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Genetic resources and selected conservation methods of tomato

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Summary

Tomato is one of the most popular vegetable crops. However, over time, the species has suffered a strong genetic diversity reduction and domestication bottlenecking. This growing trend is known as the genetic erosion. The human intervention on the genetic erosion intensification is high and has severe implications on the future programmes of management and use of *S. lycopersicum* biodiversity. The wild tomato species (especially accessions originating from the Andes to Mesoamerica) harbour many valuable genes, which have been lost among the cultivated ones. Therefore, there is an increasing interest to mine new alleles from the interspecific gene pool of *Lycopersicon* section. Sustainable genetic diversity management constitutes a basis for crop improvement, classification and protection. Moreover, conservation of plant genetic resources is crucial to food security, as well as pharmaceutical industry. There are a few strategies developed which address the preservation of tomato genetic resources. *In situ* and *ex situ* conservation are the two main complementary methods of biodiversity protection. The aim of this review is to summarise the most recent information about tomato genetic resources, genetic erosion phenomenon, as well as some traditional and modern preservation strategies.

Key words: biodiversity; DNA library; genetic erosion; *Solanum lycopersicum* L.; sustainable management; *in vitro* tissue culture

Introduction

The importance of tomato

The cultivated tomato (*Solanum lycopersicum* L.) is a dicotyledonous perennial or annual plant, which belongs to the large nightshade family – Solanaceae (Van Eck, 2018). The Solanaceae family is the third most important plant taxon, consisting of 96 genera and over 3000 species (Koo et al., 2008). Nowadays, tomato is the 2nd most important vegetable (after potato) and the 7th among most important crops in the world. It is grown on approximately 5 million hectares from the tropics to within a few degrees of the Arctic Circle (Fentik, 2017). The global tomato production is around 130 million tons, of which 88 million are destined for the fresh market and 42 million are processed (Eurofresh, 2017).

The species is of enormous economic value reaching billions of dollars (Van Eck, 2018). Due to its high consumption level, it is an important source of mineral salts, vitamins, bioflavonoids and carotenoids (Flores et al., 2017). Bhowmik et al. (2012) reported that consuming tomatoes can decrease the risk of various conditions, such as cancer, osteoporosis, neurodegenerative diseases and cardiovascular problems. Moreover, due to the presence of chlorine and sulphur, tomato has a detoxification effect in the body, helps to replace skin cells and in sun burns (Capel et al., 2017). The species importance is reflected by the enormous number of research on all aspects of the crop. Since 2000, over a thousand publications related to tomato studies have been published annually (Google Scholar, 2018).

Overtime, tomato has been adapted to different growing systems by selection and adjustment of a limited set of traits (The 100 Tomato Genome Sequencing Consortium, 2014). Application of modern biotechnological tools, such as sequencing technologies, genomic selection and multi-omic analysis, provided good results in the breeding of (a)biotic stress resistant *S. lycopersicum* plants with amended characteristics (Uluisik et al., 2016; Fentik, 2017; Zhu et al., 2018). Development of genetic linkage maps and GWAS (Genome-Wide Association Studies) made it possible to learn the chromosomal locations of QTL (quantitative trait) genes for improving yield and other complex features, such as: fruit abscission, size, flavour, texture and colour (Kulus, 2018). Site-directed mutagenesis using genetic approaches, e.g. CRISPR/Cas9 system, also can provide a wealth of resources for crop breeding, as well as for biological research by inducing precise mutation in the first and later generations (Ito et al., 2015; Pan et al., 2016). For example, CRISPR/Cas9-induced Targeted Mutagenesis and Gene Replacement were used to produce long-shelf life tomato lines, parthenocarpic plants and other (Ueta et al., 2017; Wang et al., 2017; Yu et al., 2017). Unfortunately, the reduced genetic basis that resulted from extensive inbreeding has impeded tomato improvement (The 100 Tomato Genome Sequencing Consortium, 2014).

Consequently, despite its great meaning, progress in breeding systems and development of highly efficient superior cultivars, tomato suffers a strong genetic basis reduction and domestication bottlenecking. Genetic erosion is now a common phenomenon reported in numerous crops, both locally and globally (Hoban et al., 2014). It might have severe implications on the future programmes of management and use of *S. lycopersicum* genetic resources. Therefore, to enlarge the genetic variation, breeders recently focus on introgression of genes from wild relatives into the elite cultivars, but so far, this has been quite limited and more actions are required (The 100 Tomato Genome Sequencing Consortium, 2014).

The aim of this review is to summarise the most recent information about tomato genetic resources, their meaning, genetic erosion phenomenon and development of molecular markers (from isozymes to next generation sequencing) in evaluating tomato genetic diversity, as well as some selected traditional (field collections and seed banks) and modern (*in vitro* slow-growth banks and DNA libraries) *in situ* and *ex situ* preservation methods.

Genetic diversity of tomato

Genetic diversity estimates constitute a basis for future strategies for crop improvement, sustainable use, classification and conservation. The obtained knowledge can then be applied to increase the genetic variation in base populations by crossing cultivars with a high level of genetic distance and for the introgression of exotic germplasm (Labate and Robertson, 2012).

The genetic diversity in plant species is dependent on the mating system, the domestication history, ecological factors, and the size of the sample being analysed (Mazzucato et al., 2008). The *Lycopersicon* section began its initial radiation 7 million years ago (Robertson

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and LABATE, 2006). This small monophyletic clade comprises several species, of which only *S. lycopersicum* was domesticated from *S. lycopersicum* var. *cerasiforme*, although *S. pimpinellifolium* is also casually planted for consumption (GIOVANNONI, 2018). The latter one is the closest relative of the cultivated tomato; genome sequences of both of the above-mentioned tomatoes showed only a 0.6% nucleotide divergence (GERSZBERG et al., 2015). Still, there are 15 more wild species of tomato, including *S. arcanum*, *S. cheesmaniae*, *S. chilense*, *S. chmielewskii*, *S. corneliomulleri, S. galapagense*, *S. habrochaites*, *S. huaylasense*, *S. jugandifolium*, *S. lycopersicoides*, *S. neorickii*, *S. ochranthum, S. pennellii*, *S. peruvianum, S. sitiens* (ANDERSON et al., 2010).

Since the mid-20th century, approaches based on controlled hybridisation allowed crossing between cultivated and wild tomato compartments (BAUCHET et al., 2017). However, there are some serious crossability barriers with *S. peruvianum* and *S. chilense* that are quite difficult to overcome and require biotechnological methods even in successive generations (BEDDOWS et al., 2017).

Currently the tomato germplasm is distributed throughout the following categories of: modern cultivars, obsolete cultivars, commercial breeding stocks (breeding lines), genetic stocks (e.g. monosomics, alien addition lines), landraces, heirloom varieties or primitive varieties and wild forms of cultivated species, obtained from spontaneous mutations, natural outcrossing or recombination of pre-existing genetic variation (GROUT and CRISP, 1995). In total, more than 10,000 morphologically different tomato cultivars and forms exist (GERSZBERG et al., 2015). The typical turnover time of commercial cultivars is approximately five years (BAI and LINDHOUT, 2007).

Cultivated tomato is autogamous, whereas its wild relatives, such as *S. peruvianum, S. pennellii* or *S. hirsutum*, are often facultative or obligate out-crossers (KOCHIEVA et al., 2002). This gametophytic incompatibility system contributes in greater genetic diversity (THE 100 TOMATO GENOME SEQUENCING CONSURTIUM, 2014). According to MILLER and TANKSLEY (1990), more genetic variation can be found within a single accession of one self-incompatible tomato species than among all accessions of any one of the self-compatible species, estimated at 75% versus 7%.

As for cultivated tomato, old introductions and locally developed cultivars (landraces) in the 1970s present substantially greater genetic variability than the ones produced during the 1990s (MAZZUCATO et al., 2008). Landraces usually have a higher number of rare alleles and a lower number of private alleles when compared to contemporary cultivars (CORRADO et al., 2014), although CARELLI et al. (2006) observed, that the frequency of rare alleles was similar in Brazilian commercial and landrace accessions. The high level of genetic variability within landraces is related to their plasticity (HASSAN et al., 2013).

The genetic structure of tomato

Over time, several attempts have been made to circumscribe the genetic structure of tomato (KOBAYASHI et al., 2014), including sequencing the genomes of *S. arcanum, S. habrochaites, S. lycopersicum, S. pennellii* and *S. pimpinellifolium* (THE TOMATO GENOME CONSORTIUM, 2012; THE 100 TOMATO GENOME SEQUENCING CONSORTIUM, 2014). All tomato species are diploid with 24 acrocentric to metacentric chromosomes (2n = 2x = 24), except for two natural tetraploid populations of *S. chilense* (BAUCHET and CAUSSE, 2012). They are considered to have stable genomes in which speciation has evolved primarily by genic changes rather than large-scale chromosomal rearrangements, however, small inversions have been also reported (ANDERSON et al., 2010). Genetic variation within tomato species occurs both: intraspecific – within cultivated tomato, and interspecific – between wild species (BAUCHET and CAUSSE, 2012). Domestication; i.e. selection of beneficial alleles; has evolved a number of morphological and physiological traits which distinguish cultivated tomato from its wild ancestors. This phenomenon is known as the 'domestication syndrome' and includes a more compact growth habit, increased earliness, reduction/loss of seed dispersal, and fruit shape diversity (PARAN and VAN DER KNAAP, 2007; SAUVAGE et al., 2017). Leaf variation and fruit colour observations are the easiest ways for distinguishing wild tomatoes (which have a low intraspecific phenotypic diversity) from cultivated ones (ZHOU et al., 2015). Those traits are often controlled genetically by a relatively small number of *loci* on a limited number of chromosomal regions with major phenotypic effect (FRARY and DOGANLAR, 2003). Moreover, morphological characters of tomato can be altered by environmental factors and management practice. Therefore, despite domesticated accessions show a range of morphological diversity in comparison to wild species, a strong loss of molecular diversity throughout the whole genome of cultivated tomato is observed (VAN DEYNZE et al., 2007).

Genetic erosion – causes and solutions

In tomato, the level of genetic diversity in the cultivated gene pool is substantially lower than in other crops and represents a narrow range of the species original variability due to domestication bottlenecking which took place in Europe 400 years ago (BHATTARAI et al., 2016). This is because only few tomato seeds were brought back from Mexico to Europe. Those impoverished resources were then re-introduced to America (BAUCHET and CAUSSE, 2012). In the following years, genetic diversity protection was neglected in official policy (HOBAN et al., 2014). Consequently, MILLER and TANKSLEY (1990) claim that less than 5% of the available species genetic variation exists in modern tomato cultivars.

Restricted habitat is another issue. Most of the tomato wild relatives are endemic in narrow geographical regions; their habitats are usually isolated valleys where they were adapted to particular microclimates and soil types (ALBRECHT et al., 2010). A common phenomenon in wild tomato species is founding of populations by a small number of highly homozygous individuals via seed dispersal, which causes a severe genetic bottleneck (NAKAZATO and HOUSWORTH, 2011). Moreover, due to a drastic reduction of their natural habitats, some wild species, e.g. *S. pimpinellifolium*, are now endangered (BAUCHET and CAUSSE, 2012).

As for the landraces, despite the superior flavour, yield stability in low input agricultural systems, great value for niche markets and sustainable farming, their cultivation is currently limited to gardens for personal consumption and in small-size farms for local markets. The lack of information about the origin and the relationship of landraces are the main limiting factor for their application (CORRADO et al., 2014). This is unfortunate, since those genetic resources, once lost, are very difficult to reconstitute (COSTE et al., 2015).

As for the commercial tomato cultivars, due to the evolution of highly mechanised farming systems, most of them are the F1 hybrids (KWON et al., 2009). Consequently, hybrid sterility is frequently observed. Moreover, under selection for research and breeding purposes, the initial already narrow genetic basis of the tomato is even more restricted by the development of superior but few cultivars, which replace more numerous, but less productive vintage and regional varieties (SAHU and CHATTOPADHYAY, 2017). For example, PATIL et al. (2010) observed high levels of pair wise similarity (mean = 0.838) within 17 tomato cultivars. Also ZHOU et al. (2015) noticed the high similarity coefficient of the 29 cultivated tomatoes (0.845) by applying EST-SSR (expressed sequence tag) markers. This low variability within the species was confirmed by other biochemical and molecular markers (CEBOLLA-CORNEJO et al., 2013; SHIRASAWA et al., 2015; SAHU and CHATTOPADHYAY, 2017). On the other hand,

according to SIM et al. (2012), the long history of crossing with wild relatives, breeding for various market classes and selection of distinct ideotypes for different production systems has broadened the genetic diversity in contemporary germplasm relative to vintage and landrace germplasm, although similar reports are sparse. A fair conclusion was delivered by LABATE and BALDO (2005), who claim that genetic variation in domesticated *S. lycopersium* is unevenly dispersed, with rare islands of polymorphism originating from introgression. For example, KWON et al. (2009) observed that the commercial tomato cultivars PIC (polymorphism information content) was ranging from 0.21 to 0.88. Similarly, 19 Azerbaijan genotypes had a genetic similarity ranging from 0.188 to 1.000 (SHARIFOVA et al., 2013). Nevertheless, the reduction of genetic diversity at important *loci* can limit improvements in the future (MUÑOS et al., 2011).

The increasing application of plant breeders rights also has negative implications for plant genetic diversity. Seed companies sell crop cultivars under strict legal protection (TRIPP and HEIDE, 1996). There are about a dozen tomato-breeding companies which are the main players in the world market. For example, in India private seed companies control 90% of the tomato seed market (PATIL et al., 2010). This leads towards narrowing of the biological diversity, especially in secondary centres of diversity (CEBOLLA-CORNEJO et al., 2013).

At the same time, much of the wild tomato biodiversity is still untouched in the Andes. In the 1940s, breeder Dr. Charlie Rick observed during his expeditions, that this disbanded gene pool is valuable in searching for new genes, breeding for hybrid vigour and in analysing the taxonomy and evolution of the genus (BAI and LINDHOUT, 2007). Also research conducted by WILLIAMS and ST. CLAIR (1993) showed a much higher diversity in Andean *S. pimpinellifolium* and *S. lycopersicum* var. *cerasiforme* populations (where first domestication of tomato took place) than among Mesoamerican cultivars (from where tomato was introduced to Europe). Those findings were supported by recent high density SNP (single nucleotide polymorphism) genotyping performed by BLANCA et al. (2015). They pointed Ecuadorian and Peruvian accessions to represent a pool of unexplored variation. Conservation and sustainable use of those wild tomato relatives would benefit from an understanding of genetic diversity and relationships within and between populations, as well as for sampling of the populations for genes of interest (ALBRECHT et al., 2010).

Seed mixing and pollen contamination can be performed to increase variation within the tomato (CEBOLLA-CORNEJO et al., 2013). For example, the sexually compatible wild relatives, such as *S. pimpinellifolium* or *S. habrochaites*, can be used as donors of useful genes for salt tolerance during seed germination (SIFRES et al., 2011), resistance genes to *Clavibacter michiganensis* subsp. *michiganensis*, which causes bacterial canker of tomato, and the pathogen *Oidium neolycopersici*, responsible for powdery mildew (PASSAM et al., 2007). Trichome characteristics from *S. cheesmanii* and *S. pennellii* affect the behaviour of an aphid *Myzus persicae* or resistance to infection by parasitic giant dodder (*Cuscuta reflexa*) (KRAUSE et al., 2017). The *Mi* gene found in *S. peruvianum* is controlling nematode resistance, while invulnerability to whitefly (*Bemisia* spp.) has been identified in wild populations of *S. lycopersicum* var. *cerasiforme*. The jointless *j2* allele was already introgressed from *S. cheesmanii* into many processing cultivars, allowing a large scale mechanical harvest of tomato fruits (BAUCHET et al., 2017). Good fruit quality traits (i.e. soluble solids, sugar and β-carotene content, as well as aromatic fragrance) have also been detected in *S. cheesmanii* and *S. peruvianum,* even though the fruits of those species are not usually consumed by humans (ZHOU et al., 2015; ZHANG et al., 2016).

Conservation of genetic resources

It is predicted, that the world human population will increase by 50% before 2050. At the same time the surface of arable land is progressively decreasing. Tomato characteristics must satisfy the constantly growing consumer's preference. It also must be suitable for *post*-harvest handling and marketing, even over large distances (PASSAM et al., 2007). Consequently, dramatic increase in crop production will be required (WANG et al., 2009). Plant genetic resources are crucial to food security, as well as pharmaceutical industry (EDESI et al., 2017). Therefore, it is essential to collect, preserve, evaluate and exchange the genetic variability of tomato (PETROVIĆ and DIMITRIJEVIĆ, 2012).

Preserving genetic diversity in plants has been performed for over a century (TANKSLEY and MCCOUCH, 1997). To achieve this task, the International Treaty for Plant Genetic Resources for Food and Agriculture was made within the framework of the Food and Agriculture Organization of the United Nations in 2004. Its main objectives are the conservation and sustainable use of plant genetic resources for food and agriculture production (WANG et al., 2009). There are a few fundamental strategies developed which address the conservation of plant genetic resources (Fig. 1).

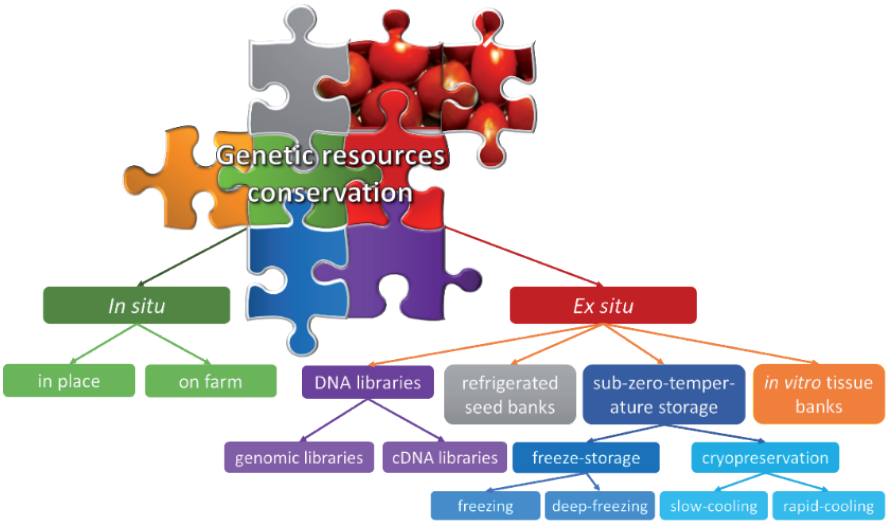

Fig. 1: There are two complementary systems of germplasm conservation; *in situ* and *ex situ* conservation. *In situ* conservation can refer to on site (in nature) preservation of wild species and on farm conservation of landraces and cultivated genetic resources. DNA libraries, seed collections, *in vitro* conservation and low-temperature storage are under the "umbrella" of *ex situ* gene banks.

In situ conservation

In situ conservation is used for both on site preservation of wild species and on farm preservation of landraces and cultivated genetic resources. Arguments in support on traditional cultivation and storage of genotypes include the importance of recognising the roles of environmental factors and the possibility to evolve under natural conditions through crossing with wild or weedy relatives (Tripp and Heide, 1996; Corrado et al., 2014). On the other hand, protection *in situ* is laborious and expensive since it requires large areas, nursing, agrotechnology, and it is threatened with loss due to (a)biotic stresses.

The tomato hosts over 200 species of pests and pathogens that can cause significant financial losses (Bai and Lindhou, 2007). The most common diseases of tomato crops include: bacterial scab, spot and wilt, as well as fungal diseases, such as powdery mildew. Other main diseases are leaf spot, early blight, leaf mould and wilts. Changes in insect's biotype and disease resistance are becoming a continuing threat to *in situ* collections (Chaudhry et al., 2010). Pathogens have to be controlled via chemical compounds, which are not always fully-effective, harmful to the environment and require compliance with chemical-use laws (Bai and Lindhou, 2007). Furthermore, the *in situ* strategy is climate-dependent; tomato is a warm season plant, requiring high light intensity and a minimum temperature of 10ºC to grow (Liza et al., 2013). As a result, other conservation strategies are also explored.

Ex situ conservation

Tomatoes are well represented in *ex situ* (off-site) working and informal collections. They are used for research and commercial purposes at the global level, with a range of dozens of thousands of accessions located in seed gene banks (mostly), *in vitro* laboratories and cryobanks, as well as in the form of DNA libraries.

Conventional gene banks

The possibility of maintaining genetic resource in gene banks is attracting great attention. They are mostly based on collections of seeds (or more rarely pollen) stored; in plastic bags, culture jars or cryovials; in controlled conditions and periodically regenerated. Gene banks are an important source of publicly available genetic material for plant breeding programmes and other research activities (Labate et al., 2009).

It is estimated that over 83,000 accessions of the wild and cultivated species of *Lycopersicon* section are maintained in germplasm banks located in over 120 countries, ranking 1st among vegetable species collected (Bauchet and Causse, 2012). The most important ones include facilities at the CM Rick Tomato Genetics Resource Center (TGRC), University of California in Davis, U.S. (with a large collection of open-pollinated cultivars) and at the United States Department of Agriculture (USDA) or Plant Genetic Resources Unit at Geneva (PGRU), U.S., as well as laboratories in the International Board for Plant Genetic Resources (IBPGR) network (Sharifova et al., 2013). The Universidade Estadual do Norte Fluminense in Rio de Janeiro (Brazil), has a tomato gene bank with accessions that have been maintained for nearly 50 years (Gonçalves et al., 2008). The Mexican government recently established a National Genetic Resources Center (CNRG) as a component of a long-term strategy for conservation and sustainable use of plant biodiversity (Arizaga et al., 2016). The World Vegetable Center (WVC), in Tainan (Taiwan) maintains one of the largest collections of *Lycopersicon* germplasm. Most of the collection (60%) was harvested from Old World regions. In the Netherlands, the Botanical and Experimental Garden maintains the most extensive *ex situ* plant collections of non-tuberous Solanaceae species (BGARD, 2018). The European Cooperative Programme for Plant Genetic Resources tomato database maintains passport information of more than 20,000 accessions of numerous tomato species. The tomato collection of European Solanaceae database is composed of about 7,000 domesticated and wild lines of *S. lycopersicum* (EU-SOL, 2018). This biological material was provided by international gene banks and by donations from private collections. The accessions were established and phenotyped, accompanied by an *ad hoc* database. Large collections of tomato germplasm are also conserved in Russia (VIR), Japan (NIAS), Peru (DHUNA), Cuba (INIFAT) and Norway (Svalbard Global Seed Vault) (Foolad, 2007). Other countries with great numbers of stored tomato accessions are: Bulgaria, Canada, China, Colombia, France, Germany, Hungary, the Philippines and Spain. They conserve mainly *S. lycopersicum* germplasm.

Large germplasm collections are typically duplicated at a second backup location in case the primary collection is lost due to mechanical breakdowns, natural disasters or political disturbance. For example, the USDA and TGRC accessions are backed up at the National Centre for Genetic Resources Preservation (NCGRP) at Fort Collins, Colorado (89 and 95% of the collections, respectively) (Robertson and Labate, 2006).

The management of tomato germplasm collections requires harvesting representative samples and performing two related activities; long-term storage of high-quality biological material, and regeneration of plants with further selection to replenish seed stocks. Therefore, most gene banks maintain also field collections (Robertson and Labate, 2006).

Gene banks include also various monogenic stocks and a large pool of tomato mutants, which were either spontaneous or induced by irradiation or chemically (Shalaby and El-Benna, 2013). For example, TGRC maintains an isogenic tomato 'mutation library' containing a total of 13,000 M(2) families (Bai and Lindhout, 2007). A miscellaneous mutant population is a fundamental resource for exploring gene function (Menda et al., 2004). TILLING (Target Induced Local Lesion In Genomes) is a mutagenesis method to generate chemically, via 0.5 - 1.0% EMS (Ethylmethane Sulphonate), or fast-neutron-induced point mutations in genomes. Unlike genetic transformation, mutagenesis is random, cost effective and is not submitted to GMO regulation. Moreover, the technique allows to rapidly transfer interesting mutations into cultivars that can improve important agronomic traits in tomato (Shirasawa et al., 2015). Collections of tomatoes carrying artificially induced genetic variants, with more than 3,000 phenotype alterations catalogued, are publicly available and can be accessed in the Solanaceae Genome Network website (Minoia et al., 2010; Okabe et al., 2011).

Traditional gene banks are useful with plant species, which produce orthodox seeds that have a low water content or will survive drying (and optional freezing) during *ex-situ* conservation. Otherwise, seeds must be often regenerated and the samples replaced with new ones. Prior to storage, tomato seeds are dried to a level of 5±1% in a room that is maintained at 20% relative humidity at 4-5 ºC. Working and active collections are stored at 5 ºC. Longer storage of base collections is possible after (deep)freezing of seeds or pollen (-20; -80 ºC). The longevity of such samples depends also on the type of storage container. It should be air-tight but not vacuum-sealed to avoid seed damage. Regeneration of plants is conducted once the danger of frosts has passed (Robertson and Labate, 2006). Further selection efficiency can be improved by genetic markers that are associated, through linkage or pleiotropy, with genes or QTLs that control the trait(s) of interest (Foolad, 2007; Kulus, 2018).

Germplasm banks, like any other facility, have some drawbacks. One of the main concerns of breeders is how to quantify the degree of variability of stored tomato plant resources, especially that during regeneration cross pollinating plants can be contaminated by foreign pollen. Furthermore, storage and regeneration steps need consider-

ably large numbers of plants and seeds, due to the intermittent viability testing to monitor the progress of viability decrease within the stored accession. To prevent genetic drift, wild or cross-pollinated tomato species require more seeds for storage and regeneration (over 50 regenerated plants per variety), than cultivated taxa (25 plants) (Robertson and Labate, 2006). The costs of characterising and cataloguing gene bank material are also considerable.

High-quality seed production requires constant laborious monitoring for diseases and pests, and timely application of pesticides followed by costly seed processing (separating of seeds from skin and pulp, washing, drying, etc.). The lack of coordination and conflicting passport data is a another drawback for an efficient *S. lycopersicum* germplasm management (Bauchet and Causse, 2012). Moreover, despite the exchange of germplasm should be free to all those who wish to use them, there is a risk that countries which host important international collections may deny another country access to them, due to political or religious reasons (Tripp and Heide, 1996). Therefore, modern biotechnology-based methods, which can help to overcome some of these problems, will be more frequently implemented for tomato genetic resources conservation. Nowadays, there is a possibility of storage under slow-growth *in vitro* conditions or of cryopreserving tissues in liquid nitrogen (-196 ºC) (FAO, 2014; Coste et al., 2015).

In vitro slow-growth storage

The development of *in vitro* culture technique has opened possibilities for various applications after its inception in the 1930s (Parmar et al., 2012). Tissue banks can be used for medium-term preservation of pathogen-free staple crops and further selection and production of stress-tolerant, high-quality plants (Al-Abdallat et al., 2017). By reducing the temperature and light intensity in the growth room, it is possible to reduce the growth pace of tomato shoots and the number of subcultures required to one per two-three years. Besides the temperature and light regimes, the success of tomato slow-growth *in vitro* system varies with the nutrient media, concentration and combination of growth regulators and osmotic agents, as well as genotype and explant related factors (Liza et al., 2013; Bhushan and Gupta, 2017).

The selected for the purpose of slow-growth biological material cannot be contaminated or excessively hydrated. In this case, source material should be cultured *de novo*, and the invalid one; immediately eliminated (FAO, 2014). Schnapp and Preece (1986) were the first to develop an *in vitro* slow-growth of tomato; i.e. decline of shoot length, rooting and callogenesis rating; when the MS (Murashige and Skoog, 1962) nutrient salts level was lowered to 25%, 50%, or 75% compared to full strength medium and when sucrose was supplied at 0.05%. Recently, *in vitro* preservation of transgenic tomato lines overexpressing the stress-responsive transcription factor SlAREB1 was studied (Al-Abdallat et al., 2017). By adding 200 - 300 mM sucrose into the MS medium, a reduction in tomato plantlets length, leaf number and rooting was reported. The increased levels of sucrose induce an osmotic stress that inhibits the growth of microshoots and extends the subculturing interval due to the restricted cell osmotic potential, reduction of water availability and decrease of cell expansion and division. The application of ABA (abscisic acid) was even more effective when compared with sucrose treatment. Severe growth retardation phenotypes were observed when microshoots were cultured on MS media supplemented with 8 or 12 µm ABA (Al-Abdallat et al., 2017).

A serious drawback of tissue banks, is the fact that tomato is susceptible to somaclonal variation occurrence (Poopola, 2015). A number of factors, viz. pre-existing genetic dissimilarities uncovered during *in vitro* tissue differentiation, stress due to the unnatural *in vitro* culture conditions, medium components and explant isolation, as well as selection for specific genotypes during plant regeneration stimulate genetic variation in regenerated microshoots (Rzepka-Plevneš et al., 2010; Ali et al., 2017). According to Gavazzi et al. (1987), regeneration from *in vitro* culture leads to a higher number of tomato mutations than application of the chemical mutagen. *In vitro*-produced *S. lycopersicum* plants may contain several monogenic mutations with morphologically visible variability, e.g. lethality, altered leaf morphology, absence of anthocyanin, reduced chlorophyll content, variegation and dwarfing (Bulk et al., 1990). A second commonly observed variation is tetraploidy, and the third is plants sterility (Ali et al., 2017).

Therefore, tissue culture system is not desirable for long-term preservation. It can be used, however, as a source of explants for cryopreservation (storage at cryogenics; at -196 ºC to -140 ºC). *In vitro* culture is used as a preparatory phase prior to storage in LN (liquid nitrogen), as well as for recovery phase after rewarming. Preservation of tissues in dewar flasks is power-independent (eco-friendly) and cost-efficient, as LN is inexpensive. Moreover, explants are stored in a small volume (2-ml cryovial per sample, comprised of even 50 tomato seeds), protected from contamination, and require very little maintenance (except for controlling the level of LN, as it is rapidly evaporating). Over time several slow- and fast-cooling cryopreservation techniques were developed (Kulus and Zalewska, 2014). However, limited data on tomato seeds, pollen and shoot tips cryopreservation are yet available (Coste et al., 2015; Al-Abdallat et al., 2017; Halmagyi et al., 2017).

DNA libraries

At the molecular level, the tomato gene pool can be stored in the form of DNA libraries: genomic or complementary DNA (cDNA). A genomic library contains DNA fragments, generated by a specific endonuclease, that represent the entire genome of an organism. As for the cDNA libraries, mRNA from a specimen are extracted and then cDNA is prepared in a multistep reaction catalysed by reverse transcriptase enzyme. Therefore, cDNA libraries contain only the coding regions (ESTs) of expressed genes with no introns or regulatory regions. The produced fragments are ligated into vector molecules and transferred into host cells (Wu et al., 2006).

The development of artificial chromosome vectors (bacterial – BAC, or yeast – YAC) and the relative ease of manipulating DNA libraries has led to the widespread use and acceptance of this approach. BACs are more efficient since they do not have the technical problems observed with YAC libraries, such as easier purification. The further development of binary-BAC (BIBAC) vectors established a new method for DNA management, plant transformation and gene identification. This technology allows to immediately introduce very large fragments of DNA, intact, into the plant genome via *Agrobacterium tumefaciens*-mediated plant transformation (Hamilton et al., 1999). Moreover, since linkage map distances are not simply related to physical distances, artificial chromosomes are used in physical mapping to determine the locations of markers on chromosomes and in map-based cloning of agronomically important genes and QTLs (Koo et al., 2008).

As for tomato, the first binary-BAC vector library was constructed by Hamilton et al. (1999) (Cornell University, U.S.). Large insert genomic libraries, containing approximately 4.6 haploid nuclear genomic equivalents, were constructed for *Solanum lycopersicum* 'Mogeor' and *S. pennellii* LA716. The *S. lycopersicum* DNA library has an average insert size of 125 kb and is comprised of 42,272 individual colonies stored as frozen cultures in a 384-well format (108 plates). The *S. pennellii* genomic library has an average insert size of 90 kb and is comprised of 53,760 individual clones (140,384-well plates). In 2003, Qu et al. constructed large-insert genomic libraries of tomato, based on a new TAC (transformation-competent

artificial chromosome) vector. The library contains 96,996 clones (28.3-38.5 kb insert size) and has 3.18 haploid genome equivalents. TAC vectors proved to be useful to rapidly isolate important genes in tomato. A detailed physical map of *S. pennellii* genomic regions including two genomic libraries (in a form of BAC/cosmid clones) was screened with 104 collocated markers from five selected genomic regions by KAMENETZKY et al. (2010). This gave a genome-wide map of a nondomesticated tomato species, which covers 10% of the physical distance of the selected regions corresponding to approximately 1% of the wild tomato genome. To accelerate the progress of tomato genomics studies, recently a full-length cDNA library has been established for the cultivar 'Micro-Tom' (KOBAYASHI et al., 2014).

Development of molecular markers in evaluating tomato genetic diversity

Knowledge about genetic distances is essential for optimum organisation of gene banks, and for identifying parental combinations which produce progenies with maximum genetic diversity. Genetic variation can be investigated via various methods, e.g. through morphological and biochemical traits, pedigree analysis or by molecular markers (CORRADO et al., 2014). Because the tomato phenotype can be easily altered by environmental factors, thus quantification of genotypic variation by phenotypical markers, despite being intuitive and practical, is not always possible. Molecular markers are recognised as a more reliable method for fingerprinting the accessions in gene banks (ZHOU et al., 2015). In the past three decades, various molecular markers (including isozymes, seed proteins and PCR-based markers) were employed, either individually or in combination, for genetic diversity analyses in tomato (OHYAMA et al., 2017; SHARIFOVA et al., 2017; CHAUDHRY et al., 2018). Development of isozymes allowed for the first evaluation of wild tomato germplasm (RICK and FOBES, 1975). However, isozyme marker scarcity and their low polymorphism was a serious limitant.

The narrow genetic base of tomato cultivars makes it difficult to distinguish them via many contemporary genetic markers (ZHOU et al., 2015). Commonly used identification methods, based on the amplification of a limited number of pre-selected barcoding regions, are often inapplicable due to DNA degradation, low amplification success or low species discriminative power of selected genomic regions (RAIME and REMM, 2018). The lack of sufficient marker systems in the tomato was a bottle neck in genetic and linkage studies for many years. Fortunately, nowadays, specific DNA fragment-based markers can identify tomato cultivars despite high levels of monomorphism and low polymorphism information content values (LABATE and BALDO, 2005). For *S. lycopersicum*, $(GATA)_4$, $(CCTA)_4$ and $(GGAT)_4$ oligonucleotide motifs are suitable for the differentiation of cultivars and breeding lines that are otherwise difficult to distinguish (GARCÍA-MARTÍNEZ et al., 2013). Moreover, $(GATA)_4$ fingerprinting generated hierarchical classifications consistent with the history of tomato cultivation (KAEMMER et al., 1995). In 2005, TAM et al. found that SSAP (Sequence-Specific Amplification Polymorphism) is more corresponding for inferring overall genetic variation and relationships, while microsatelites (short tandem repeats; SSRs) have the capability to detect specific genetic relationships. Recently, SSRs have become the most popular source of tomato genetic markers owing to their multi-allelic nature, high reproducibility, wide dispersion in eukaryotic genomes, and co-dominant inheritance. Due to variations in repeat copy number; possibly caused by slippage during replication; they show a higher level of polymorphism than any other genetic marker (OHYAMA et al., 2017). For example, BREDEMEIJER et al. (2002) documented, that despite STMS (Sequence Tagged Microsatellite Site) polymorphism in tomato was relatively low (the number of alleles per *locus* ranged from 2 to 8), though, more than 90% of 508 analysed popular tomato cultivars had different microsatellite profiles. Unfortunately, microsatellite development is expensive and time consuming, so other markers are also used. RUIZ et al. (2005) compared SRAP (Sequence-Related Amplified Polymorphism) and SSR marker systems in order to analyse the genetic variability of some traditional tomato cultivars of Spain (including commercial cultivars, local cultivars and a few wild species). SRAP is a reliable and simple PCR-grounded dominant marker system, designed to detect mostly coding sequence polymorphisms. The system is based on a combination of two types of primers. The forward primer amplifies exonic regions, while the reverse primer amplifies intronic and promoters regions. In the study by RUIZ et al. (2005), both marker types (SSR and SRAP) sorted out the cultivars from different groups, but SSR failed to distinguish some of those classified within the same group. This can be explained by the fact, that despite microsatellites regions present higher variability than other genomic regions, the SRAP system has a higher multiplexing ratio and analyses a much higher number of *loci*. Therefore, this system can also be recommended for evaluating tomato genetic diversity.

The finding of SNPs as bi-allelic molecular markers, first described in ESTs (LABATE and BALDO, 2005) then in non-coding tomato sequences (LABATE et al., 2009), delivered access to a high level of polymorphism as they exhibit less homoplasy than markers based on fragment-size. SNPs are highly abundant in plants (they represent even 90% of the genetic variation in any species), and can be assayed cost-effectively (VAN DEYNZE et al., 2007). It should be mentioned tough, that SNPs can be divided in two clusters: SNPs of which both forms are present in the wild tomato relatives and in domesticated ones (originating from common ancestors), and SNPs unique for the domesticated tomato (originating from after the domestication event) (VÍQUEZ-ZAMORA et al., 2013). According to THE 100 TOMATO GENOME SEQUENCING CONSORTIUM (2014), in wild tomato species the number of SNPs exceeds 10MM, i.e. 20-fold higher than found in most of the cultivated accessions. Therefore, screening of SNPs through *de novo* sequencing is inefficient within cultivated tomato, as the sequencing error rate is over ten-fold higher than the polymorphism rate. Fortunately, the development of DNA microarray method, ESTs and COS (Conserved Orthologous Set) data; the number of which reaches now several hundred thousand; has made it possible to discover putative SNPs *in silico*, prior to experimental verification (SIM et al., 2012; OHYAMA et al., 2017).

As sequencing costs continue to decline, researchers are developing new technologies, e.g. genotyping by sequencing (or next generation sequencing; NGS). The availability of high-throughput sequencing tools, accompanied by the development of array-based genotyping platforms, has provided unparalleled ability to determine genome diversity across entire clades, at both the structural and genotype level by rapid scoring of several thousand markers in parallel (THE 100 TOMATO GENOME SEQUENCING CONSORTIUM, 2014; LIU et al., 2018). For example, the 152 SNPs (obtained via custom-made Illumina SNP-panel) were able to clearly distinguish 75 landraces from a set of 25 contemporary Italian varieties (CORRADO et al., 2014). VÍQUEZ-ZAMORA et al. (2013) identified a set of 6,000 SNPs and 5,528 of them (1,980 originated from 454-sequencing, 3,495 from Illumina Solexa sequencing and 53 were additional known markers) were used to evaluate tomato germplasm at the level of species, varieties and segregating populations. Such genotyping techniques have enabled high-density molecular map construction and genome-wide association analysis (CELIK et al., 2017). Another novelty, based on plastid genome sequences, was described by RAIME and REMM (2018), who identified over 800 *Solanum lycopersicum* specific DNA k-mers (32 nucleotides in length) from 42 different chloroplast genome regions. The chloroplast DNA is high copy and has a small, generally stable and mechanical breakdown resistant, circular form as compared to nuclear DNA (KIM et al., 2015). Moreover, the chlo-

roplast genome is endemic to plants and may help to bypass DNA contamination from chloroplast-free organisms (Dong et al., 2014). Therefore, plastid genome sequencing can be considered as a valuable tool allowing for rapid identification of plant taxa directly from raw sequencing reads without aligning, mapping or assembling the reads.

Bioinformatic data extrapolation can increase the efficiency of molecular markers discovery (Pailles et al., 2017). The development of nanotechnologies in the so called "*post*-genomics" era opens new perspectives in terms of genetic diversity management, toward conservation and survey of large populations (Muños et al., 2011; Giovannoni, 2018). The use of computer simulations and multivariate statistical algorithms (such as: principal component analysis, canonical variable analysis, clustering methods, *etc*.) is an important strategy to quantify the degree of (dis)similarity or number of (rare) alleles in plant genetic resources (Gonçalves et al., 2008). After previous cloning and sequencing, genetic markers from database sequences (e.g. BLAST, EMBL or Genbank) can be screened *in silico* and utilized to create diversity profiles in tomato, especially for cultivars with limited genetic variation and in other accessions of the *Lycopersicon* section (Yang et al., 2014; Shirasawa et al., 2016).

Conclusions

Despite new donor segments have been introduced from more variable related species into the cultivated tomato germplasm, typically of self-pollinated crops, the species severely lacks genetic diversity (Shirasawa et al., 2015). The conservation and utilisation of crop biodiversity is of particular importance, especially to the least developed countries, where modern plant breeding has had much less success.

The Polytechnic of Agriculture and Cattle Husbandry at the University of Manabi (Ecuador) is conducting a project aiming at collecting, characterising and conserving the genetic resources of wild tomato species (including rare and endangered accession). The project involves characterisation of *in situ* environmental conditions in which plants grow, as well as *ex situ* morphological description and molecular analysis of collected specimens. Moreover, the establishment of a wild tomato seed cryobank is planned (Zevallos et al., 2014). Projects, such as the one described above, are necessary for plant biodiversity protection, and thus, efficient future breeding and food security. However, the maintenance, characterisation and management of all accessions within a collection is economically demanding. According to Corredo et al. (2014), almost all the genetic diversity at the specific *loci* can be caught by a limited number of lines. Therefore, it is recommended to extract subsets of individuals (with a 15-25% sampling intensity) that represent the diversity conserved in the entire germplasm collection. High-resolution molecular profiling can be applied to generate core subsets, ensuring that alleles present with low frequencies are not lost, dodging the need to phenotypically characterise and maintain the entire collection.

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