genotypes significantly differ for the average level of MDF (Table 4.11) despite they explain a small part of the total variance (5.7%) giving a H^2 estimate of 0.128, that is a low value. Moreover, the interaction genotype × year was also significant with components (4.9%) is of the same magnitude of the genotypic effect (Table 4.11).

When the two years were considered separately (Table 4.12), the H^2 estimated under field conditions (2012) resulted 2.5 fold higher than that estimated under greenhouse conditions, being 0.30 and 0.13,

respectively. The correlation between the cultivar means across the two years was significant despite weak (Pearson r = 0.293, P =0.0013; Spearman ϱ = 0.212, P = 0.0103; n = 117).

Table 4.11: Results of REML procedure combining 2012 and 2013 years for the trait density of mines on the fruit (MDF).

Random effect	C.I. 95%		% of the total
	Lower	Upper	
Year	0.000244	0.0005187	55.525
Genotype	4.9161e-6	2.311e-5	5.672
Genotype × Year	2.9566e-6	2.1153e-5	4.879
Block[Year]	-9.111e-7	2.0455e-6	0.230
Residual	7.5019e-5	0.0000929	33.695
Total			100.000

C.I. 95% = confidence interval (95%) for the genotype variance component; % of total = variance components as % of the total variance.

Table 4.12: Results of REML procedure for the two years separately and for the trait density of mines on the fruit (MDF).

Trait[a]	Mean	SD	Min	Max	C.I. 95%		% of the total			H^2
					Lower	Upper	Genotype	Block	Error	
MDF_{2012}	0.006	0.006	0	0.051	0.0000176	3.9239e-5	30.826	0.623	68.551	0.310
MDF_{2013}	0.2314	0.0076	0.0066	0.0391	7.144e-7	3.2338e-5	11.432	0.405	88.162	0.115

SD = standard deviation; Min = minimum value; Max = maximum value; C.I. 95% = confidence interval (95%) for the genotype variance component; % of total = variance components as % of the total variance; H^2 = broad sense heritability.

Sampling times not connected by same letter are significantly different ($P < 0.05$) based on Friedman's rank test.

[a] MDF, fruit mines density (mines/cm^2).

The results of Tukey-Kramer HD multiple comparison test based on two-years data is presented in Table 4.13.

Two very small groups of tomato individuals that are well differentiated for the MDF were identified. One comprised two susceptible accessions (with 0.043 and 0.029 mines/cm^2) and the second included five cultivars with a low level of attack (range: 0.0040-0.0072 mines/cm^2). Thus, there is one order of magnitude of difference between the two most contrasting genotypes (0.043 versus 0.0041 mines/cm^2). The most susceptible accession was 'Local (CGN15895)', followed by 'I6121'; these are landraces from Portugal and Guatemala, respectively. The most resistant accession is a Sardinian landrace, 'Tamatta kaki', followed by four Sardinian landraces ('Tamatta sarda rosa', 'Grosso appiattito costoluto', 'Trammatta marmande', and 'Cuore di bue piriciola').

Figure 4.7 compares the distribution of the trait MDF within the sample of tomato cultivars analyzed for the two growing conditions separately. The position of the wild species used as control in the experiment is also evidenced. First, it is of note that the average susceptibility of the tested wild species was lower than the average susceptibility of the cultivated species. Second, there are differences among the tested wild species. Indeed, *S. pimpinellifolium* showed a high low susceptibility in 2012 and the highest in 2013; *S. lycopersicum* var. *cerasiforme* showed intermediate levels of attack in both years, whereas Cocktail, *S. habrochaites* and *S. pennellii* showed a high resistance level. Third, and most important, there were some cultivated accessions for which the level of resistance by antixenosis was close to the most resistant wild species.

Table 4.13: Rank of the tomato cultivars based on the least square means (LSM) for mines density on the fruits (MDF). Levels not connected by same letter are significantly different ($P < 0.05$) based on Tukey-Kramer HD test.

Accession		LSM	Accession		LSM
Local (CGN15895)	A	0.04309899	Limachino	B C D	0.0139975
I 6121	A B	0.02882361	Portocala	B C D	0.01384572
Tamatta tipo san marzano	A B C	0.02760281	Pomodoro genovese	B C D	0.0137128
Tramatta 'e prutone	A B C	0.02619861	Pumatta antiga	B C D	0.01334573
Nepal 32	A B C	0.02489263	Tramatta tunda C	B C D	0.01323692
PI 118778	A B C D	0.02470413	Tamatta cor'e boi	B C D	0.01292646
San Marzano tipo Lampadina	A B C D	0.02449519	Butirra (a pera)	B C D	0.01273198
PI 114969	A B C D	0.02431528	Pumatta tipu cirio	B C D	0.01258793
San Marzano Lampadina	A B C D	0.02274473	Cachi	B C D	0.01256981
Congolese Tomaat	A B C D	0.02250827	Lorighittas	B C D	0.01253511
Arracadas	B C D	0.02210833	Campu perdu	B C D	0.01253194
Tramatta tunda B	B C D	0.02187129	Eterogeneo	B C D	0.01250087
Costoluto fiorentino	B C D	0.02138048	Tramatta tunda	B C D	0.01243645
MI 1/81	B C D	0.02075976	cv Chico III	B C D	0.01226093
Tondo	B C D	0.0204368	Precoce	B C D	0.01220889
SM scatolato	B C D	0.02013249	Tramatticasa tundasa a siccu	B C D	0.01178981
Local	B C D	0.02004467	Ammelasa ammelasa	B C D	0.01174952
MII 1/81	B C D	0.02001378	Ganti	B C D	0.01163427
Cuor di bue di Albenga	B C D	0.01993956	cv Ailsa Craig	B C D	0.01157897
I 6604	B C D	0.01984522	Tondo liscio piccolo	B C D	0.01149869
Rybka	B C D	0.01931788	Trematta	B C D	0.01144037
Pyongyang Bun Hong	B C D	0.01919752	Cuore di bue	B C D	0.01130863
Appimpirilloddi	B C D	0.0191814	Tamatta sarda	B C D	0.01128379
Local	B C D	0.01904691	Grosso appiattito costoluto	B C D	0.01124386
Piccolo marmande	B C D	0.01904588	Cor'e boi	B C D	0.01119548
Tramatta tunda A	B C D	0.01890883	Chvatikovo Uslechtile	B C D	0.01117289
I 6582	B C D	0.0187677	Kujawski	B C D	0.01108656
I 7113	B C D	0.01864689	Hu Shi Zi Feng Ding	B C D	0.01100842
Cuore di bue	B C D	0.0186214	cv M82	B C D	0.01093171
cv VFNT	B C D	0.01773921	Cuore di bue	B C D	0.01075593
Cor'e' boi afriscilonada	B C D	0.01757344	Cor'e boi	B C D	0.01073729
Plum (local)	B C D	0.01753161	Lider 165	B C D	0.01070885
5	B C D	0.01749639	Rio Grande	B C D	0.01046602
Lorigheddas de appiccai	B C D	0.01727989	Cor'e boi	B C D	0.01025907
Cuore di Bue	B C D	0.01722302	Tamatta sarda	B C D	0.01006722
Gondol	B C D	0.01685894	Bakonycsernye 1	B C D	0.0099364
Scatolone di Bolsena	B C D	0.01683487	Local tomato	B C D	0.00993121
Costoluto	B C D	0.0168252	Balady	B C D	0.00973509
PI 127824	B C D	0.01658309	Grande costoluto	B C D	0.00970601
Zaghebacka Kasna	B C D	0.01640609	Tamatta	B C D	0.0095758
Local	B C D	0.01631245	Grosso appiattito costoluto	B C D	0.00957206
A peperone	B C D	0.01612959	Acme	B C D	0.00940284
Costoluto Genovese	B C D	0.01584931	Grosso appiattito costoluto	B C D	0.00933474
Eterogeneo	B C D	0.01567516	Pruno II	B C D	0.00930348
Broccolittu da mensa	B C D	0.01564659	Tamatta siccada 2	B C D	0.0091111
Sant'Isidoro 2	B C D	0.0156013	Cor'e boi	B C D	0.00898534
Sant'Isidoro 3	B C D	0.01545648	Tamatta siccada	B C D	0.00833318
Ostravske Rane	B C D	0.01508889	Tonda	B C D	0.00818191
Sant'Isidoro 1	B C D	0.01507783	Yellow Pear	B C D	0.00810213
Ovale	B C D	0.01504118	Tommatis mannu (de bachis)	B C D	0.00807058
Pantano romanesco	B C D	0.01502835	Domates Edremit	B C D	0.00805411
I 6137	B C D	0.01480699	Tondo	B C D	0.00797147
Principe Borghese	B C D	0.01473201	Skopski Jabucar	B C D	0.00781199
Tamatta sarda	B C D	0.01471921	Cuore di bue piriciola	C D	0.00722378
Tolna Megyei TF.	B C D	0.0146903	Tramatta marmande	C D	0.0071914
Tamatta	B C D	0.0145436	Grosso appiattito costoluto (P82)	C D	0.00657672
Super Precoce Marmande	B C D	0.01449651	Tamatta sarda rosa	C D	0.00635545
Local	B C D	0.01413787	Tamatta kaki	D	0.00406604
Tamatta a forma di cachi	B C D	0.01408979			

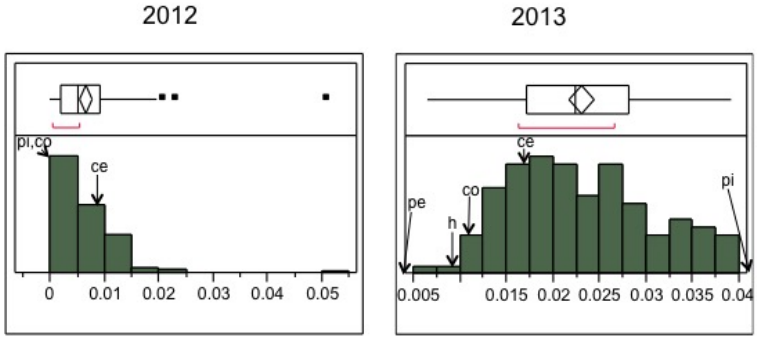

Figure 4.7: Frequency distribution for density of mines on the fruits (MDF) in 2012 and 2013 among cultivated accessions. Distributions have been evaluated using accession means for the traits. The arrows indicate the values of the wild species included as references: co, Cocktail; ce, *Solanum lycopersicum* var. *cerasiforme*; pi, *Solanum pimpinellifolium*; pe, *Solanum pennellii*; h, *Solanum habrochaites*.

4.4 Discussion

Despite *Tuta absoluta* is one of the major pests of tomato, relatively few studies have been carried out to exploit the natural variation in resistance to this insect. Moreover, these studies have been mainly concentrated in South American countries (Ecole et al., 2001; Resende et al., 2006; Oliveira et al., 2009). For this reason, the aim of the present study was to explore a wide tomato germplasm collection for resistance to *T. absoluta* by antixenosis in a typical Mediterranean environment like in Sardinia island. Moreover, to the best of our knowledge, the present study is the first that studies antixenosis resistance in *T. absoluta* also at the fruit level.

The main outcome of the present study is that the screening of germplasm collection for antixenotic resistance against *Tuta absoluta* can potentially be fruitful. Several observations coherently supported this conclusion.

The results obtained at foliar level evidenced that the possibility to detect antixenotic effect is strictly dependent upon the presence of an adequate abundance of the insect. This is indicated by a sort of "replicated evidence". Indeed, genotypic differences among adult plants emerged in 2013 under glasshouse conditions and where a stronger infestation was present, but not in 2012 under field conditions and with low infestation. Moreover, during 2013 there was a clear parallelism between the abundance of the insect and the H^2 for mine density on the leaf. While it was not possible do directly measured the abundance of the insect, Cocco et al. (2014) found that the correlations between the number of mines per leaf and the number of mines with larvae is highly significant and with high coefficient of determination, indicating that the count of mines in the field was a reliable density estimation of larvae on leaves.

Moreover, the pattern observed in 2013 can be directly related to the within plant distribution of the pest and it can be the direct consequence of the increase in oviposition preference moving from the bottom to the upper part of the canopy. Indeed, antixenosis acts mainly for oviposition whereas antibiosis prevents larval development and egg hatching (Dias et al., 2013).

Gomide et al. (2001) studied this aspect in Brazil, and observed that larvae and mines were associated with expanded leaves in the medium part of the canopy, while high egg counts tended to be associated with expanded leaves in the apical part of the plant. However, Torres et al. (2001) found that after

flowering, the first three larval stages are distributed equally between the apical and the middle parts of the plant in all phenological stages. However, other studies such as that of Leite et al. (1995) reported that *Tuta absoluta* prefers oviposition site in the median third of the canopy. Haji et al. (1988) also observed higher proportion of eggs in the apical leaves. Mature larvae, however, are distributed evenly over the entire plant, including the basal portion. In other words the insect could prefer the upper part of the canopy and among the various genotypes present to choose the "best upper part", leading to the increase of H^2 from the bottom to the top of the canopy.

Leite et al. (2001) found that *S. habrochaites* presents an increase in the leaf levels of tridecan-2-one present in leaf glandular trichomes. The higher levels of tridecan-2-one associated with older plants of *S. habrochaites*, are related to a slower larval development of *T. absoluta* when compared with insects reared on *S. lycopersicum*. It is suggested that commercial varieties of tomato originating from *S. habrochaites* may be more resistant to the leafminer since the greater attack by this insect occurred on the apical and medium parts of the plants (Leite et al., 1995; Picanço et al., 1995).

Generally, the heritability of the resistance to arthropods-pests does not show high heritability values due to the difficulty of the environmental control in an evaluation system that embodies not only the plant, but also the arthropod-pest (Resende et al., 2002). Under this scenario, an heritability around 0.30 is not trivial.

Even if overall it was not possible to evidence very strong effects, significant genotypic differences in different growing stages (young plants/adult plants) and for different organs (leaves/fruits) were found. Moreover, when factoring out the effect of the features of the plant architecture (different leaf type, size, foliar density, and yield components) differences among genotypes in the density of mines are still significant, indicating a possible role for additional less obvious traits (i.e. trichome density, production of volatile compounds, etc.) (Gilardón et al., 2001; Ecole et al., 2000).

Different studies suggest that a high number of small mines may indicate that the insect could not find an adequate food source on the host plant (Leite et al., 2001). If the relative number of big and small mines is measuring a combination of resistance by antixenosis and antibiosis, it should be expected that when the number of mines on the leaf decreases, the percentage of small mines increases. Encouragingly, this is indeed what was observed even if the effect, that is significant, is quite small. However, this is due to the fact that individuals with a high number of mines on the leaves tend to have a low percentage of small mines but it is not true that the plants with a low number of mines have the highest percentage of small mines. This could be due to the fact that when the number of mines is low the variance of the estimate of the percentage of small mines increases. Nonetheless, some authors suggest that, when considering resistance, the number of small mines should be evaluated together with the number of large mines (Ecole et al., 2001; Suinaga et al., 2004). In fact, mines which were classified as small at the time of evaluation could have been formed recently. These mines probably would evolve into larger mines with time, possibly due to the absence of deterrent compounds (Suinaga et al., 2004).

Moreover, mine density on the fruit showed a significant (despite very low) correlation years. This is however interesting as the two environments represent very different growing conditions: open field during spring to summer versus greenhouse from winter to summer, pest scarcity versus pest abundance,

'complex' versus 'simplified ecosystem', etc.

Cropping season is one of the factors that affect tomato resistance to insects (Ecole et al., 2001). Several research groups have put forward the hypothesis that the glandular trichome density and production of exudates and sesquiterpenes is affected by the cropping season, in particular high densities were observed at medium-high temperatures and long photoperiod, affecting consequently the resistance to insects (Gianfagna et al., 1992; Pérez-Estrada et al., 2000; Nihoul, 1993; Wilkens et al., 1996). Ecole et al. (2001) showed that *S. habrochaites* f. *typicum*, cultivated in a greenhouse in Brazil, was more resistant to the leafminer during the autumn/winter cropping season than during the summer, whereas the susceptibility of *S. lycopersicum* to this insect was similar in both seasons.

The resistance characteristics of wild species are better highlighted in 2013. *S. habrochaites* and *S. pennellii* showed the highest resistance. In particular, different studies suggest that the accession LA177 of *S. habrochaites* is a promising source of resistance for breeding purposes to tomato pests by antixenosis and antibiosis (Ecole et al., 2001; Weston et al., 1989; Channarayappa et al., 1992; Eigenbrode and Trumble, 1993; Eigenbrode et al., 1996; Krishna Kumar et al., 1995). Noteworthy, at foliar level accessions of cultivated tomato showed interesting level of resistance in comparison to wild species. The first three more resistant accessions for TML were 'cv VFNT', 'Sant'Isidoro 2', 'Tamatta'e prutone' while the first three for DML were 'Sant'Isidoro 2', 'Tamatta'e prutone' and 'Kujawski'. At fruit level interesting accession are 'Tamatta kaki' and 'Tamatta sarda rosa'.

Overall data indicate that some of the domesticated tomatoes investigated might have interesting level of antixenotic resistance and that the present collection of landraces and cultivars studied is suitable for the study of the resistance to this insect. Moreover this encourage the screening of even larger populations as also deeper and focused study involving the most interesting genotypes identified in this work.

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Appendices

APPENDIX A

List of accessions

Table A.1: Data of all tomato accessions studied.

Name	Code	Group[a]	Location[b]	Supplier[c]	2012	2013[d]	2013[e]
Arracadas	P01	L-SAR	Ozieri	Caragliu	✓	✓	✓
Lorighittas	P02	L-SAR	Ales	Ladoni	✓	✓	✓
Grande costoluto	P03	L-SAR	Ales	Ladoni	✓	✓	✓
Tramatticasa tundasa a siccu	P04	L-SAR	Giba	Mura	✓	✓	✓
Lorigheddas de appiccai	P05	L-SAR	Giba	Mura	✓	✓	✓
Tamatta sarda	P07	L-SAR	Gesico	Schirru	✓	✓	✓
Tamatta sarda rosa	P07R	L-SAR	Gesico	Schirru	✓	✓	✓
Tamatta siccada	P08	L-SAR	Gesico	Schirru	✓	✓	✓
Tamatta siccada 2	P08S2	L-SAR	Gesico	Schirru	✓	✓	✓
Pumatta antiga	P09	L-SAR	Laerru	Carta	✓	✓	✓
Pumatta tipu cirio	P10	L-SAR	Laerru	Carta	✓	✓	✓
Tamatta a forma di cachi	P16	L-SAR	Gavoi	Satta	✓	✓	✓
Tamatta sarda	P17S	L-SAR	Gavoi	Satta	✓	✓	✓
Cuore di Bue	P17C	L-SAR	Gavoi	Satta	✓	✓	✓
Tamatta	P18	L-SAR	Olzai	Porcu	✓	✓	✓
Appimpirilloddi	P19	L-SAR	Galtellì	Fronteddu	✓	✓	✓
Tamatta tipo san marzano	P21	L-SAR	Bonnanaro	Zamburri	✓	✓	✓
Tramatta marmande	P23	L-SAR	Bosa	Mannu	✓	✓	✓
Trematta	P25	L-SAR	Cuglieri	Casule	✓	✓	✓
Tramatta tunda	P26	L-SAR	Scano Montiferro	Cambula	✓	✓	✓
Tramatta tunda A	P27A	L-SAR	Villagrande Strisaili	Seoni	✓	✓	✓
Tramatta tunda B	P27B	L-SAR	Villagrande Strisaili	Seoni	✓	✓	✓
Tramatta tunda C	P27C	L-SAR	Villagrande Strisaili	Seoni	✓	✓	✓
Tramatta 'e prutone	P28	L-SAR	Villagrande Strisaili	Seoni	✓	✓	✓
Tamatta	P29	L-SAR	Sadali	Deplano	✓	✓	✓
Ammelasa ammelasa	P30	L-SAR	San Nicolò Gerrei	Porcu	✓	✓	✓
Cor'e boi	P31	L-SAR	San Nicolò Gerrei	Porcu	✓	✓	✓
Tamatta kaki	P32	L-SAR	San Nicolò Gerrei	Porcu	✓	✓	✓
Tommatis mannu (de bachis)	P33	L-SAR	Mamoiada	Solinas	✓	✓	✓
Cachi	P34	L-SAR	Villanova Franca	Caria	✓	✓	✓
Butirra (a pera)	P36	L-SAR	Villanova Franca	Caria	✓	✓	✓
Cor'e boi	P37	L-SAR	Villanova Franca	Caria	✓	✓	✓
Cor'e boi	P38	L-SAR	Sant'Andrea Frius	Serra	✓	✓	✓
Tonda	P39	L-SAR	Sant'Andrea Frius	Serra	✓	✓	✓
Cocktail	P40	W	Ortisei		✓	✓	✓
Sant'Isidoro 1	P41	L-SAR	Quartucciu	Piras	✓	✓	✓

[a] L-SAR = Sardinian landraces, L-IT = Italian landraces, L-EXOT = exotic landraces, C = vintage cultivars, W = wild species, IL = introgression lines.

[b] DR Congo = Democratic Republic of the Congo; Congo = Republic of the Congo; DPR Korea = Democratic People's Republic of Korea; RF = Russian Federation; USSR = Union of Sovietic Socialist Republic.

[c] VU = Viterbo University; AGRIS = AGRIS Sardegna, Agenzia per la Ricerca in Agricoltura; SMP = COOP Santa Maria la Palma; CGN = Centre for Genetic Resources, the Netherlands; TGRC = Tomato Genetics Resource Center, University of California, Davis.

[d] Trial carried out in Ottava, Sassari, Sardinia; [e] Trial carried out in Oristano, Sardinia.

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Table A.1 – *Continued from previous page*

Name	Code	Group[a]	Location[b]	Supplier[c]	2012	2013[d]	2013[e]
Sant'Isidoro 2	P42	L-SAR	Quartucciu	Piras	✓	✓	✓
Sant'Isidoro 3	P43	L-SAR	Quartucciu	Piras	✓	✓	✓
Tamatta groga de appiccai	P44	L-SAR	Escolca	Atzeni		✓	✓
5	P45	L-SAR		Mallica	✓	✓	✓
Tamatta cor'e boi	P46	L-SAR	Gonnoscodina	Pia	✓	✓	✓
Broccolittu da mensa	P47	L-SAR		AGRIS	✓	✓	✓
Cuore di bue piriciola	P48	L-SAR		AGRIS	✓	✓	✓
Cuore di bue	P49	L-SAR		AGRIS	✓	✓	✓
Cor'e boi	P50	L-SAR	Burcei	AGRIS	✓	✓	✓
Cuore di bue afesciara	P51	L-SAR		AGRIS		✓	✓
Cor'e boi afriscilonada	P52	L-SAR		AGRIS	✓	✓	✓
A peperone	P53	L-IT	Bolsena	VU	✓	✓	✓
SM scatolato	P54	L-IT	Bolsena	VU	✓	✓	✓
Costoluto fiorentino	P55	L-IT		VU	✓	✓	✓
Canestrino	P56	L-IT	Lucca	VU		✓	✓
Pantano romanesco	P57	L-IT	Bavicchi	VU	✓	✓	✓
Scatolone di Bolsena	P58	L-IT	Bolsena	VU	✓	✓	✓
Cuor di Bue di Albenga	P59	L-IT	Olter	VU	✓	✓	✓
cv Ailsa Craig	P60	C		VU	✓	✓	✓
cv VFNT	P61	C		VU	✓	✓	✓
cv Chico III	P62	C		VU	✓	✓	✓
S. lycopersicum var. *cerasiforme*	P63	W		VU	✓	✓	✓
S. pimpinellifolium	P64	W		VU	✓	✓	✓
cv M82	P65	C		VU	✓	✓	✓
Tondo	P72	L-SAR	Villanova Forru	SMP	✓	✓	✓
Tondo	P73	L-SAR	Escolca	SMP	✓	✓	✓
Costoluto	P74	L-SAR	Sestu	SMP	✓	✓	✓
Tondo	P75	L-SAR	Sorgono	SMP	✓	✓	✓
Cuore di bue	P76	L-EXOT	Corsica	VU	✓	✓	✓
Tamatta sarda	P77	L-SAR	Terraseo	Pisci	✓	✓	✓
Cuore di bue	P78	L-SAR	Alà dei Sardi		✓	✓	✓
Pomodoro genovese	P80	L-SAR		Arghittu	✓	✓	✓
Campu perdu	P81	L-SAR		Falqui	✓	✓	✓
Grosso appiattito costoluto	P82	L-SAR	Villamar	SMP	✓	✓	✓
Grosso appiattito costoluto	P83	L-SAR	Furtei	SMP	✓	✓	✓
Grosso appiattito costoluto	P84	L-SAR	Siddi	SMP	✓	✓	✓
Grosso appiattito costoluto	P85	L-SAR	Collinas	SMP	✓	✓	✓
Ovale	P86	L-SAR	Tiana	SMP	✓	✓	✓
Tondo liscio appiattito	P87	L-SAR	Ussassai	SMP		✓	✓
Piccolo marmande	P88	L-SAR	Sestu	SMP	✓	✓	✓
Eterogeneo	P89	L-SAR	Sanluri	SMP	✓	✓	✓
Tondo liscio piccolo	P90	L-SAR	Sanluri	SMP	✓	✓	✓
Eterogeneo	P91	L-SAR	Sanluri	SMP	✓	✓	✓
Rio Grande	P101	C		Ingegnoli	✓	✓	✓
San Marzano Lampadina	P102	C		Ingegnoli	✓	✓	✓
Costoluto Genovese	P103	C		Ingegnoli	✓	✓	✓

[a] L-SAR = Sardinian landraces, L-IT = Italian landraces, L-EXOT = exotic landraces, C = vintage cultivars, W = wild species, IL = introgression lines.

[b] DR Congo = Democratic Republic of the Congo; Congo = Republic of the Congo; DPR Korea = Democratic People's Republic of Korea; RF = Russian Federation; USSR = Union of Sovietic Socialist Republic.

[c] VU = Viterbo University; AGRIS = AGRIS Sardegna, Agenzia per la Ricerca in Agricoltura; SMP = COOP Santa Maria la Palma; CGN = Centre for Genetic Resources, the Netherlands; TGRC = Tomato Genetics Resource Center, University of California, Davis.

[d] Trial carried out in Ottava, Sassari, Sardinia; [e] Trial carried out in Oristano, Sardinia.

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Table A.1 – *Continued from previous page*

Name	Code	Group[a]	Location[b]	Supplier[c]	2012	2013[d]	2013[e]
Cuore di Bue	P104	C		Ingegnoli	✓	✓	✓
Super Precoce Marmande	P105	C		Ingegnoli	✓	✓	✓
Principe Borghese	P106	C		Ingegnoli	✓	✓	✓
Chvatikovo Uslechtile	CGN14408	L-EXOT	Czech Republic	CGN	✓	✓	✓
Local	CGN14459	L-EXOT	Madagascar	CGN	✓	✓	✓
Nepal 32	CGN14469	L-EXOT	Nepal	CGN	✓	✓	✓
Bakonycsernye 1	CGN15258	L-EXOT	Hungary	CGN	✓	✓	✓
Ganti	CGN15262	L-EXOT	Hungary	CGN	✓	✓	✓
MI 1/81	CGN15311	L-EXOT	DR Congo	CGN	✓	✓	✓
MII 1/81	CGN15317	L-EXOT	DR Congo	CGN	✓	✓	✓
Balady	CGN15396	L-EXOT	Lebanon	CGN	✓	✓	✓
Congolese Tomaat	CGN15413	L-EXOT	Congo	CGN	✓	✓	✓
Local	CGN15460	L-EXOT	Spain	CGN	✓	✓	✓
Pyongyang Bun Hong	CGN15470	L-EXOT	DPR Korea	CGN	✓	✓	✓
Local tomato	CGN15517	L-EXOT	Ghana	CGN	✓	✓	✓
Local	CGN15522	L-EXOT	Peru	CGN	✓	✓	✓
PI 127824	CGN15822	L-EXOT	Bolivia	CGN	✓	✓	✓
Gruntovyi Gribovskii 01180	CGN15855	L-EXOT	RF	CGN	✓	✓	✓
Pruno II	CGN15894	L-EXOT	Peru	CGN	✓	✓	✓
Local	CGN15895	L-EXOT	Portugal	CGN	✓	✓	✓
Plum (local)	CGN15924	L-EXOT	Thailand	CGN	✓	✓	✓
PI 114969	CGN15925	L-EXOT	India	CGN	✓	✓	✓
I 6582	CGN15949	L-EXOT	Honduras	CGN	✓	✓	✓
I 6604	CGN15951	L-EXOT	Costa Rica	CGN	✓	✓	✓
Skopski Jabucar	CGN16325	L-EXOT	Macedonia	CGN	✓	✓	✓
Zaghebacka Kasna	CGN16332	L-EXOT	Yugoslavia	CGN	✓	✓	✓
Domates Edremit	CGN16760	L-EXOT	Turkey	CGN	✓	✓	✓
Limachino	CGN16762	L-EXOT	Chile	CGN	✓	✓	✓
Acme	CGN17058	L-EXOT	United States	CGN	✓	✓	✓
Kujawski	CGN17079	L-EXOT	Poland	CGN	✓	✓	✓
Hu Shi Zi Feng Ding	CGN17089	L-EXOT	China	CGN	✓	✓	✓
Ostravske Rane	CGN17125	L-EXOT	Czechoslovakia	CGN	✓	✓	✓
Portocala	CGN17129	L-EXOT	Romania	CGN	✓	✓	✓
Tolna Megyei TF.	CGN18387	L-EXOT	Hungary	CGN	✓	✓	✓
I 6121	CGN18410	L-EXOT	Guatemala	CGN	✓	✓	✓
I 6137	CGN18412	L-EXOT	Guatemala	CGN	✓	✓	✓
I 7113	CGN18415	L-EXOT	Guatemala	CGN	✓	✓	✓
Gondol	CGN19157	L-EXOT	Indonesia	CGN	✓	✓	✓
Lider 165	CGN23998	L-EXOT	Ukraine	CGN	✓	✓	✓
Rybka	CGN23999	L-EXOT	USSR	CGN	✓	✓	✓
Uzb 1999	CGN24026	L-EXOT	Uzbekistan	CGN	✓		
Yellow Pear	CGN24030	L-EXOT	Mexico	CGN	✓	✓	✓
Local	CGN24205	L-EXOT	Peru	CGN	✓	✓	✓
PI 118778	CGN24209	L-EXOT	Brazil	CGN	✓	✓	✓
San Marzano tipo Lampadina	CGN24453	L-EXOT	Italy	CGN	✓	✓	✓
Precoce	CGN24465	L-EXOT	France	CGN	✓	✓	✓

[a] L-SAR = Sardinian landraces, L-IT = Italian landraces, L-EXOT = exotic landraces, C = vintage cultivars, W = wild species, IL = introgression lines.

[b] DR Congo = Democratic Republic of the Congo; Congo = Republic of the Congo; DPR Korea = Democratic People's Republic of Korea; RF = Russian Federation; USSR = Union of Sovietic Socialist Republic.

[c] VU = Viterbo University; AGRIS = AGRIS Sardegna, Agenzia per la Ricerca in Agricoltura; SMP = COOP Santa Maria la Palma; CGN = Centre for Genetic Resources, the Netherlands; TGRC = Tomato Genetics Resource Center, University of California, Davis.

[d] Trial carried out in Ottava, Sassari, Sardinia; [e] Trial carried out in Oristano, Sardinia.

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Table A.1 – *Continued from previous page*

Name	Code	Group[a]	Location[b]	Supplier[c]	2012	2013[d]	2013[e]
S. pennellii	LA0716	W		TGRC			✓
S. habrochaites	LA1777	W		TGRC			✓
cv E-6203	LA4024	C		TGRC			✓
TA1258-1	LA3913	IL		TGRC			✓
TA523-1	LA3914	IL		TGRC			✓
TA1229-1	LA3915	IL		TGRC			✓
TA1223-1	LA3916	IL		TGRC			✓
TA1535-1	LA3917	IL		TGRC			✓
TA1127-1	LA3918	IL		TGRC			✓
TA1128-1	LA3919	IL		TGRC			✓
TA1536-1	LA3920	IL		TGRC			✓
TA1105-2	LA3921	IL		TGRC			✓
TA1266-2	LA3922	IL		TGRC			✓
TA1537-3	LA3923	IL		TGRC			✓
TA1538-2	LA3924	IL		TGRC			✓
TA1111-3	LA3925	IL		TGRC			✓
TA1276-3	LA3926	IL		TGRC			✓
TA1277-3	LA3927	IL		TGRC			✓
TA1540-3	LA3928	IL		TGRC			✓
TA1541-3	LA3929	IL		TGRC			✓
TA1133-4	LA3930	IL		TGRC			✓
TA1280-4	LA3931	IL		TGRC			✓
TA1562-4	LA3932	IL		TGRC			✓
TA1542-4	LA3933	IL		TGRC			✓
TA1459-4	LA3934	IL		TGRC			✓
TA517-4	LA3935	IL		TGRC			✓
TA1475-4	LA3936	IL		TGRC			✓
TA1473-4	LA3937	IL		TGRC			✓
TA1287-5	LA3938	IL		TGRC			✓
TA1293-5	LA3939	IL		TGRC			✓
TA1112-5	LA3940	IL		TGRC			✓
TA1543-5	LA3941	IL		TGRC			✓
TA1117-5	LA3942	IL		TGRC			✓
TA1544-5	LA3943	IL		TGRC			✓
TA1539-6	LA3944	IL		TGRC			✓
TA1545-6	LA3945	IL		TGRC			✓
TA1546-6	LA3946	IL		TGRC			✓
TA1559-6	LA3947	IL		TGRC			✓
TA1303-7	LA3948	IL		TGRC			✓
TA1304-7	LA3949	IL		TGRC			✓
TA1547-7	LA3950	IL		TGRC			✓
TA1312-7	LA3951	IL		TGRC			✓
TA1315-8	LA3952	IL		TGRC			✓
TA1316-8	LA3953	IL		TGRC			✓
TA1548-8	LA3954	IL		TGRC			✓
TA1320-8	LA3955	IL		TGRC			✓

[a] L-SAR = Sardinian landraces, L-IT = Italian landraces, L-EXOT = exotic landraces, C = vintage cultivars, W = wild species, IL = introgression lines.

[b] DR Congo = Democratic Republic of the Congo; Congo = Republic of the Congo; DPR Korea = Democratic People's Republic of Korea; RF = Russian Federation; USSR = Union of Sovietic Socialist Republic.

[c] VU = Viterbo University; AGRIS = AGRIS Sardegna, Agenzia per la Ricerca in Agricoltura; SMP = COOP Santa Maria la Palma; CGN = Centre for Genetic Resources, the Netherlands; TGRC = Tomato Genetics Resource Center, University of California, Davis.

[d] Trial carried out in Ottava, Sassari, Sardinia; [e] Trial carried out in Oristano, Sardinia.

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Table A.1 – *Continued from previous page*

Name	Code	Group[a]	Location[b]	Supplier[c]	2012	2013[d]	2013[e]
TA1324-9	LA3956	IL		TGRC			✓
TA1325-9	LA3957	IL		TGRC			✓
TA1330-9	LA3958	IL		TGRC			✓
TA1331-9	LA3959	IL		TGRC			✓
TA1550-10	LA3960	IL		TGRC			✓
TA1551-10	LA3961	IL		TGRC			✓
TA1552-10	LA3962	IL		TGRC			✓
TA1337-10	LA3963	IL		TGRC			✓
TA1339-10	LA3964	IL		TGRC			✓
TA1555-11	LA3965	IL		TGRC			✓
TA1554-11	LA3966	IL		TGRC			✓
TA1342-11	LA3967	IL		TGRC			✓
TA1350-12	LA3968	IL		TGRC			✓
TA1121-12	LA3969	IL		TGRC			✓

[a] L-SAR = Sardinian landraces, L-IT = Italian landraces, L-EXOT = exotic landraces, C = vintage cultivars, W = wild species, IL = introgression lines.

[b] DR Congo = Democratic Republic of the Congo; Congo = Republic of the Congo; DPR Korea = Democratic People's Republic of Korea; RF = Russian Federation; USSR = Union of Sovietic Socialist Republic.

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[d] Trial carried out in Ottava, Sassari, Sardinia; [e] Trial carried out in Oristano, Sardinia.

APPENDIX B

List of markers

Table B.1: Details of SSR markers used for genetic analysis.

Locus	Type[a]	Chr	Sequence 5'-3'	Ta[b]	Reference
LE20592	NQ-SSR	11	F: CTGTTTACTTCAAGAAGGCTG R: ACTTTAACTTTATTATTGCCACG	54.5	Smulders et al. (1997)
LE21085	NQ-SSR	4	F: CATTTTATCATTTATTTGTGTCTTG R: ACAAAAAAAGGTGACGATACA	55.0	Smulders et al. (1997)
LELE25	NQ-SSR	10	F: TTCTTCCGTATGAGTGAGT R: CTCTATTACTTATTATTATCG	50.0	Smulders et al. (1997)
LELEUZIP	NQ-SSR	8	F: GGTGATAATTTGGGAGGTTAC R: CGTAACAGGATGTGCTATAGG	55.1	Smulders et al. (1997)
LEMDDNa	NQ-SSR	5	F: ATTCAAGGAACTTTTAGCTCC R: TGCATTAAGGTTCATAAATGA	54.5	Smulders et al. (1997)
Tom 47-48	NQ-SSR	3	F: CAAGTTGATTGCATTACCTATTG R: TACAACAACATTTCTTCTTCCTT	48.0	Suliman-Pollatschek et al. (2002)
Tom 162-163	NQ-SSR	1	F: TCTCAACCACTTAATCAATCTC R: CCCCAAGTAGCAACATAAATCT	48.0	Suliman-Pollatschek et al. (2002)
SLM12-29	NQ-SSR	12	F: AAGGAAAGGGAAAGGGGAAT R: CCTTGGTGAAAATCCTGCAT	55.0	Geethanjali et al. (2011)
SLM6-35	NQ-SSR	6	F: GTGCAACGCACGTTTTCG R: CCGCAAGCTCAACTAAACCT	50.0	Geethanjali et al. (2011)
EST245053	NQ-SSR	1	F: CCATTTAAATGACCCTATGCT R: AATCAAAAAGAATCTAAGCCCT	58.0	Areshchenkova and Ganal (2002)
TMS42	Q-SSR	11	F: AGAATTTTTTCATGAAATTGTCC R: TATTGCGTTCCACTCCCTCT	55.0	Areshchenkova (2000)
Tom 59-60	Q-SSR	3	F: TAACACATGAACATTAGTTTGA R: CACGTAAAATAAAGAAGGAAT	48.0	Suliman-Pollatschek et al. (2002)
TMS52	Q-SSR	12	F: TTCTATCTCATTTGGCTTCTTC R: TTACCTTGAGAATGGCCTTG	55.0	Areshchenkova and Ganal (2002)
Tom 236-237	Q-SSR	9	F: GTTTTTTCAACATCAAAGAGCT R: GGATAGGTTTCGTTAGTGAACT	47.0	Suliman-Pollatschek et al. (2002)
SLM6-14	Q-SSR	6	F: TCCGTAATAAGTTGAGGAACCA R: TCACAAGAATATTTGCCGTCAT	55.0	Geethanjali et al. (2010), Saliba-Colombani et al. (2001), Van der Knaap and Tanksley (2003)
TMS59	Q-SSR	8	F: TGAACGGGCCTTCTGTTATC R: ATCATCATTATAGTTCTTAAGTGAT	55.0	Areshchenkova and Ganal (2002)
TMS63	Q-SSR	1	F: GCAGGTACGCACGCATATAT R: GCTCCGTCAGGAATTCTCTC	60.0	Areshchenkova and Ganal (2002)
EST253712	Q-SSR	6	F: GAAATGAAGCTCTGACATCAAA R: TCATTGCTTGCATATGTTCATG	55.0	Areshchenkova and Ganal (2002)
EST258529	Q-SSR	5	F: AACACCCTTTATTCAGATTCC R: GCATAAAAATGTTAAAGGGG	50.0	Areshchenkova and Ganal (2002)

[a] Q-SSR = marker associated with known QTLs; NQ-SSR = marker without a known linkage with QTLs of interest.

[b] Annealing temperature.