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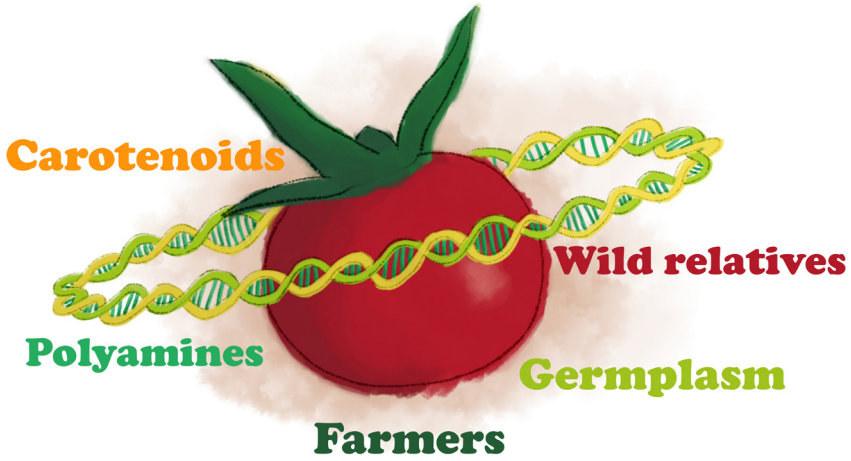
Bolivian tomatoes

genetic diversity, quality traits and value chains

Evelyn Elizabeth Villanueva Gutierrez



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Cover: Tomato fruit surrounded by topics addressed in the thesis.
Illustration by Paola Ariana Moreira Villanueva

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Bolivian tomatoes - genetic diversity, quality traits and value chains

Abstract

Bolivia is considered one of the centres of origin and distribution of tomatoes, and wild relatives harbouring potential genetic diversity and quality traits are preserved in germplasm collections. Tomato is the most commonly grown vegetable world-wide, but in Bolivia, tomatoes are low-yielding and have short shelf-life. This thesis studied genetic diversity of 20 accessions and eight cultivars using 11 simple sequence repeat markers, quality traits and bioactive compounds in fruits of 29 accessions and eight cultivars using HPLC techniques. Fruit quality along the tomato value chain (TVC) was assessed in case studies in four provinces.

Genetic diversity was found to be low and party influenced by plant growth type, geographical origin, fruit shape and size, and stage of maturity. A UPGMA tree distributed the 28 accessions into six groups, with separation between indeterminate and determinate plant growth type. Quality traits and polyamines (putrescine, spermidine, spermine) concentrations showed inconsistent variations between six maturity stages studied. At maturity, only two the 29 Bolivian accessions showed significant differences in polyamines concentrations. However, the accessions differed significantly in carotenoids and vitamin C content, but no interaction between polyamines and carotenoids concentrations was observed.

Five critical factors within the Bolivian TVC were identified as affecting tomato quality: i) landscape and land access; ii) cultural practices; iii) harvesting process; iv) packing process; and v) transportation system.

The new information on Bolivian genetic resources, bioactive compounds and desirable traits presented in this thesis can be used by breeders to develop new cultivars. In a broader perspective, the thesis identified constraints and actors involved in tomato quality determination in a developing country.

Key words: Germplasm, polyamines, carotenoids, population genetics, vitamin C

Bolivianska tomater - genetisk mångfald, kvalitetsegenskaper och värdekedjor

Abstract

Bolivia anses vara ett av tomatens ursprungs- och distributionscentra, och vilda släktingar som hyser potentiell genetisk mångfald och kvalitetsegenskaper bevaras i genbanker. Tomat är den mest odlade grönsaken i världen, men i Bolivia ger tomater låg avkastning och har kort hållbarhet. Denna avhandling studerade genetisk mångfald i blad från 20 accessioner och åtta sorter med hjälp av 11 enkla sekvensupprepningsmarkörer. Även kvalitetsegenskaper och bioaktiva föreningar studerades i frukter från 29 accessioner och åtta sorter med hjälp av HPLC-tekniker. Fruktkvaliteten längs tomaternas värdekedja bedömdes i fallstudier i fyra provinser.

Den genetiska mångfalden visade sig vara låg och delvis påverkad av växtsätt, geografiskt ursprung, fruktens form och storlek samt mognadsstadium. Ett UPGMA-träd fördelade de 28 accessionerna i sex grupper, med separation mellan obestämda och bestämda tillväxtmönster. Kvalitetsegenskaper och polyaminkoncentrationer (putrescin, spermidin, spermin) visade inkonsekventa variationer mellan de sex mognadsstadierna som studerades. Vid mognad uppvisade endast två av de 29 bolivianska accessionerna signifikanta skillnader i polyaminkoncentrationer. Accessionerna skiljde sig dock signifikant i halter av karotenoider och C-vitamin, men ingen interaktion mellan polyaminer och karotenoidkoncentrationer observerades.

Fem kritiska faktorer inom den bolivianska värdekedjan identifierades som påverkar tomatkvaliteten: i) landskap och marktillgång; ii) Odlingsmetoder; iii) skördeprocess; iv) packningsprocess och v) transportsystem.

Den nya informationen om bolivianska genetiska resurser, bioaktiva föreningar och önskvärda egenskaper som presenteras i denna avhandling kan användas av förädlare för att utveckla nya sorter. Ur ett bredare perspektiv identifierade avhandlingen begränsningar och faktorer som är involverade i bestämning av tomatkvalitet i ett utvecklingsland.

Nyckelord: genbanker, polyaminer, karotenoider, populationsgenetik, vitamin C

Dedication

To my beloved children Paola and Adrian. *You are the light of my life*

To my parents Ruddy and Elizabeth: *You give everything*

"Without EDUCATION people perish, without EDUCATION nobody will be able to survive, without EDUCATION woman cannot overcome poverty, will not be able to take care of themselves, will not be able to move forward on the planet"

Oprah Winfrey

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Evelyn E. Villanueva-Gutierrez*, Eva Johansson, Maria Luisa Prieto-Linde, Alberto Centellas Quezada, Marie E. Olsson, and Mulatu Geleta *(2022). Simple sequence repeat markers reveal genetic diversity and population structure of Bolivian wild and cultivated tomatoes (*Solanum lycopersicum* L.). *Genes* 13 (1505), 1-25.

II. Evelyn E. Villanueva Gutierrez, Eva Johansson*, Alberto Centellas Quezada, Karl-Erik Gustavsson, and Marie E. Olsson (2021). Genotype and maturity stage affect the content and composition of polyamines in tomato - possible relations to plant and human health. *Horticulturae* 7 (300), 1-15.

III. Evelyn E. Villanueva Gutierrez*, Karl-Erik Gustavsson, Alberto Centellas Quezada, Marie E. Olsson, Mulatu Geleta, and Eva Johansson* (2022). Delving into the bioactive compounds in Bolivian accessions of tomato (*Solanum lycopersicum* L) fruits: impact of genetic, phenotypic and origin factors (manuscript).

IV. Evelyn E. Villanueva Gutierrez *, Geovana Mercado*, Alberto Centellas Quezada, Marie E. Olsson, Mulatu Geleta, and Eva Johansson *(2022). Critical points for quality assurance in Bolivian smallholders' tomato value chains (manuscript).

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*Corresponding author

The contribution of Evelyn Elizabeth Villanueva Gutierrez to the papers included in this thesis was as follows:

I. Participation in design and execution of the experiment, selection of SSRs, elaboration of the first draft, collaboration on research visualisation, paper submission and paper improvement for publication.

II. Polyamine quantification, statistical analysis, results analysis, elaboration of first draft, submission preparation and correction.

III. Participation in design and execution of the experiments, quantification of bioactive compounds, statistical analysis, interpretation of results and elaboration of the manuscript.

IV. Participation in survey design, development of participatory observation guidelines, visits to Bolivian farms to perform case studies in tomato-growing regions, data compilation and partial analysis, visualisation, and writing the manuscript.

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Abbreviations

AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
AVC	Agriculture value chain
CS	Case study
PUT	Putrescine
SPD	Spermidine
SPM	Spermine
SSR	Simple sequence repeat
PGT	Plant Growth Type
TA	Titratable acidity
TPA	Total polyamines
TSS	Total soluble solid
TVC	Tomato value chain

1. Introduction

Cultivated tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family and the genus *Solanum*, with over 2000 species identified (Kaunda & Zhang, 2019). It originated in the western part of South America, but wild relatives are distributed from Mexico to Guatemala, Ecuador, Peru, Bolivia and Chile (Acquaah, 2012; Bauchet & Causse, 2012; Blanca *et al.*, 2012). Tomato consumption has existed in South America since 500 BC, while the tomato's culinary introduction to Europe occurred after the Spanish conquest of the Aztec area in Mexico (Bergougnoux, 2014). In Europe, tomato breeding started narrowing the genetic diversity, resulting in the modern tomato with its many sizes, shapes and colour variations, affecting the evolutionary process of tomato species (Lin *et al*., 2014). Wild relatives and landraces are also distributed across Europe and efforts have been made to establish national collections of tomato relatives, *e.g.* in Spain, Greece and Italy. At present, tomato is the first most common vegetable produced in the world (FAO, 2020), with a great variety of shapes and colours. It serves as a source of bioactive compounds available for fresh consumption and in processed form (Barrett *et al*., 2010; Guil-Guerrero & Rebolloso-Fuentes, 2009; Perveen *et al*., 2015). Open-field production of tomatoes in favourable climate conditions is typically performed by self-employed smallholders (Chaudhary *et al*., 2018; Rowles *et al*., 2018).

The intention with the work in this thesis was to provide new information on wild tomato relatives in Bolivia and to assess the complexity of tomato production from the field to the consumer. Four research areas were identified: (i) the genetic diversity of wild tomato relatives in Bolivia compared with modern cultivars; (ii) polyamine content and variation among tomato genotypes at different maturity stages when grown in controlled conditions; (iii) content of bioactive compounds present in mature wild

tomato relatives; and (iv) critical points in the tomato value chain and their effects on tomato quality parameters. A brief description of the background to each of these research areas and relevant information about the methods employed in fulfilling the objectives are provided in the following paragraphs.

Despite the fact that Bolivia is part of the origin and diversity centre of wild tomatoes, there have been few analyses of the genetic diversity of Bolivian tomatoes (Torrico *et al*., 2015). Such information is crucial to understand how genetic diversity was shaped and to assess the level of genetic diversity of tomato material conserved *ex situ* in the Bolivian gene bank. It can also indicate the possible effects of geographical position and domestication status on quality traits (Nakazato & Housworth, 2011).

Determining the level and distribution of genetic variation in plant genetic resources can play a crucial role in efficient conservation of genetic resources and selection of germplasm for use in plant breeding programmes (Priyadarshan & Jain, 2022). Therefore, the first step in this thesis was to explore the genetic diversity and population structure of Bolivian tomato accessions and to compare their genetic variation against variations in phenotypic traits and in their geographical origins. The approach used was to select representative accessions from the tomato core germplasm of Bolivia, based on their passport data, and to assess genetic diversity and population structure. The accessions targeted in the analysis comprised modern cultivars well accepted by Bolivian farmers, advanced breeding lines, landraces and wild populations. DNA extracted from 279 tomato plants representing 28 accessions was used for the genetic diversity analyses, based on previously published simple sequence repeat (SSR) markers (Frary *et al*., 2005; Geethanjali *et al*., 2010; Korir *et al*., 2014; Smulders *et al*., 1997) and also newly developed SSR markers.

From a holistic point of view, bioactive compounds confer protection against plant stressors and benefit overall consumer health (Ali *et al*., 2020). Among the bioactive compounds, polyamines are common in all organisms (Lenis *et al*., 2017). A previous study on changes in the concentrations of the polyamines putrescine, spermidine and spermine in tomato plants under biotic or abiotic stress reported that these confer additional protection, contributing to the survival of the plants (Sánchez-Rodríguez *et al*., 2016). Polyamines also have beneficial effects on human health following

consumption (Hirano *et al*., 2021). However, changes in polyamine concentrations during tomato maturation in a controlled environment have been largely unexplored, despite the valuable effects of polyamines in plants under stress conditions (Romero *et al*., 2018). In addition, there have been few simultaneous assessments of changes in polyamines depending on accession and maturity stage in tomatoes grown in a controlled environment (Yahia *et al.*, 2001). Therefore, HPLC-DAD-MS analysis was used to evaluate the effect of tomato maturity stages and tomato accessions on the composition and levels of polyamines and their interaction with other quality traits in tomato fruits. Additional quality parameters, including total soluble solid contents, titratable acidity and vitamin C, were also evaluated, in an attempt to identify patterns in quality parameters and potential inter-relationships.

Information on tomato quality traits in the Bolivian core germplasm is currently limited (Choque, 2014; The GRIN Global Project, 2022). In particular, concentrations of polyamines, carotenoids and vitamin C content have not been explored. Availability of information on the content of bioactive compounds is an asset for future choice of accessions in plant breeding programmes, due to the importance of these compounds in plant stress protection and their positive effects on human health (Abreu & Fernández, 2020; Anwar *et al*., 2015; Berni *et al*., 2018; Sequera-Mutiozabal *et al*., 2016; Shah *et al*., 2020). To overcome this lack of information, concentrations of bioactive compounds in the fruits of Bolivian tomato accessions were evaluated in order to gain a better understanding of the interaction between bioactive compounds, natural niches and phenotypic traits at mature stage under controlled conditions. Analysis of a larger sample of tomato accessions was necessary to reveal the effects of accession on the variation in bioactive compounds. In addition, a comprehensive bioactive compound profile for unstudied accessions was created, to provide initial key information for proper selection of germplasm for tomato breeding programmes. To examine the differences between accessions, identification and quantification of polyamines, carotenoids and vitamin C was conducted using HPLC-MS-LC on mature tomatoes of 29 Bolivian accessions.

To identify critical aspects in the value chain and their impact on quality parameters in tomato production in Bolivia, secondary information was first evaluated. This revealed that previous studies conducted in Bolivia provide little information regarding changes in quality traits from open field production to the consumer (Da Silva-Ovando et al., 2021; Thompson & Wainwright, 1991). Identification of critical aspects in the tomato value chain in Bolivia is essential to create innovative alternatives and protect the quality achieved in the field, as the product passes through multiple actors such as smallholders and traders to consumers (Norton, 2017). A fair and transparent value chain is crucial in terms of attaining the United Nations Sustainable Development Goals (SDGs) of no poverty, zero hunger, decent work, reduced inequality between farmers and traders, and responsible consumption, which are part of Agenda 2030 (United Nations, 2015). Further, open-field production is a source of self-employment in which farmers are the suppliers of local food to rural and urban areas, so their participation in research is essential to ensure food security (FAO, 2016). In Bolivia, the boundaries between production and distribution in the value chain are vague, and the impacts of the value chain on quality traits have not yet been determined (Geoffrey *et al*., 2014). Thus, qualitative methods were used in this thesis to elaborate a tomato value chain from production to transportation, to overcome the issue of having limited information available (Creswell, 2013; Yin, 2009). Information was collected from farmers and key actors, and in direct observation of tomato production in four case studies in four regions of Bolivia.

2. Aims

Limited knowledge of tomatoes from Bolivia, one of the centres of origin of the species, limits the possibilities of utilising germplasm collected in different ecological regions. Therefore, the overall aim of this thesis was to obtain more information on tomatoes in two perspectives: genetic diversity and fruit quality traits in Bolivian core germplasm and modern cultivars, and value chain constraints that negatively affect quality traits under open field conditions.

Specific objectives were to:

Assess the genetic diversity and population structure of Bolivian tomato and associate the findings with variation in phenotypic traits and geographical origin (Paper I).

Assess the effect of tomato maturity stage and genotype on the composition and levels of polyamines present and their effect on other quality traits in tomato fruits (Paper II)

Analyse the levels of bioactive compounds in the fruits of Bolivian tomato accessions and determine their correlation with natural niches and phenotypic traits (Paper III).

Identify critical stage of the value chain and their impact on quality parameters in tomato production in Bolivia (Paper IV).

3. Methods

3.1 Quantitative methods

3.1.1 Plant material and sample preparation

Three large experiments were conducted in a greenhouse of the Department of Plant Breeding on the premises of SLU-Campus Alnarp, in order to collect young leaf samples (Paper I) and tomato fruits at different maturity stages (Papers II and III). Details of plant material used in this thesis are presented in Table 1 and in Figures 1 and 2.

Young leaf tissue was collected from 9-10 plants per accession for genetic diversity and population structure analyses of 28 tomato accessions. The accessions were of three major groups: Bolivian landrace and wild accessions, modern cultivars, and advanced breeding lines. Treatment of each sample and analysis of data are described in detail in Paper I.

To analyse bioactive compounds and other quality traits in tomato fruit, samples were taken as follows: After total fruit development, sampling started by collecting 3-4 fruits per replication at different maturity stages (green, break, turning, pink, light red and red) in Paper II and only fruits at the mature (red or yellow) stage in Paper III. For each replication examined in Papers II and III, data were recorded for different traits, including weight, size, colour and firmness. Additional measurements of total soluble solids (TSS) content and titratable acidity (TA) were performed in Paper II.

Table 1. *Description of 41 tomato accessions from Bolivian germplasm, cultivars and advanced lines*

Accession	Sampling region	Breeding status	Altitude, masl	Fruit shape	Fruit size	Fruit colour	Plant growth type
BOL-8222-HT[I,III]	Cbba	CU	2858	HI RO	INT	Red	DET
BOL-8223-HT[I,III]	Sucre	CU	2201	HI RO	VS	Yellow	SDET
BOL-8225-HT[I,III]	Sucre	CU	1165	SI FL	VS	Pink	SDET
BOL-8224-HT[I]	Santa Cruz	CU	300	HI RO	SS, INT	Red	DET
BOL-8226-HT[I,III]	Sucre	CU	1143	RO	VS	Yellow	SDET
BOL-8242-HT[III]	Santa Cruz	CU	NR	RO	VS	Yellow	SDET
BOL-8277-HT[III]	La Paz	W	NR	RO	VS	Yellow	SDET
BOL-8279-HT[III]	Beni	W	NR	RO	VS	Red	SDET
BOL-8281-HT[I,III]	Beni	W	227	RO	VS	Red	INDET
BOL-8282-HT[I,III]	Beni	CU	227	SI FL	VS	Red	INDET
BOL-8284-HT[I,III]	Beni	CU	259	SI FL	VS	Red	INDET
BOL-8288-HT[I]	La Paz	W	498	RO	VS	Red	INDET
BOL-8290-HT[I,III]	La Paz	W	594	SI FL	VS	Yellow	SDET
BOL-8292-HT[I,III]	La Paz	W	1676	SI FL	VS	Yellow	SDET
BOL-8295-HT[I,III]	La Paz	W	599	SI FL	INT	Red	DET
BOL-8297-HT[III]	La Paz	W	599	RO	VS	Yellow	INDET
BOL-8306-HT[III]	La Paz	W	1734	CY	INT	Red	DET
BOL-8311-HT[III]	La Paz	CU	478	RO	VS	Red	SDET
BOL-8313-HT[III]	La Paz	W	805-870	RO	INT	Red	DET
BOL-8316-HT[I,III]	La Paz	CU	961-1030	SI FL	INT	Red	SDET
BOL-8320-HT[III]	La Paz	CU	1726-1700	CY	INT	Red	DET
BOL-8322-HT[I]	La Paz	CU	1725- 1690	SIFL	VS	red	SDET
BOL-8328-HT[I,III]	La Paz	W	1853- 1870	FL	VS	Red	SDET
BOL-8329-HT[III]	La Paz	CU	1855-1860	RO	VS	Red	SDET
BOL-8330-HT[I,III]	La Paz	W	1716-1720	SI FL	VS	Red	SDET
BOL-8331-HT[III]	La Paz	CU	1671-1680	RO	VS	Red	SDET
BOL-8333-HT[III]	La Paz	CU	1128- 1170	RO	VS	Red	SDET
BOL-8335-HT[I,III]	La Paz	W	1124-1190	SI FL	VS	Red	SDET
BOL-8340-HT[I,III]	La Paz	W	1492- 1550	SI FL	VS	Red	SDET
BOL-8348-HT[I,III]	La Paz	W	2021-2012	RO	VS	Yellow	SDET
BOL-8349-HT[I]	La Paz	W	2021-2010	RO	VS	red	SDET

BOL-8352-HT[III]	La Paz	W	1993-2010	RO	VS	Red	SDET
BOL-8354-HT[III]	La Paz	W	1926-1930	RO	VS	Red	SDET
Lia[I,II]	Israel	CU	NR	CY	INT	red	DET
Shanty[I, II]	Israel	CU	NR	CY	INT	red	DET
Huichol[I,II]	Thailand	CU	NR	CY	INT	red	DET
Rio Grande[I, II]	Cbba	CU	2548	CY	INT	red	DET
HT36[I, II]	Cbba	ADL	2548	RO	VL	red	DET
HT37[I, II]	Cbba	ADL	2548	RO	LL	red	DET
HT23[I, II]	Cbba	ADL	2548	CY	INT	red	DET
HT25[I, II]	Cbba	ADL	2548	CY	INT	red	DET

Germoplasm provider = INIAF-CNPSH, Bolivia. Accession description sources is in the website http://germoplasma.iniaf.gob.bo/. To find information for each accession, do not include the suffixes BOL- -HT (accessed on 1 September 2022). W (wild), CU (cultivated), ADL (Advanced line), DET (Determinate), SDET (Semi-determinate), INDET (Indeterminate), HIRO (High rounded), SIFL (Slightly flattened), RO (Rounded), CY (Cylindrical), FL (Flattened), VS (very small), SS (Small), INT (Intermediate), LL (Large size), VL (Very large), NR (not reported).

Wild


BOL-8279-HT


BOL-8281-HT

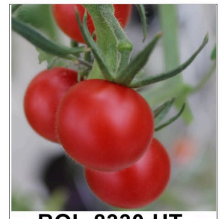

BOL-8295-HT


BOL-8306-HT


BOL-8288-HT


BOL-8313-HT

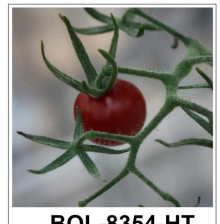

BOL-8328-HT


BOL-8330-HT


BOL-8335-HT


BOL-8340-HT


BOL-8352-HT


BOL-8354-HT

Cultivated


BOL-8222-HT

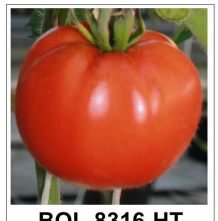

BOL-8225-HT


BOL-8331-HT


BOL-8282-HT


BOL-8284-HT


BOL-8311-HT


BOL-8316-HT


BOL-8320-HT

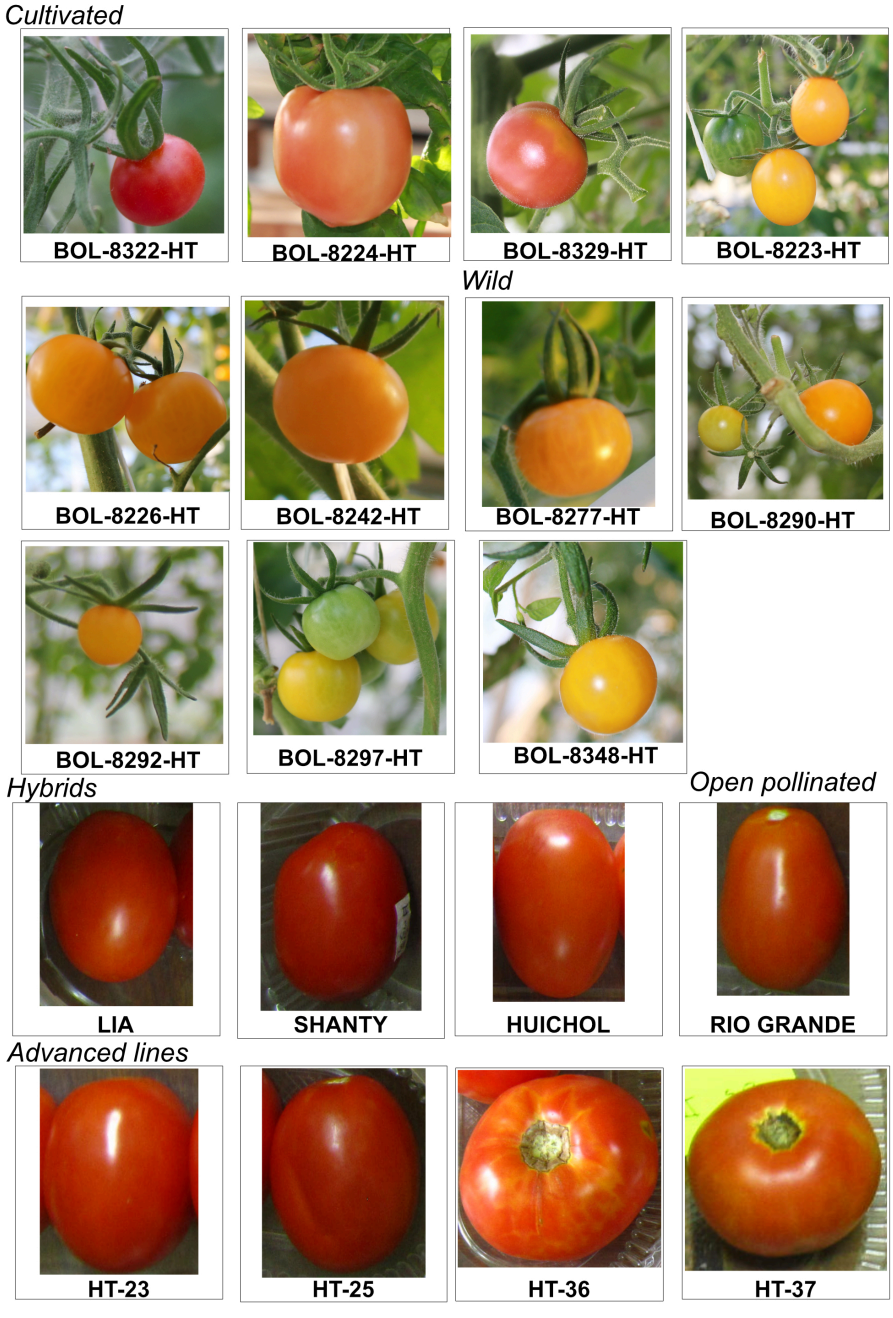


Figure 1. Tomato fruits grown from seeds obtained from the Bolivian core tomato collection, common commercial cultivars and advanced lines from the Bolivian breeding programme.

Figure 2. Geographical distribution of the tomato accessions collected by Bolivian Gen bank and analysed in this thesis.

3.1.2 Analysis of qualitative traits

Fruit size, weight, colour and firmness

Fruit size, weight, colour, and firmness were evaluated immediately after harvesting for each accession, with 3-4 tomato fruits used per replication. Evaluation and data analysis of these quality traits is described in Papers II and III.

Total soluble solid (TSS), titratable acidity (TA) and TSS/TA ratio

To predict sweetness, acidity and flavour, the parameters TSS and TA and TSS/TA ratio were evaluated in samples of tomato fruit juice for each accession representing different fruit maturity stages. Sample evaluation and data analysis are described in detail in Papers II and III.

Polyamines

Detection and quantification of putrescine, spermidine and spermine was performed using previously reported polyamine analysis methods (Buranaphalin, 2009; Eerola *et al*., 2013; Flores & Galston, 1982). Samples were analysed by high performance liquid chromatography-diode array detection-mass spectrometry (HPLC-DAD-MS), using a 1260 HPLC-DAD-MS Infinity system from Agilent Technologies (CA, USA). A detailed description of sample extraction, quantification and data analysis is provided in Papers II and III.

Carotenoids

Detection and quantification of carotenoids in 29 tomato accessions were performed based on a previously published method (Saad *et al*., 2017). Carotenoid detection was performed by HPLC (1100 Series HPLC, Agilent Technologies, CA, USA) with a diode array detector (DAD G4212), and quantification was based on lycopene standard quantification with a spectrophotometer (MultiskanGo Thermo Scientific, Finland). A detailed description of method modifications and data analysis is presented in Paper III.

Vitamin C

Vitamin C analyses were based on a previously published method (Bergquist *et al*., 2006) with specific modifications according to sample requirements. Samples from green to mature stages were evaluated in an HPLC Infinity System 1260 (Agilent Technologies, CA, USA) in Paper II, and samples only at mature stage by HPLC-DAD in Paper III. Extraction, quantification and data analysis are described in detail in Paper II.

3.2 Qualitative methods

3.2.1 Case studies

A qualitative approach was chosen to study the current situation in the Bolivian tomato value chain from production to the last step prior to commercialization. Mixed methods research, combining qualitative methods, such as case studies, and quantitative methods, are gaining increasing attention (Creswell, 2013; Yin, 2009). Then, the qualitative studies allow collection of information in a more profound form, which may result in an exploration of communal participation in a specific area and time-frame and in efforts to understand the details of a complex system (Creswell & Poth, 2016; Martella *et al*., 2013). For gathering reliable information from multiple actors and places to address research questions, qualitative methods use different strategies, including case studies, interviews and direct observations (Darlington & Scott, 2002; Denzin & Lincoln, 2011).

Case studies in four Bolivian provinces (Punata, Narciso Campero, La Florida, Chapare) were performed to explore the smallholder tomato value chain (Figure 3). Punata, Narciso Campero and La Florida are traditional tomato-producing provinces, while Chapare was included due to its growing importance in tomato production during the dry season in recent years.

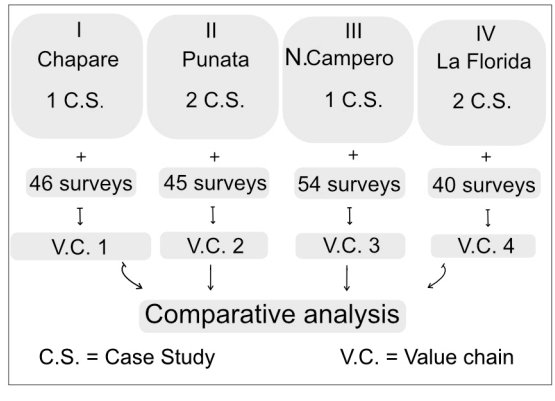


Figure 3. Diagram showing case study areas, surveys and value chain analysis.

3.2.2 Critical points identification

Three approaches were used to explore the critical points in the tomato value chain. The first was to elaborate the value chain by mapping it in each province, through on-site visits, document analysis and interviews. The second approach was to conduct semi-structured interviews in each province based on a guide with multiple-choice and open questions options. In this approach, 185 farmers were interviewed, with 40-56 individuals in each province. The third approach was participatory observation, including direct observations of farmers working on their farms. A combination of these three approaches was used in the four case studies described in Paper IV.

4. Results and discussion

4.1 Genetic diversity analyses of Bolivian tomato germplasm and their relationships with cultivars and advanced lines

Paper I in this thesis analysed the genetic variation in unexplored tomato germplasm from Bolivia, as one of the centres of origin and distribution of tomato. Simple sequence repeat markers were used to measure genetic diversity and population structure, as briefly summarised below.

4.1.1 Simple sequence repeat marker performance

The genetic diversity and population structure of 28 accessions were analysed based on alleles recorded at 11 simple sequence repeat (SSR) loci. Four of the SSR markers were chosen from among those previously published (Frary *et al*., 2005; Geethanjali *et al*., 2010; Korir *et al*., 2014; Smulders *et al.*, 1997) based on their level of polymorphism, while seven markers were newly developed in Paper I, resulting in a total of 11 SSR markers. These SSR markers detected 2-5 alleles per locus and 33 alleles across all loci. The most informative SSR marker was SLM6-11, with a polymorphism information content (PIC) of 0.65, followed by LE20592 and TOMSatX11-1 with PIC of 0.55 and 0.49, respectively (Table 2 in Paper I).

4.1.2 Population genetic analysis: polymorphism information content, fixation index and genetic differentiation

The PIC values of the SSR markers varied from 0.05 to 0.65, with a mean of 0.29. Comparison with previous studies showed that similar SSR marker PIC values have been reported for inbred populations (Benor *et al*., 2008). Higher

PIC values have been reported in studies that included hybrids, modern cultivars and landraces (Gonias *et al*., 2019), and samples from different countries (Meng *et al*., 2010). Fixation index (F_{ST}) and estimated genetic differentiation (G_{ST}) between the accessions was 0.80 and 0.77, respectively, with a G_{ST} p-value of 0.001 indicating significant genetic differentiation between the accessions (Table 2 in Paper I).

4.1.3 Observed heterozygosity and expected heterozygosity

Observed heterozygosity (Ho) is generally lower than expected heterozygosity (He) at neutral loci in self-pollinated plant species, including tomatoes. The Ho value of an accession indicates the level of inbreeding, while the He value can be viewed as a measure of genetic diversity within each accession (Blanca *et al*., 2012; Causse *et al*., 2020; Nakazato *et al*., 2012). In Paper I, the Ho values were lower than the He values at over 50% of the loci (see Table 3 in Paper I). However, the He values were lower than those reported in some previous studies (Ranc *et al*., 2008; Rao *et al*., 2012), suggesting lower genetic diversity in Bolivian tomato germplasm, despite the fact that the country is part of the centre of origin of tomatoes.

In hybrid accessions, Ho values tend to be larger than He values (Flint-Garcia *et al*., 2009; Krieger *et al*., 2010). In Paper I, the hybrid accessions Lia, Shanty and HT-36 had higher Ho values than He values, leading to an F value of -1. Two other hybrid accessions (Huichol and HT-25) also had negative F values (-0.33 and -0.17, respectively), although these values are higher than -1, suggesting that the accessions probably were reproduced via open pollination following the initial hybridisation. The hybrids HT-23 and HT-37 were different in that their F values were positive (F = 0.47 and 1.0, respectively), probably due to a series of self-pollination events after the initial hybridisation (Table 3 in Paper I).

The 20 Bolivian accessions were divided into three subgroups based on He (expected heterozygosity). These were: inbred (He = 0); extremely low diversity (He = 0.01-0.08); and low to medium diversity (He = 0.11-0.21) (Table 3 in Paper I). The variation was explained to between 16.75% and 22.53% by geographical region of origin, cultivation status, fruit shape, fruit size and plant growth type. The inbred group with He = 0 most likely have cleistogamous flowers, and hence exhibit strict self-pollination and inhibited pollen movement. Individual genotypes within this group that have desirable characteristics could be used as inbred parents for crossbreeding, resulting in

genetically uniform F1 hybrids. The accessions with extremely low diversity (He = 0.01 to 0.08) and with fixation index (F_{ST}) = 1 are also self-pollinating types, so the very low He values might be the result of an unintentional gene flow in the form of seeds. The subgroup with low to medium diversity (He = 0.11-0.21) and a high percentage of polymorphic loci (PPL) are most likely open-pollinating types.

4.1.4 AMOVA

Analysis of molecular variance (AMOVA) demonstrated that 77.3% of the total genetic variation found was between accessions, while the remaining 15.6% was within accessions, indicating substantial differentiation between the accessions (Table 4 in Paper I). The AMOVA results obtained on grouping the accessions into different subgroups according to their geographical region of origin, cultivation status, fruit shape, fruit size and plant growth type showed significant differences between these subgroups in all cases. However, these significant differences only explained 16-23% of the variation.

4.1.5 Pairwise comparison of accessions, and cluster and population structure analyses

Genetic differences between accessions were visualised using three graphical methods. The first was a heatmap of the average number of pairwise differences (above the diagonal) and Nei's distance (below the diagonal) between accessions (Figure 4). The second was an unweighted pair group method with arithmetic mean (UPGMA) dendrogram (Figure 5), which showed the grouping of the accessions into six clusters. The third method was a graph displaying population structure based on an optimum number of three genetic populations (Figure 6).

The Nei's distance heatmap revealed low, but statistically significant, genetic differentiation between the accessions. However, four accessions (BOL8281-HT, BOL-8284-HT, BOL-8335-HT and BOL-8349-HT) were the exception (darker green in Figure 4). In the diagram, variation within the population is displayed in the middle (purple squares) corresponding to He values ranging from 0 to 0.21 and Shannon index (I) values ranging from 0 to 0.29. Accessions HT-25, BO-8288-HT, BOL-8326-HT and BOL-8348-HT displayed the greatest variation (Figure 4, Table 3 in Paper I).

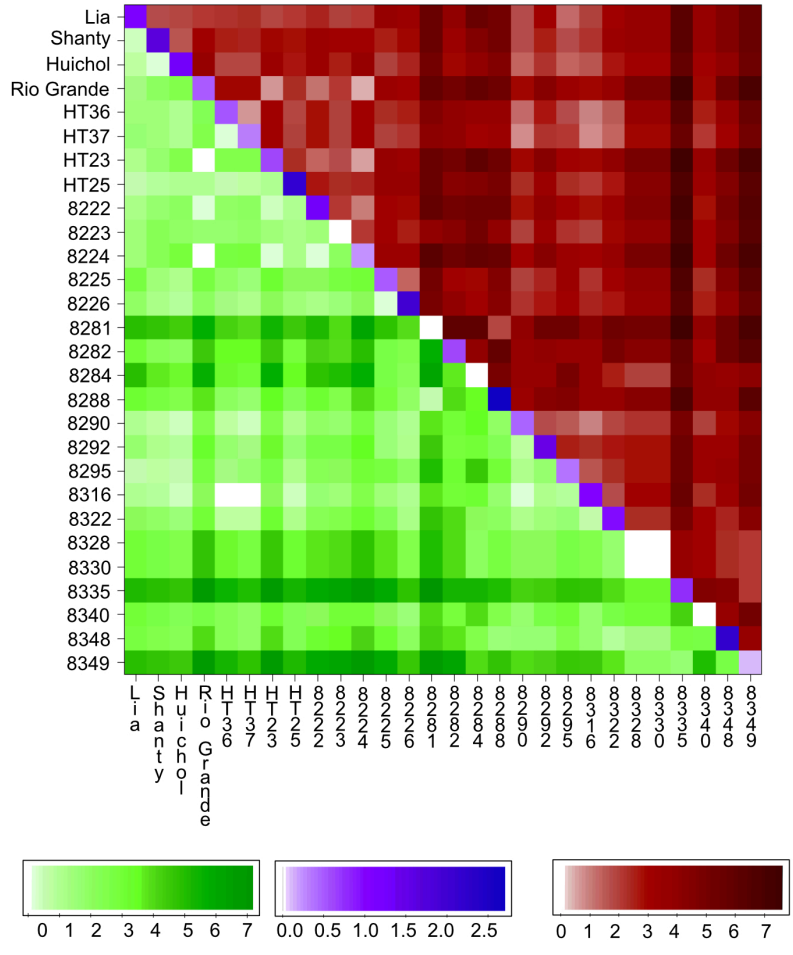


Figure 4. Heatmap of the average number of pairwise differences (ANPD) between the 28 Bolivian tomato accessions. Nei's distance (PiXY-(PiX+PiY)/2; below the diagonal), ANPD within the corresponding accession (PiX; the diagonal), and ANPD between the accessions (PiXY; above the diagonal). Pairwise differences were estimated using the number of different alleles as a distance method.

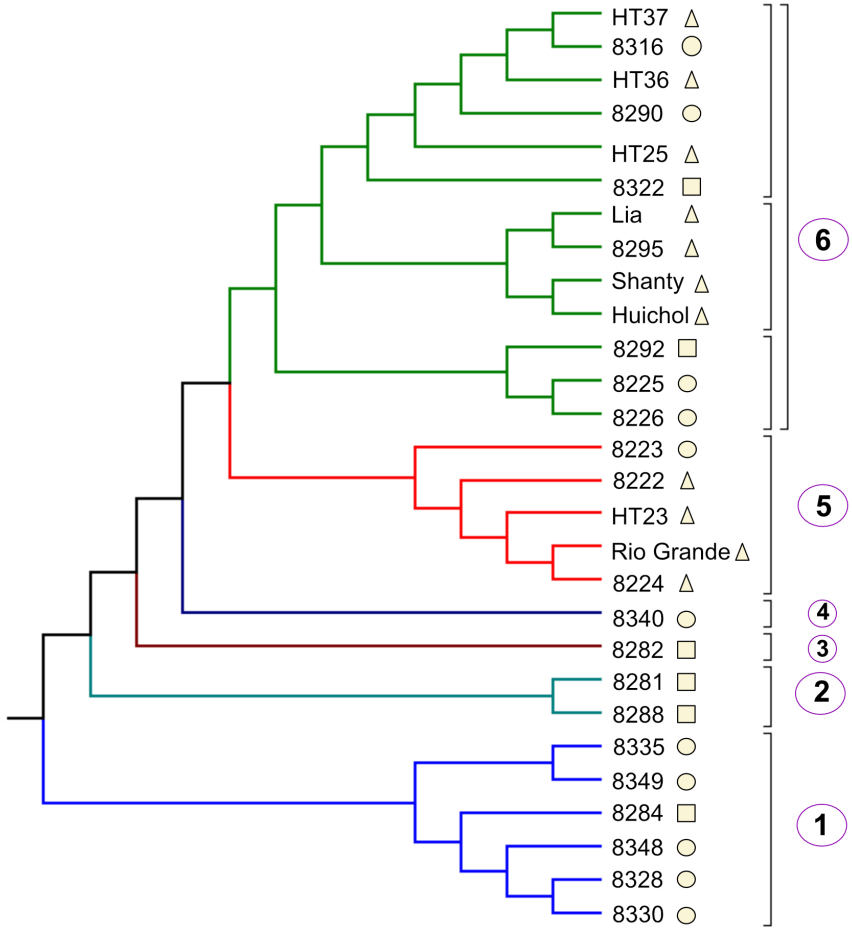


Figure 5. Unweighted pair group method with arithmetic mean (UPGMA) dendrogram depicting the clustering pattern of the 28 Bolivian tomato accessions. Different line colours represent different clusters (and two solitary accessions, BOL-8390-HT and BOL-8340-HT). Shapes next to accession represents the plant growth type (PGT): Triangle is determinate, round is semi.determinate and square is indeterminate PGT.

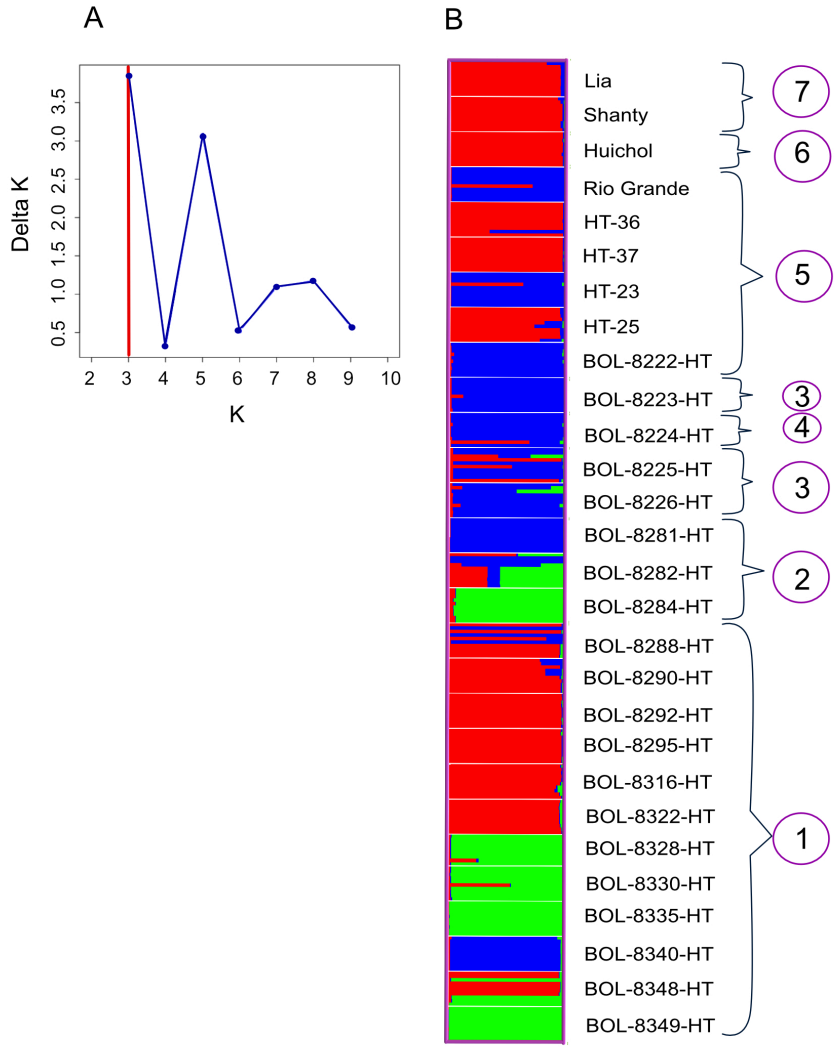


Figure 6. A) Delta-K plot showing the optimum number of genetic populations (K = 3) representing the 28 tomato accessions. B) Graphical display of the optimal genetic structure of the 28 accessions, each of which is represented by 9-10 individual genotypes. Red, blue and green colours represent different genetic populations (clusters). Each accession is delineated by white lines, and the proportion of colours in each accession represents the average proportion of alleles from different genetic populations. The sources of the accessions are as follows: 1 = La Paz, 2 = Beni, 3 = Sucre, 4 = Santa Cruz, 5 = Cochabamba, 6 = Thailand, and 7 = Israel.

The UPGMA clusters and the structure graph in Figure 5 showed genetic differentiation between tomato accessions with indeterminate and determinate plant growth type (PGT). Accessions with indeterminate PGT were mainly located in clusters 1 to 4 of the UPGMA dendrogram, whereas accessions with determinate PGT were located in clusters 5 and 6. In the structure graph (Figure 6-B), the indeterminate PGT accessions BOL-8328-HT, BOL-8330-HT, BOL-8335-HT and BOL-8349-HT had very low genetic admixture, represented by green bars. Accessions with determinate PGT appeared to have more admixture, although they were mainly represented by red bars. These results agree with findings in another study on tomato, which showed less genetic admixture in landrace and wild populations than in cultivars (Corrado *et al*., 2013). One of the causes of genetic admixture is crossbreeding of genotypes with different genetic backgrounds for trait improvement (Bai & Lindhout, 2007). Hence, crossbreeding could have contributed to the increased genetic admixture seen in accessions with a determinate plant growth type.

The UPGMA dendrogram clustered very closely the accessions BOL-8328-HT and BOL-8330-HT, Rio Grande and HT-23, and HT36 and HT37, suggesting a "duplication" of genetic material. The identification of duplicate accessions through genetic diversity analyses helps reduce the number of *ex situ* conserved accessions in gene banks (Razdan, 2006).

Unlike previous genetic research on Bolivian tomatoes (Torrico *et al*., 2015), in this thesis novel genetic diversity data were produced by only targeting *Solanum lycopersicum* accessions comprising both cultivated and wild populations. This analysis shed light on the genetic relationship between the 20 Bolivian accessions (wild and landrace) and modern cultivars preferred by Bolivian farmers due to their adaptability to open-field conditions under drought and higher temperatures, as well as breeding lines from breeding programmes intended to develop new cultivars that are well-adapted to challenging climate conditions.

Analysis of quality traits in tomato fruit

External tomato fruit traits and internal tomato quality traits involved in plant survival, fruit quality and in positive effects on health were explored under controlled conditions in Papers II and III. Empirical evidence of variations in quality traits during tomato maturation, and of the accession effect, was collected in Paper II, where only cultivars and advanced lines were assessed. Profiling of polyamines, carotenoids, and vitamin C concentrations, their relationship with common quality traits and possible explanations for observed differences were explored for 29 Bolivian accessions at mature stage in Paper III.

4.2.1 ANOVA

Analysis of variance (ANOVA) of eight accessions at six fruit maturity stages (Paper II) revealed significant differences between the accessions, maturity stage and their interaction for almost all quality traits evaluated. These included external quality traits (weight, firmness, colour), internal quality traits (total soluble solid, titratable acidity, and TSS/TA ratio), and bioactive compounds (putrescine, spermidine, spermine and total polyamines). This significant variation due to accession and maturity stages on quality traits was also reported in other crops (Johansson et al., 2014; Vagiri et al., 2013).

Another ANOVA analysis at mature stage in 29 accessions (Paper III) showed significant differences between accessions regarding beta-carotene, total carotenoids, trans-cis lycopene, lycoxanthin, vitamin C, colour a* value (redness), weight and firmness at maturity. A strong influence of accession has been reported previously for lycopene and beta-carotenoids (Bhandari *et al*., 2016; Roselló *et al*., 2011) and for polyamine content (Yahia *et al*., 2001).

4.2.2 Bioactive compounds

Accessions BOL-8226-HT, BOL-8295-HT and BOL-8306-HT displayed significantly higher concentrations of lycoxanthin than accession BOL-8311-HT (Figure 7A). Beta-carotene and lycopene concentrations also differed significantly between the accessions (Figure 7B). For example, accession BOL-8222-HT had significantly higher levels of lycopene than the yellow accessions BOL-8348-HT, BOL-8292-HT, BOL-8223-HT, BOL-

8297-HT, BOL-8277-HT, BOL-8290-HT and BOL-8226-HT. On the other hand, the beta-carotene concentration in the all yellow accessions was statistically than the red accessions BOL-8281-HT, BOL-8329-HT, BOL-8331-HT, BOL-8333-HT, and BOL-8354-HT.

Comparison of concentrations of the main polyamines across accessions at different maturity stages showed multiple patterns that were highly dependent on accession in Paper II, but little differences at mature stage in the 29 accessions in Paper III. For example, during maturation accessions Huichol, Rio Grande (RG) and HT-36 exhibited an increase in PUT, while in accessions HT-37 and HT-25 no significant differences were detected (Figure 3B in Paper II). Accessions Huichol and HT-23 showed a considerable decrease in spermidine concentration during maturation, while no significant differences were detected for RG or HT-37 (Figure 3C in Paper II). On the other hand, at mature stage, only BOL-8313-HT showed significant differences compared with five accessions in PUT concentration (BOL-8282-HT, BOL-8311-HT, BOL-8223-HT, BOL-8226-HT and BOL-8290-HT), and BOL-8354-HT had higher levels of SPD compared with other two accessions (BOL-8313-HT and BOL-8292-HT) (Figure 7D).

The total carotenoid concentration in the red accession BOL-8222-HT was significantly different to that in all yellow accessions, but was similar to that in all red accessions except BOL-8328-HT (Figure 7C). Comparison of total carotenoid concentrations (Figure 7C) and vitamin C concentrations (Figure 7E) showed that the accession effect was more significant than red versus yellow colour skin subgrouping. Higher levels of vitamin C were found in the wild yellow accessions BOL-8277-HT and BOL-8348-HT, and these were significantly different from the yellow accessions BOL-8223-HT, and BOL-8226-HT and seven red accessions (BOL-8295-HT, BOL-8222-HT, BOL-8313-HT, BOL-8225-HT, BOL-8316-HT, BOL-8340-HT and BOL-8320-HT). A previous study developed a cultivar richer in vitamin C through with an interspecific cross between wild tomato relative (*S. peruvianun*) and cultivated tomato (Stevens & Rick, 1986).

Although variation during maturation has been reported in previous studies, these tested only a few accessions at the same time (Anwar *et al*., 2019; Nambeesan *et al.*, 2010; Yahia *et al*., 2001) or showed differences across accessions but only at the mature stage (Goyal *et al*., 2016; Nishibori *et al*., 2007). The results presented in this thesis show the importance of understanding the variation depending on accession combined with maturity

stage, with accession significantly affecting polyamine content during maturation (see Figure 3 in Paper II) but being almost irrelevant in the mature stage (Figure 7D).

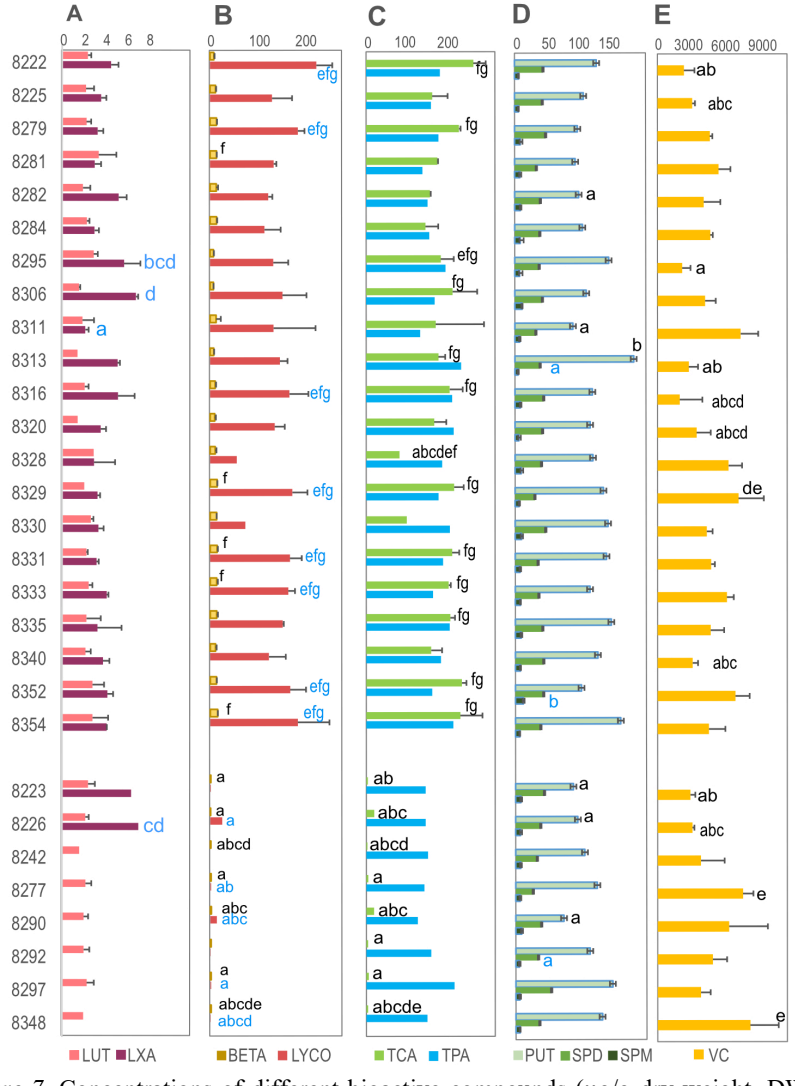


Figure 7. Concentrations of different bioactive compounds (µg/g dry weight, DW) at mature stage in 29 tomato accessions from the Bolivian germplasm collection. A) lutein (LUT) and lycoxanthin (LXA). B) Beta-carotene (BETA) and trans-cis-lycopene (LYCO). C) Total carotenoids (TCA), total polyamines (TPA). D) Putrescine (PUT), spermidine (SPD) and spermine (SPM). F) Vitamin C (VC). Difference among accessions was evaluated with Tuckey test. Different letters indicate statistically significant differences between accessions (p<0.05, Tukey test).

Principal component analysis (PCA) in Paper II revealed component differentiation along maturity stages, where quality parameters such as colour (a*, b* (blueness), Chroma value (brightness)), TSS and TA were in the positive quadrant. Firmness, vitamin C and L* value (lightness) were in the negative quadrant of principal component 1 (PC1) during maturation (Figure 8). This is in agreement with findings in previous studies of changes in colour, TSS and TA during maturity (Arias *et al*., 2000; Clément *et al.*, 2008; Zsom-Muha *et al.*, 2008). In PC2, total polyamines (TPA) and putrescine (PUT) were located on the positive side, and spermidine (SPD) and spermine (SPM) on the negative side (Figure 8), which suggests that polyamine concentrations are affected by maturation. Previous studies have demonstrated that spermidine can delay maturation (Nambeesan *et al*., 2010) and that an abundance of putrescine is present in mature stages (Yahia *et al*., 2001). However, in Paper II the sum of PC1 (34%) and PC2 (20%) only explained 54% of the variation, leaving 46% of variation attributable to other unknown factors.

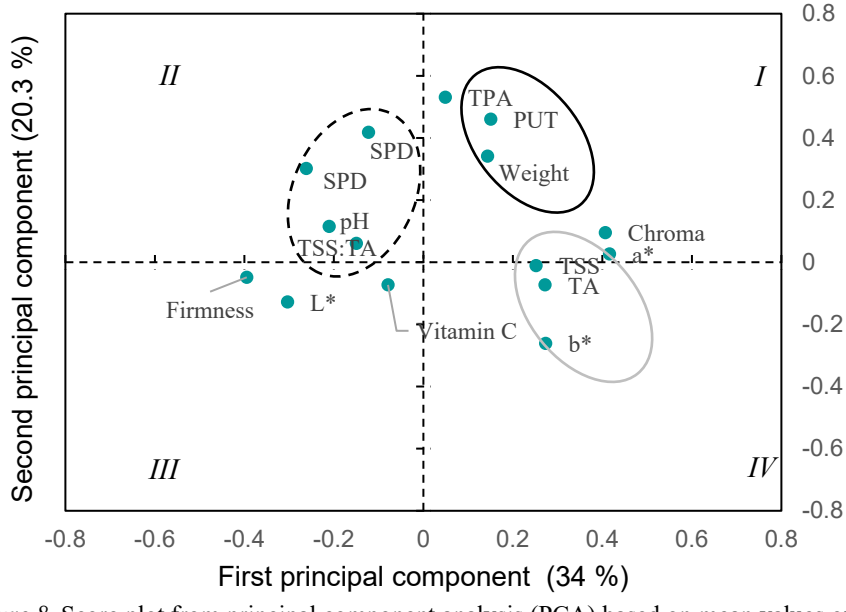


Figure 8. Score plot from principal component analysis (PCA) based on mean values of external fruit quality traits: Weight, firmness, colour (Chroma (brightness); a* value (redness), b* value (blueness), L* value (lightness)). Bioactive compounds: vitamin C, total polyamines (TPA), putrescine (PUT), spermidine, (SPD), and spermine (SPM)). Sensory parameters: Total soluble solids (TSS), titratable acidity (TA), TSS/TA ratio and pH.

In the PCA analysis of multiple bioactive compounds in mature tomatoes in Paper III, colour a* value, representing redness (López Camelo & Gómez, 2004), total carotenoids and total lycopene clustered together in the upper right of the PCA plot (Figure 9). The a* value was also found highly correlated (p<0.001) with total carotenoid concentration (r = 0.87), and with lycopene contents (r = 0.84) (Figure 5 in Paper III).

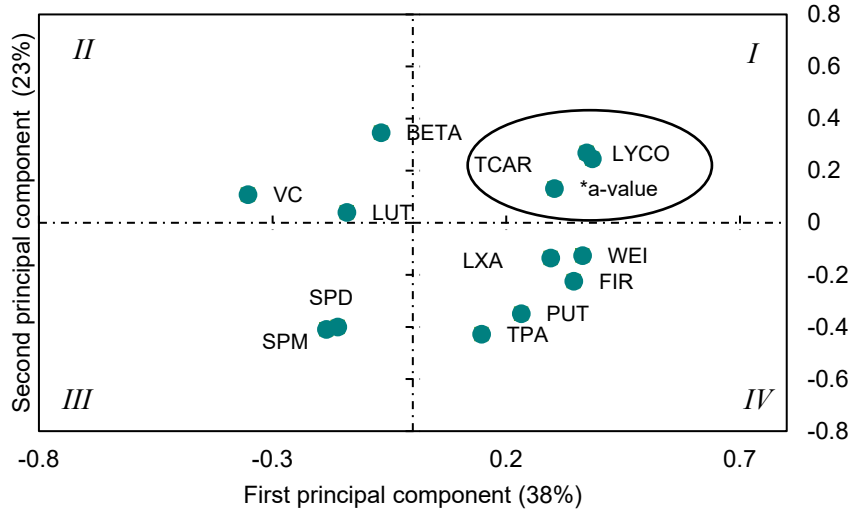


Figure 9. Score plot from principal component analysis (PCA) with the first component (PC1) explaining 38% and the second component (PC2) explaining 23% of the variation in bioactive compound concentrations and quality traits in 29 tomato accessions at mature stage: vitamin C (VC), spermine (SPM), spermidine (SPD), lutein (LUT), beta-carotene (BETA), total polyamines (TPA), putrescine (PUT), lycoxanthin (LXA), total carotenoids (TCAR), total lycopene (LYCO), a* value (redness), weight (WEI) and firmness (FIR).

The variation seen in bioactive compound concentrations between accessions indicates a strong effect of accession on polyamine content during maturation and on carotenoid content, vitamin C content, firmness, size and weight. It also suggests a disassociation of polyamines content with carotenoids, and of vitamin C with carotenoids, under no-stress conditions. However, artificial enhancement of polyamines leads to an increase in carotenoid concentrations (Neily *et al*., 2011; Stommel, 2007). Carotenoid accumulation can also lead to an increase in vitamin C (Gupta *et al*., 2019), demonstrating that an external force may induce a cascade of biochemical changes in the plant compared with controlled conditions where plants are

protected from stressors. Identifying accessions rich in putrescine, carotenoids and vitamin C would be useful for breeding programmes, which could target any of those bioactive compounds.

4.3 Analysis of tomato value chains in traditional and new production regions in Bolivia

Using data obtained in four case studies, 185 interviews and six direct observation campaigns, a tomato value chain map was developed in which critical points, common obstacles and differences between case study regions that influenced tomato quality traits from production to consumer were identified (Paper IV). The four case studies showed limited integration between vertical links (production, commercialization and consumption) and horizontal links (farmers, supply providers, associations, pesticide companies, wholesalers, local and national representatives) in the value chain. For example, there is currently little coordination in managing production to reach the final consumer effectively, due to lack of knowledge and poor time management. As a result, farmers do not have the power to influence the price setting process. This affects farmers' perception of tomato abundance or scarcity and, based on this limited perception, farmers make decisions on future investments. Hence, national and local policies for tomato value chain establishment must seek to integrate the sectors.

Along the tomato value chain, the following five critical aspects were identified: i) landscape and land access; ii) cultural practices; iii) harvesting process; iv) packing process; and v) transport system/s. The combination of these aspects affected tomato shelf-life and overall quality parameters. Figure 10 shows the tomato value chain in the four case study regions in Paper IV and significant findings in each case study.

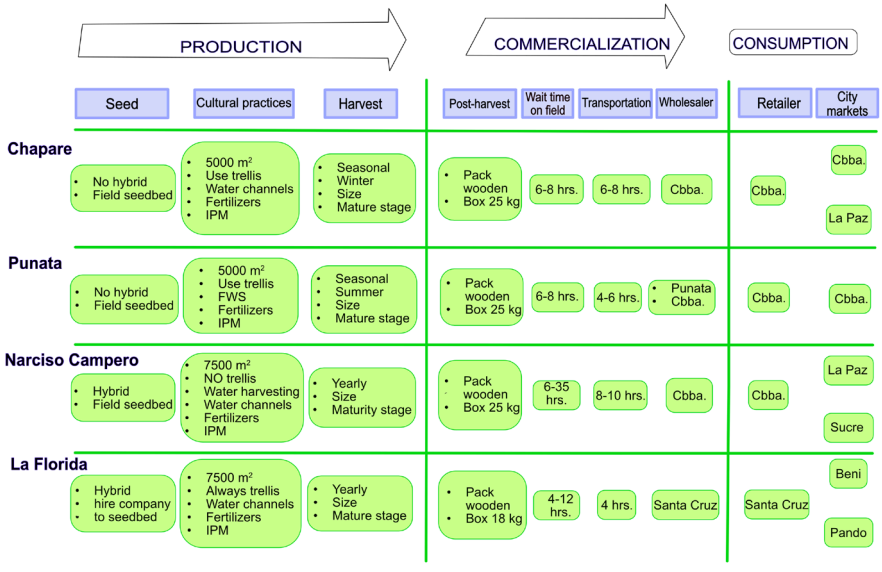


Figure 10. Diagrammatic representation of case studies on the Bolivian tomato value chain (TVC) in four provinces. IPM integrated pest management, CBBA Cochabamba, trellis (support for growing tomatoes), water harvesting (hole with water collected from rain), water channels (water channelled from rivers), FWS (fresh water spring).

4.3.1 Cultural practices

Cultural practices differed between farmers depending on their experience of tomato production, including seed choices, seedbed preparation, use of trellis, application of fertilisers and pesticides and watering the plants (Figure 10). All these factors affect final tomato quality at harvesting. Seed choice is part of quality assurance and hybrid seeds have become highly relevant due to their potential vigour (Cheema & Dhaliwal, 2004). Farmers who participated in the case studies in Chapare and Punata provinces prefer to use open pollinated seeds, whereas for farmers who participated in the La Florida case study hybrid seeds are the first choice. In addition, La Florida farmers usually know the seed producer and they hire nursery seedling expertise as a regular practice. The Narciso Campero case study revealed that hybrid seeds are a common choice for more than 50% of the farmers surveyed, while open pollination, seeds from the previous harvest or a combination of seed sources are the common approach for the other 50% of farmers interviewed (Figure 11).

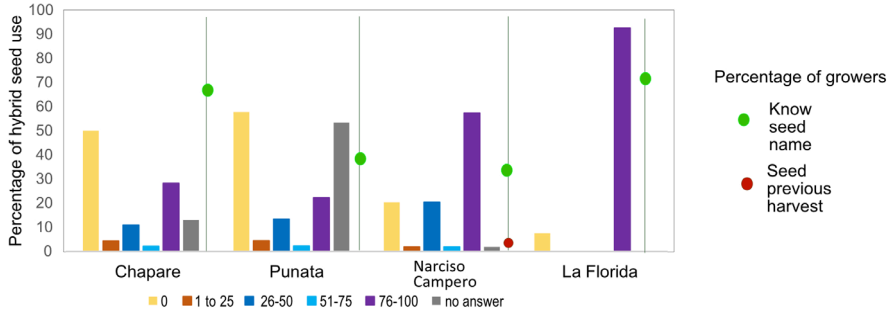


Figure 11. Bar graph showing seed choices of farmers in the four case study areas. Each bar represents farmers' preference for using hybrid seeds (UHS) on a scale of 0 100%: only open pollinated seeds (yellow bars); 1-25% UHS (orange bars); 26-50% UHS (dark blue bars); 51-75% UHS (sky blue); 100% UHS (purple bars). Green dots indicate acknowledgment of seed producer, and red dots indicate use of seeds from previous harvest according to the farmers.

Use of trellis, the right amount of fertilisers, shoot pruning and other plant protection techniques ensure high quality and high yield (Rathore *et al*., 2021). Paper IV revealed significant variation in cultural activities between the case studies. For example, in Chapare, Punata and La Florida, trellising was the most common method of supporting tomato plants, while using trellis as a support structure was considered a waste of time and resources in Narciso Campero. Fertilisation methods and pesticide applications were similar in the four case study areas. Fertiliser was applied once or twice during the production cycle, and pesticides were applied regularly, starting from the appearance of disease symptoms and continuing until harvesting of the tomatoes, with an average of 6-7 days between applications. There is growing evidence of overuse of pesticides and negative consequences for farmers and the environment. Correct application of pesticides is highly dependent on farmers' expertise in spotting and differentiating between diseases (Bempah *et al.*, 2011; Ciancio & Mukerji, 2008; Danielsen & Kelly, 2010; Elgueta *et al*., 2020; Montaño Riveros, 2009; Paudel *et al*., 2016; Simione, 2018).

Access to water during the dry season is crucial to maintain fruit quality (Sato *et al*., 2004; Saure, 2014). In Bolivia, the use of water resources is managed through communities or associations. In Chapare, farmers

occasionally irrigate their plants using water from rivers. In Punata, farmers use pits and water resources in turn, in order to ensure fair distribution of water among neighbours. In Narciso Campero, farmers hire private tanks and use water harvesting holes, while farmers in La Florida mainly use rivers to irrigate their tomato crops (Table 4 in Paper IV). Farmers from Punata, Narciso Campero and La Florida reported water scarcity and more challenging plant-growing conditions in the past decade. Similar findings have been reported in India, where water shortage is becoming a common problem (Hans *et al*., 2021). Tomato production is seasonal in Chapare (from April to September) and Punata (from October to March). Production is year-round in Narciso Campero and La Florida, with fluctuations according to temperature. Most of the farmers interviewed harvest tomatoes once the fruits mature, while a few farmers from Narciso Campero had decided not to harvest based due to low prices caused by overproduction. Overproduction causes a drop in market prices due to a lack of channels in the value chain to process fresh tomatoes into different food products. This results in inefficient contributions of tomato cultivation to food security and lower income for tomato farmers, especially on farms exclusively dedicated to tomato production (Hans *et al*., 2021; Karki *et al*., 2021).

4.3.2 Harvesting process

Tomato is a climacteric fruit where ethylene production plays a key role in maturation (Sammi & Masud, 2009), affecting firmness and change of colour (Iqbal *et al*., 2017). Harvesting may induce a burst of ethylene production, increasing the risks of deterioration and over-ripening (Ansari & Tuteja, 2015). The case studies in Paper IV revealed that containers used at harvest and to transfer tomatoes to the packing station generate pressure, and heat within the tomatoes, which might trigger overproduction of ethylene synthesis. Another factor was labour availability in high harvesting season, which determined working speed, increasing dramatically the time from harvesting to packing.

4.3.3 Packing process

Female workers in the case study areas usually sort and pack tomatoes by appearance, maturity stage and size, in line with previous reports (Thompson & Wainwright, 1991). Tomatoes are packed in wooden boxes with or without surface protection. Box capacity varies between 24 and 29 kg in Chapare, Punata and Narciso Campero, while in La Florida the maximum capacity is slightly lower (20 kg). Boxes are overloaded to reach maximum box capacity (Figure 6 in Paper IV) and they are piled up outdoors exposed to the sun, which negatively affects fruit quality (Woolf & Ferguson, 2000).

4.3.4 Transportation system

Once ready to transport, boxes with harvested tomatoes wait up to eight hours in the field in Chapare and Punata, while in Narciso Campero they can wait up to 24 hours before being loaded onto a truck. In the truck, tomato boxes are piled up to six high in multiple columns, overloading the truck to travel from Chapare and Punata to Cochabamba city (8 and 4 hours, respectively), and eight hours from Narciso Campero to Cochabamba (Figure 11). In La Florida, it takes 4 to 12 hours between packing and starting to transport the fruits, and a further four hours to arrive in Santa Cruz de la Sierra city in eastern Bolivia (Figure 7 in Paper IV).

The poor type of packing, long waiting time for transportation, transportation with overloaded tracks without a cooling system and long time it takes to reach the market place drastically constrain fruit quality in all four case study areas. The longer the distance from farm to market place, the lower the percentage of marketable tomatoes. In La Florida and Punata, tomatoes endure a 16-18 awaiting period and transportation hours (APTH), reducing marketable tomato volume from 100% to 85 and 80%, respectively. In Chapare and Narciso Campero (APTH up to 18 or 45 hours, respectively), the reduction in marketable tomatoes is even greater, from 100% to 75% and 70%, respectively, inducing accelerated ripping of the fruits and deterioration of their physical appearance. Evaluations of tomato quality after transportation showed different damage levels on tomato skin and different degrees of deterioration in appearance due to packing and transportation procedures. Tomatoes collected at the turning stage showed

greater firmness and lower TSS content than mature tomatoes in all four case study areas (Table 6 in Paper IV), as reported also in other studies (Kader, 2008; Nunes, 2008). However, in Narciso Campero TSS contents was significantly altered due to water stress during production, showing the effects of the stress conditions (Lahoz *et al.*, 2016).

5. Conclusions

This thesis studied genetic diversity, population structure, and composition and concentrations of bioactive compounds in cultivated and wild populations of tomatoes sampled from the core germplasm collection of Bolivia. Bolivia is part of the centre of origin and distribution of wild tomato relatives, but relatively low genetic variation was observed within accessions from the germplasm collection. However, genetic differentiation between accessions was high, with over 75% of the total variation attributable to accession. There was also significant genetic differentiation between accessions from different geographical regions of origin, accessions with different plant growth type, wild and cultivated accessions, and accessions with different fruit characteristics.

Profiling of polyamine, carotenoid and vitamin C content in 29 accessions at the mature stage revealed that accession was a determining factor in the relative concentrations of these bioactive compounds. Concentrations of the carotenoids lycoxanthin, beta-carotene and trans-cis-lycopene and vitamin C content were also highly affected by accession. Accessions with a high content of vitamin C, carotenoids or putrescine were identified, but no accession was found to contain high levels of all compounds analysed. No correlation was found between polyamines and carotenoids, or between polyamines and vitamin C, in tomatoes grown under controlled conditions. Plant breeding programmes can apply this extensive information on bioactive compounds in different Bolivian tomato accessions to include desirable traits in plant breeding.

Polyamines content and composition were examined further to determine the interaction between tomato maturation stage and accession effects on putrescine, spermidine and spermine content. Comparisons of accessions and maturation stages showed that putrescine was the most abundant polyamine

in all accessions at the mature stage, while spermidine was nearly five times more abundant than spermine. Spermidine and spermine concentrations were correlated, with typically higher contents in immature tomatoes in all accessions. However, putrescine, spermidine and spermine concentrations fluctuated during maturation and no common pattern was identified, confirming the need to profile individual polyamines in each accession. Common tomato fruit quality traits, such as deep colour, higher total soluble solid contents and high titratable acidity, were associated with more mature stages. Considering the previous lack of data on Bolivian core germplasm, this is valuable new empirical information on the bioactive compound profile in tomatoes, which is part of a coping mechanism in response to stressors. The results can be used to develop new accessions with germplasm already adapted to challenging climate conditions.

Analysis of Bolivia's tomato value chain from farm to market and the impact on quality traits revealed several critical stages responsible for inducing a deterioration in tomato quality. During the production stage, seed selection, cultural practices, water access, overuse of pesticides and inappropriate harvesting affected yield, size, appearance and disease-free fruits. During the transport stage, overloaded and stacked boxes, overloaded trucks, long time from farm to market and little or no connection between actors along the tomato value chain induced further quality deterioration before the products reached the consumer.

In case studies of the tomato value chain in four provinces of Bolivia, the principal actor identified was the tomato farmer, who has to grow a challenging crop, but also deal with external forces such as rising temperatures and water shortages during production. Farmers were also found to be involved in harvesting and packing, and some in transportation and marketing, activities that consumed valuable time for the farmers, who need to develop or update skills during tomato production. The case studies also revealed that there is currently little coordination in managing primary production to match consumer demand, due to lack of knowledge and poor time management among farmers. Farmers therefore do not have the power to influence the price setting process and secure their income. Overall, the results demonstrated a need for more research on whether the concentrations of bioactive compounds, such as polyamines, in tomato fruits are affected by the drought conditions commonly attributed by farmers to climate change,

and on improving stages in the tomato value chain affecting the quality of the marketed product.

6. Future perspectives

The work in the present thesis have resulted in a number of ideas that needs further attention as described below;

Time and resources should be invested to complete the genetic diversity, population structure and bioactive compound profile for all wild tomato relatives from the Bolivian germplasm bank.

Content of polyamines, carotenoids and vitamin C in accessions from genetically diverse plant material should be evaluated and be utilized to carry out pre-breeding research on drought tolerant accessions.

Popular science reports should be produced in Spanish for the public, to spread knowledge on critical points during the tomato value chain. The value chain analysis performed in the present thesis should be presented to local authorities, tomato farmers, associations and farmers, to identify alternatives to protect the quality and secure food supply.

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Popular science summary

Bolivia is part of the centre of origin and distribution of tomatoes and the country maintains a core germplasm collection for food security purposes. Tomato is the most commonly grown vegetable worldwide. In developing countries with a suitable climate, such as Bolivia, open-field production of tomatoes is a source of employment and income in rural areas. However, field-grown tomatoes suffer different challenges, including pest and disease attacks, heat, drought and flooding. Important quality traits of tomatoes include external traits, such as fruit colour, size, flavour and texture, and internal attributes, such as sweetness, acidity, nutrient content and content of bioactive compounds. Consumption of bioactive compounds is known to provide positive health effects, *e.g.* it enhances protection against cardiovascular diseases and prevents different types of cancer. Polyamines and carotenoids are important bioactive compounds in tomatoes and their concentrations increase in stress conditions, such as those found in open-field production.

This thesis examined genetic diversity and fruit quality traits in Bolivian tomatoes from the national germplasm collection and assessed the tomato value chain (TVC) from primary production to market. Analysis of genetic diversity and population structure in 20 accessions, four cultivars and four advanced linesrevealed low genetic diversity in wild relatives collected in Bolivia. However, some of the accessions analysed differed significantly. Studies on tomato quality traits at different maturity stages under uniform growing conditions showed that polyamine concentrations, colour, sweetness, acidity, firmness and weight depended strongly on the accession. A more extensive analysis on 29 accessions and eight cultivars revealed no differences in polyamine content apart from in two accessions (BOL-8313-HT and BOL-8352-HT), but carotenoid and vitamin C concentrations

depended significantly on the accession. These differences might result from geographical region of origin, fruit colour and size.

Analysis of the tomato value chain in Bolivia identified five critical factors that combine to shorten tomato shelf-life and decrease the profitability of tomato production. These are: i) landscape and land access; ii) cultural practices; iii) harvesting process; iv) packing process; and v) transportation system/s.

The new information provided in this thesis can help breeders to utilise genetic diversity resources to develop new cultivars with high concentrations of bioactive compounds. Field studies also revealed the complexity of tomato production in a developing country and the different factors affecting the final quality of produce offered to consumers.

Populärvetenskaplig sammanfattning

Bolivia utgör en del av centrat för ursprung och distribution av tomater, och landet har engenbank för säker livsmedelsförsörjning. Tomat är den mest odlade grönsaken i världen. I utvecklingsländer med ett lämpligt klimat, som Bolivia, är produktion av tomater på friland en källa till sysselsättning och inkomst på landsbygden. Frilandsodlade tomater lider dock av olika utmaningar, inklusive angrepp av skadedjur och sjukdomar, värme, torka och översvämningar. Viktiga kvalitetsegenskaper hos tomater inkluderar yttre egenskaper, såsom fruktfärg, storlek, smak och konsistens, och inre egenskaper, såsom sötma, surhet, näringsinnehåll och bioaktiva föreningar. Konsumtion av bioaktiva föreningar är känd för att ge positiva hälsoeffekter, t.ex. ökat skydd mot hjärt-kärlsjukdomar och förebyggande av olika typer av cancer. Polyaminer och karotenoider är viktiga bioaktiva föreningar i tomater, och deras koncentrationer ökar under stressförhållanden, vilka finns vid produktion på friland.

Denna avhandling undersökte genetisk mångfald och fruktkvalitetsegenskaper hos bolivianska tomater från den nationella genbanken och bedömde tomaternas värdekedja från primärproduktion till marknad. Analys av genetisk mångfald och populationsstruktur i 20 accessioner och åtta sorter avslöjade låg genetisk mångfald hos vilda släktingar som samlats in i Bolivia. Vissa av de analyserade accessionerna skiljde sig dock betydligt åt. Studier av tomatkvalitetsegenskaper vid olika mognadsstadier under enhetliga odlingsförhållanden visade att polyaminkoncentrationer, färg, sötma, surhet, fasthet och vikt starkt berodde på accessionen. En mer omfattande analys av 29 accessioner och åtta sorter visade inga skillnader i polyamininnehåll, förutom i två accessioner (BOL-8313-HT och BOL-8352-HT), men karotenoid- och vitamin C-

koncentrationerna berodde signifikant på accessionen. Dessa skillnader kan bero på det geografiska ursprungsområdet, fruktens färg och storlek.

Analys av värdekedjan för tomater i Bolivia identifierade fem kritiska faktorer som tillsammans förkortar tomaternas hållbarhet och minskar lönsamheten i tomatproduktionen. Dessa är i) landskap och marktillgång; ii) Odlingsmetoder; iii) skördeprocess; iv) packningsprocess; och v) transportsystem.

Den nya informationen som tillhandahålls i denna avhandling kan hjälpa förädlare att använda genetiska mångfaldsresurser för att utveckla nya sorter med höga koncentrationer av bioaktiva föreningar. Fältstudier avslöjade också komplexiteten i tomatproduktion i ett utvecklingsland och de olika faktorer som påverkar den slutliga kvaliteten på de produkter som erbjuds konsumenterna.

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Article

Simple Sequence Repeat Markers Reveal Genetic Diversity and Population Structure of Bolivian Wild and Cultivated Tomatoes (*Solanum lycopersicum* L.)

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Abstract: The western part of South America is a centre of diversity for tomatoes, but genetic diversity studies are lacking for parts of that region, including Bolivia. We used 11 simple sequence repeat (SSR) markers (including seven novel markers) to evaluate genetic diversity and population structure of 28 accessions (four modern cultivars, four advanced lines, nine landraces, 11 wild populations), and to compare their genetic variation against phenotypic traits, geographical origin and altitude. In total, 33 alleles were detected across all loci, with 2–5 alleles per locus. The top three informative SSRs were SLM6-11, LE20592 and TomSatX11-1, with polymorphism information content (PIC) of 0.65, 0.55 and 0.49, respectively. The genetic diversity of Bolivian tomatoes was low, as shown by mean expected heterozygosity (He) of 0.07. Analysis of molecular variance (AMOVA) revealed that 77.3% of the total variation was due to variation between accessions. Significant genetic differentiation was found for geographical origin, cultivation status, fruit shape, fruit size and growth type, each explaining 16–23% of the total variation. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree and principal coordinate analysis (PCoA) scatter plot both revealed differentiation between accessions with determinate flowers and accessions with indeterminate flowers, regardless of cultivation status. The genetic profiles of the accessions suggest that the Bolivian tomato gene pool comprises both strictly self-pollinating and open-pollinating genotypes.

Keywords: core germplasm; AMOVA; UPGMA; landraces; advanced lines; modern cultivars

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1. Introduction

Bolivia, Chile, Ecuador, the Galapagos Islands and Peru together constitute the centre of origin and distribution of tomatoes (*S. lycopersicum* L.) [1], although early domestication of tomatoes occurred in both the Andean region and Mexico [2]. Wild tomato relatives are still distributed naturally in the Andean region [3] and may harbour an untapped diversity of genes, which might be useful in diversifying the current cultivated tomato gene pool. In South America, the first domestication, affecting weight and fruit shape, occurred in Ecuador and Peru [4]. In Bolivia, tomato relatives grow in a wide range of ecosystems [5] and public Bolivian institutions are responsible for utilisation, conservation and characterisation of Bolivian core germplasm [6,7]. Some progress has been made in classifying the genetic diversity of Bolivian tomatoes by evaluation and comparison of quality traits among selected accessions. For example, there has been an evaluation and characterisation of 28 tomato accessions from Bolivian core germplasm [8] and a genetic study of 31 accessions from various subspecies of *S. lycopersicum* [9]. A wide range of phenotypic differences has been reported for Bolivian germplasm, despite a lack of

evaluations using molecular markers [2,9,10]. However, lack of knowledge on genetic variation in Bolivian tomatoes and their wild relatives impedes the successful use of Bolivian germplasm in breeding programmes.

Recent advances in genomics and molecular technologies have enabled the characterisation of genetic diversity and its effective use in commercial breeding [11]. The genome sequence of the tomato has recently been made available by the Tomato Genome Sequencing Consortium [12,13], greatly facilitating development of genome-based tomato breeding using specific markers. The increasing availability of sequencing data, together with the whole-genome reference sequence, also provides opportunities to develop and use molecular markers for population genetics analyses, including genetic variation within and among tomato populations [14]. Among the molecular markers currently available for population genetics studies, simple sequence repeats (SSR), also referred to as microsatellites, are among the most popular. SSRs are co-dominant markers mainly found outside genes and in non-coding regions of genes [15,16]. These markers have been used to determine polymorphism in tomato landraces in studies with different objectives, such as identifying differences between cultivars [17], conserving representative plant material [18], identifying desirable quantitative traits [19] and promoting pyramidal marker-assisted selection to build disease resistance [20].

Current knowledge on the genetic variation in Bolivian core germplasm is insufficient. Further research is needed to understand the genetic relationship between Bolivian tomato germplasm and the domestication process, and also to unveil desirable quality and agronomic traits [3]. The aim of the present study was thus to determine the genetic diversity of Bolivian tomatoes and their level of relatedness. This was done by analysing 28 accessions of tomato, consisting of four cultivars, four advanced breeding lines and 20 accessions from the Bolivian core germplasm collection. A further aim was to identify possible relationships between the genetic variation of the accessions and variations in their phenotypic traits (fruit size, shape and colour), cultivation status, growth type, geographical region of origin and growing site altitude.

2. Materials and Methods

2.1. Plant Material, Planting, Sampling and DNA Extraction

The cultivated and wild tomato germplasm obtained from different sources for this study are referred to hereafter as "accessions" for the sake of simplicity. The 28 tomato accessions analysed represented cultivars, advanced lines, landraces and wild populations (Table 1 and Figure 1). Three hybrid cultivars ('Lia', 'Shanty', 'Huichol') commonly used by tomato farmers in Bolivia were purchased from a local market in Cochabamba, Bolivia. Cultivars 'Lia' and 'Shanty' are marketed by Hazera Seeds Ltd. and 'Huichol' by Seminis. An open-pollinated cultivar ('Rio Grande') and four advanced breeding lines were obtained from the Bolivian National Center for Horticultural Seed Production (CNPSH) [16]. The remaining 20 accessions (landraces and wild populations, see Table 1), which were originally collected from the geographical positions shown in Figure 2, were selected from the accessions held at the Horticultural Germplasm Bank—National Germplasm Center—(BGH-BNG) located in CNPSH. The accessions held at the BGH-BNG representing the Bolivian tomato core-germplasm were collected by the personnel involved in the establishment and management of a tomato gene bank. Of the 162 registered accessions at CNPSH collected between 1938 and 2010, only 119 wild relatives or landraces were originally collected in Bolivia (in Beni (7), Cochabamba (44), La Paz (63), Santa Cruz (2), and Sucre (3)) [5]. Of the 44 accessions from the Cochabamba region, only one cultivated accession was included in the present analysis. This is because the wild accessions collected share the exact same geographical location [5]. Pre-selection criteria such as significant geographical distance between accessions combined with region further narrowed the study sample to the most representative 20 accessions from the five regions (Table 1).

Table 1. Description of the 28 tomato accessions used in this study.

Accession/Commercial Name	Germ-Plasm Provider	Country of Origin	Region of Sampling Site in Bolivia	Domestication/ Breeding Status	Geographical Position of Sampling Site	Altitude of Sampling Site (Masl)	Fruit Shape	Fruit Size	Fruit Colour	Plant Flowering Type
Lia	Hazera	Israel	–	Cultivated	Not applicable	Not applicable	Cylindrical	Intermediate	Red	Determinate
Shanty	Hazera	Israel	–	Cultivated	Not applicable	Not applicable	Cylindrical	Intermediate	Red	Determinate
Huichol	Seminis	Thailand	–	Cultivated	Not applicable	Not applicable	Cylindrical	Intermediate	Red	Determinate
Rio Grande	CNPSH	Bolivia	Cochabamba	Cultivated	17°26′24″ S; 66°20′47″ W	2548	Cylindrical	Intermediate	Red	Determinate
HT-36	CNPSH	Bolivia	Cochabamba	Advanced line	17°26′24″ S; 66°20′47″ W	2548	Rounded	Very large	Red	Determinate
HT-37	CNPSH	Bolivia	Cochabamba	Advanced line	17°26′24″ S; 66°20′47″ W	2548	Rounded	Large	Red	Determinate
HT-23	CNPSH	Bolivia	Cochabamba	Advanced line	17°26′24″ S; 66°20′7″ W	2548	Cylindrical	Intermediate	Red	Determinate
HT-25	CNPSH	Bolivia	Cochabamba	Advanced line	17°26′24‴′ S; 66°20′47″ W	2548	Cylindrical	Intermediate	Red	Determinate
BOL-8222-HT	BGH-BNG	Bolivia	Cochabamba	Cultivated	17°23′03″ S; 66°08′05″ W	2858	High rounded	Intermediate	Red	Determinate
BOL-8223-HT	BGH-BNG	Bolivia	Sucre	Cultivated	19°17′43″ S; 64°22′33″ W	2201	High rounded	Very small	Yellow	Semi-determinate
BOL-8224-HT	BGH-BNG	Bolivia	Santa Cruz	Cultivated	17°24′00″ S; 63°53′00″ W	300	High rounded	Small, intermediate	Red	Determinate
BOL-8225-HT	BGH-BNG	Bolivia	Sucre	Cultivated	19°44′26″ S; 63°52′24″ W	1165	Slightly flattened	Very small	Pink	Semi-determinate
BOL-8226-HT	BGH-BNG	Bolivia	Sucre	Cultivated	19°48′26″ S; 64°00′29″ W	1143	Rounded	Very small	Yellow	Semi-determinate
BOL-8281-HT	BGH-BNG	Bolivia	Beni	Wild	14°52′10.7″ S; 61°04′42.3″ W	227	Rounded	Very small	Red	Indeterminate
BOL-8282-HT	BGH-BNG	Bolivia	Beni	Cultivated	Not reported	227	Slightly flattened	Small	Red	Indeterminate
BOL-8284-HT	BGH-BNG	Bolivia	Beni	Cultivated	15°08′47.4″ S; 61°02′15″ W	259	Slightly flattened	Very small	Red	Indeterminate
BOL-8288-HT	BGH-BNG	Bolivia	La Paz	Wild	15°47′32″ S; 60°58′41″ W	498	Rounded	Very small	Red	Indeterminate
BOL-8290-HT	BGH-BNG	Bolivia	La Paz	Wild	15°48′28″ S; 61°37′27″ W	594	Slightly flattened	Very small	Yellow	Semi-determinate
BOL-8292-HT	BGH-BNG	Bolivia	La Paz	Wild	16°15′47″ S; 61°41′44″ W	1676	Slightly flattened	Very small	Yellow	Semi-determinate

Table 1. *Cont.*

Accession/Commercial Name	Germ-Plasm Provider	Country of Origin	Region of Sampling Site in Bolivia	Domestication/ Breeding Status	Geographical Position of Sampling Site	Altitude of Sampling Site (Masl)	Fruit Shape	Fruit Size	Fruit Colour	Plant Flowering Type
BOL-8295-HT	BGH-BNG	Bolivia	La Paz	Wild	16°11′21.6′′ S; 67°43′29′′ W	599	Slightly flattened	Small, Intermediate	Red	Determinate
BOL-8316-HT	BGH-BNG	Bolivia	La Paz	Cultivated	15°58′12′′ S; 67°27′44′′ W	961–1030	Slightly flattened	Intermediate	Red	Semi-determinate
BOL-8322-HT	BGH-BNG	Bolivia	La Paz	Cultivated	16°11′53′′ S; 67°42′16′′ W	1725–1690	Slightly flattened	Very small	Red	Semi-determinate
BOL-8328-HT	BGH-BNG	Bolivia	La Paz	Wild	16°15′31′′ S; 67°41′32′′ W	1853–1870	Flattened	Very small	Red	Semi-determinate
BOL-8330-HT	BGH-BNG	Bolivia	La Paz	Wild	16°11′19′′ S; 67°43′29′′ W	1716–1720	Slightly flattened	Very small	Red	Semi-determinate
BOL-8335-HT	BGH-BNG	Bolivia	La Paz	Wild	16°20′21′′ S; 67°26′38′′ W	1124–1190	Slightly flattened	Very small	Red	Semi-determinate
BOL-8340-HT	BGH-BNG	Bolivia	La Paz	Wild	16°26′09′′ S; 67°28′26′′ W	1492–1550	Slightly flattened	Very small	Red	Semi-determinate
BOL-8348-HT	BGH-BNG	Bolivia	La Paz	Wild	16°28′35′′ S; 67°26′59′′ W	2021–2012	Rounded	Very small	Yellow	Semi-determinate
BOL-8349-HT	BGH-BNG	Bolivia	La Paz	Wild	16°28′35′′ S; 67°26′59′′ W	2021–2010	Rounded	Very small	Red	Semi-determinate

Note: Accession description sources can be retrieved without suffixes BOL-HT from the website http://germoplasma.iniaf.gob.bo/ (accessed on 27 July 2022).

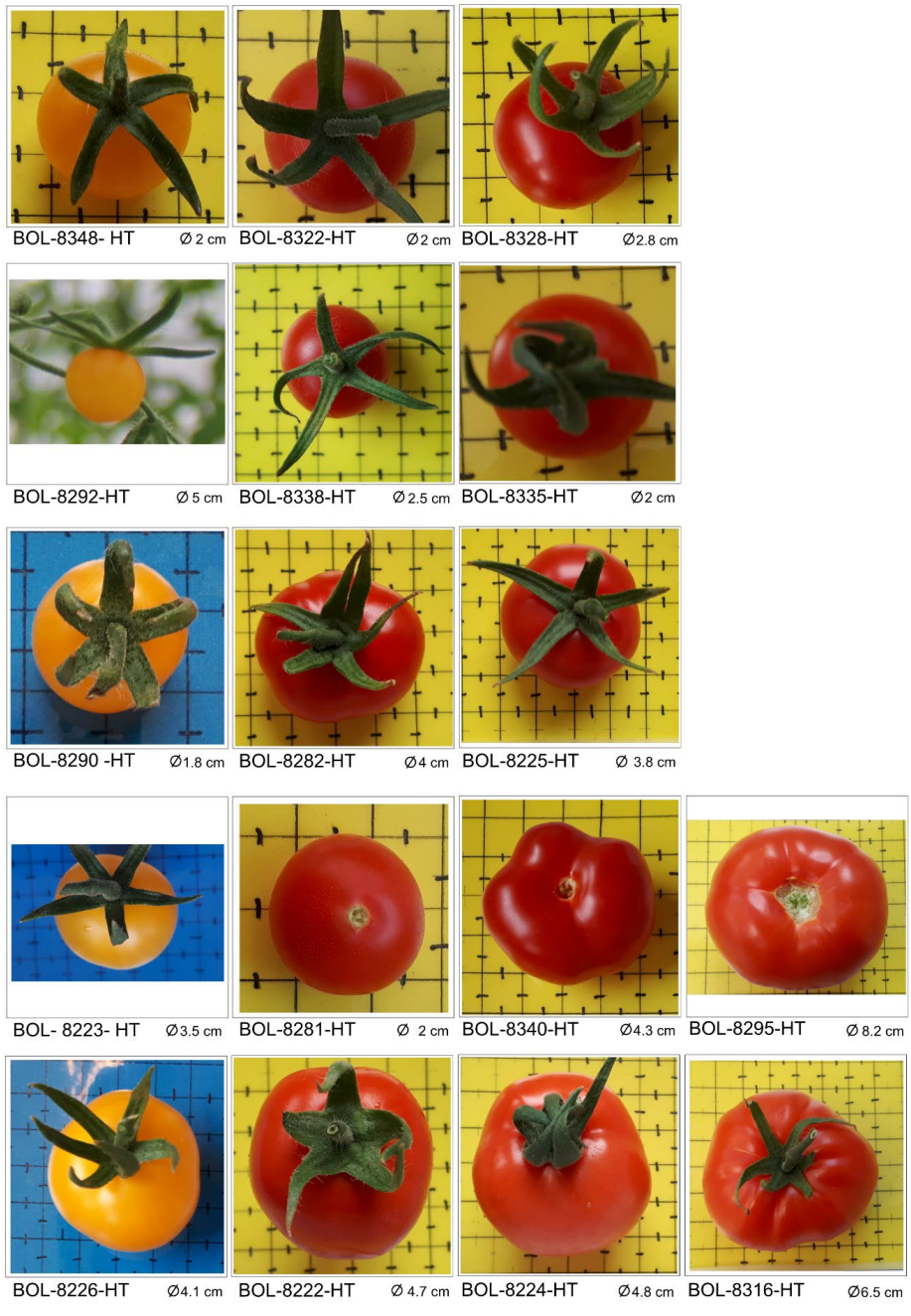

Figure 1. Images of 17 mature tomato fruits from Bolivian core germplasm.

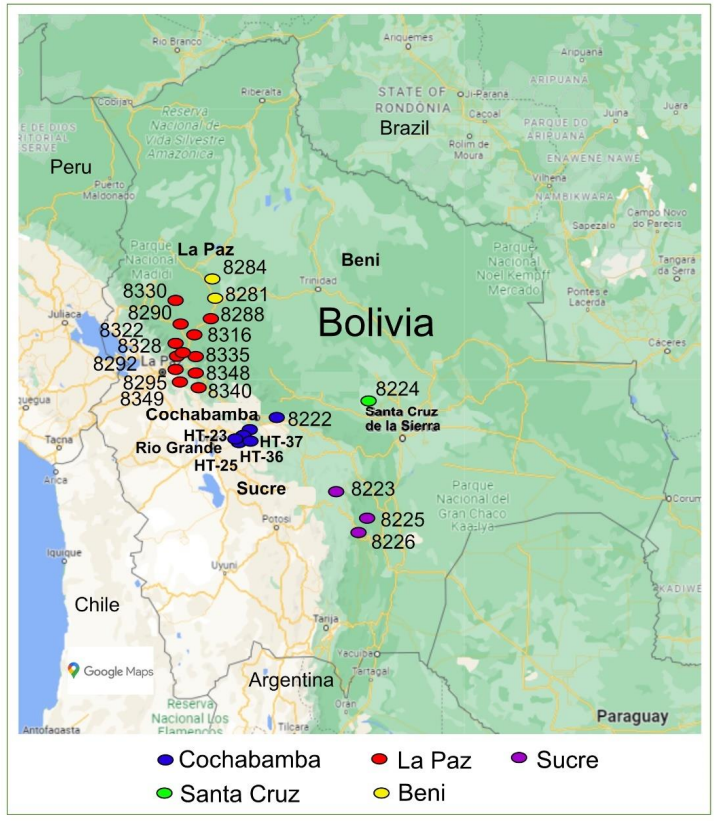

Figure 2. Geographical source of the 25 accessions collected or developed in Bolivia.

Seeds of the 28 accessions were planted in 4.5-L plastic trays filled with nutrient-rich soil (0.08 L per plug) in a greenhouse at the Department of Plant Breeding, Swedish University of Agricultural Sciences (SLU). The emerging seedlings were grown under day/night temperature of ±24 °C/19 °C, 16 h of light and 60% relative humidity until sampling of leaf tissue for DNA extraction at one month after planting. Young leaf tissue was sampled separately from 10 individual plants of all accessions except one, which was represented by nine plants (279 samples in total). The fresh leaf tissue taken from each plant was placed in a separate 2-mL Eppendorf tube containing two glass beads with diameter 3 mm, and immediately dipped in liquid nitrogen. The frozen samples were stored at −80 °C until DNA extraction.

The frozen leaf samples were homogenised for 1 min at 30 Hz in a Mixer Mill (MM400-Retsch GmbH, Haan, Germany). Then 400 µL of CTAB-based buffer (0.1 M Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, pH 7.5) were added to each sample, followed by incubation for 15 min at 56 °C and centrifugation (using Eppendorf 5427 R, Hamburg, Germany) at 9000× *g* for 3 min. A 200 µL subsample of the supernatant of each sample was transferred to a 96-well plate and DNA extraction was performed with a QIAamp DNA kit, using a QIAcube HT extraction robot (Qiagen, Hilden, Germany). The integrity of the extracted DNA was evaluated by agarose gel electrophoresis (1.5% *w*/*v*) and the quantity was determined using a NanoDrop spectrophotometer (ND-1000, Saveen Werner, Sweden). All extracted DNA samples were kept at −20 °C until polymerase chain reaction (PCR) analysis, while the working solutions (5 ng/µL) were kept at 4 °C for up to 24 h.

2.2. Identification of SSRs in the Tomato Genome and Primer Design

The genome of the *S. lycopersicum* cultivar 'Heinz 1706' (SL3.0 reference Annotation Release 103; (https://www.ncbi.nlm.nih.gov/assembly/GCF_000188115.4 (accessed on 27 July 2022)) was used to identify SSRs in the tomato genome, which were then utilised as new genomic analysis resources for tomato. First, genomic regions of 400 bp to 1200 bp, representing the 12 tomato chromosomes, were randomly sampled. These sequences were searched for identification of dinucleotide and trinucleotide repeat motifs, using WebSat, internet-based software developed for SSR identification [17]. Sequences containing the target SSRs were then further screened based on the suitability of the SSR positions in the sequences for primer design. Next, these sequences were compared against the tomato reference genome at the National Center for Biotechnology Information (NCBI) database, to identify those that are single copy (unique) in the tomato genome, using the Basic Local Alignment Search Tool (BLAST). This resulted in selection of 22 single-copy sequences (two sequences per chromosome) and two highly similar sequences (corresponding to TomSat9-2a and TomSat-2b SSR loci, see Supplementary Tables S1 and S2. The Primer3 program [18–20], targeting these SSRs, was used for primer design.

Ten genotypes were selected from the different tomato accessions for a first testing of the newly designed primer-pairs in amplifying the target SSR loci under optimised PCR conditions (described below). Twelve of the primer-pairs amplified extra fragments in addition to the target loci, and were therefore excluded, while the remaining primer-pairs amplified only their target loci. To confirm that they matched the target sequences, the amplified products of the 12 primer-pairs were purified and sequenced. Thereafter, the PCR products were purified using E.Z.N.A. Cycle Pure Kit V-spin (Omega Bio-tek; Norcross, GA, USA) and 2 µL of 10 µM sequencing primer and 15 µL of 1 ng/µL purified PCR product for each sample were mixed and sent to Eurofins Genomics Sequencing GmbH (Anzinger Str. 7, 85560 Ebersberg, Germany), where Sanger sequencing was conducted. Each amplified product was sequenced with both the forward and reverse primers used for the PCR. The DNA sequences of the PCR products were then aligned with their corresponding reference sequences using CLUSTAL X version [21], which confirmed amplification of the target loci.

In parallel with the development of the new SSR markers, 40 primer-pairs previously reported to amplify polymorphic SSR loci [22–25] were screened to determine the quality of their amplified products under optimised PCR reaction conditions. Four of these primer-pairs (a–d in Table 2) were selected for use in this study, together with the 12 newly developed primer-pairs (Table S1).

2.3. PCR Amplification and Electrophoresis

The 16 selected primer-pairs were used to amplify the target loci of 279 individual genotypes from the 28 tomato accessions. The 5′-end of the forward primers was labelled with either 6-FAM or HEX fluorescent dye (Sigma-Aldrich, St. Louis, USA), for detection of amplified products during capillary electrophoresis. A GCTTCT hexamer was added to the 3′-end of the reverse primers (PIG tailing) to prevent the Taq polymerase from adding non-template sequences to the PCR products, as described in Ballard et al. [26]. The PCR reaction solution was prepared for each sample using the following reagents from Thermo Fisher Scientific GmbH (Waltham, MA, USA) (V.A. Graciuno 8. LT-02241 Vilnius, Lithuania): 2.5 µL Dream Taq buffer (KCl, $(NH_4)_2SO_4$ and 20 mM $MgCl_2$), 0.3 µL dNTPs (25 mM), 7.5 µL of each forward and reverse primer (10 µM), 0.2 µL DreamTaq DNA Polymerase (5 U/µL), and 5 µL of 5 ng/µL DNA template. A negative control reaction, replacing the DNA template with sterile Millipore water, was also included.

The PCR analysis was carried out using a Bio-Rad thermal cycler S1000 (Hercules, CA, USA) with the following cycling parameters: initial denaturation for 3 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 3–5 °C below the primer's melting temperature and primer extension for 40 s at 72 °C, and a final primer extension for 20 min at 72 °C. After each PCR run, electrophoresis was performed using 1.5% agarose containing GelRed on randomly selected amplified products using a 50 bp

Gene Ruler ladder (Thermo Fisher Scientific GmbH, Dreieich, Germany) as size standard, followed by scanning with a BioDoc-It Imaging System (Upland, CA, USA). This led to exclusion of four of the 12 newly developed SSRs, because a significant number of samples failed to be amplified. Hence, the amplified products of 12 SSR loci were used in capillary electrophoresis, as described below.

The PCR products of the 12 SSR loci were multiplexed into four panels following the criteria described in Geleta et al. [27], except that each PCR product was diluted 1:10 in Millipore ultrapure water before multiplexing. This was followed by mixing each multiplexed PCR product (0.5 µL) with Hi-Di™ Formamide (9 µL) (Thermo Fisher Scientific, Waltham, MA, USA) and Size Standard Gene Scan—600 LIZ (9.7 µL) (Thermo Fisher Scientific, Austin, TX, USA), heating at 96 °C for 3 min and cooling on ice. Multiplexed PCR products were then separated by capillary electrophoresis as described in Andersson et al. [28], using an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA), at the Department of Plant Breeding, SLU, Sweden.

2.4. Data Analysis

The capillary electrophoresis step was followed by peak identification using GeneMarker version 2.7.0 (SoftGenetics, LLC, State College, PA, USA) software with the default settings [29]. Each peak was regarded as an allele and its size was determined using the GS600 size standard. Among the 12 SSR loci, locus TomSatX1-1 was monomorphic across the 28 accessions studied, and hence was excluded from the final data analysis. The alleles of each sample for the remaining 11 polymorphic SSR loci (Table 2) were exported to Excel and converted to genotypic data for subsequent statistical analysis.

Various genetic diversity parameters were estimated for each locus across all accessions and for each accession across all loci. POPGENE version 1.32 [30] was used to determine observed number of alleles (Na), effective number of alleles (Ne) and percentage of polymorphic loci (%PL). GeneAlEx 6.41 [31] was used to determine number of private alleles (NPL), number of locally common alleles (NLCA), expected heterozygosity (He), observed heterozygosity (Ho), Shannon information index (I), genetic differentiation (G_{ST}) and fixation index (F). Polymorphism information content (PIC) of each locus was calculated as described in Botstein et al. [32].

Analysis of molecular variance (AMOVA) was conducted to determine the variance within and between accessions and the variance at higher hierarchal level, using Arlequin version 3.5.2.2 [33]. Matrices of pairwise F_{ST} and average pairwise differences between and within accessions were used to generate graphs using a series of R scripts within Rcmd, a console version of the R statistical package, by triggering the command button added to Arlequin version 3.5.2.2 toolbar.

Nei's unbiased genetic distance between the 28 accessions was calculated using GeneAlEx 6.41 and then used as input data for Unweighted Pair Group Method with Arithmetic Mean (UPGMA)-based cluster analysis using MEGA7 software, where the optimal tree with the sum of branch length −2.13486148 is shown [34,35]. The genetic distance value was also used for principal coordinate analysis (PCoA) using GeneAlEx 6.41 to visualise the genetic relationship between the accessions. Bayesian statistics-based population structure analysis was conducted using STRUCTURE version 2.3.4 [36], based on the admixture model implementing 100,000 burn-in periods and 200,000 Markov chain Monte Carlo chain iterations for K (number of genetic populations) ranging from two to 15 (with 10 independent runs at each K). The optimum K was then determined using STRUCTURESELECTOR [37], a statistical program based on the STRUCTURE output, following the ΔK approach [38]. A β version of CLUMPACK [39] integrated into the STRUCTURESELECTOR was used to visualise the population structure after the optimal K was determined.

3. Results

3.1. SSR Markers

The total number of alleles recorded across the 11 SSR loci was 33, with the number of alleles per locus varying from two to five (Table 2). Five of the 11 SSR loci had only two alleles per locus. SLM6-11 and TomSat11-1 were the most polymorphic loci, with five alleles each. The average number of alleles observed per population (Na) for each locus varied from 1.04 (for TomSatX2-2 and TomSatX7-2) to 1.57 (for SLM6-11), with an overall mean of 1.21. The effective number of alleles (Ne) ranged from 1.02 (for TomSatX2-2) to 1.305 (for SLM6-11), with an overall mean of 1.12 (Table 2). The polymorphism information content (PIC) of the loci ranged from 0.05 (for TomSatX2-2) to 0.65 (for SLM6-11), with an overall mean of 0.29. The most informative locus among the 11 loci was SLM6-11 (PIC = 0.65), followed by TomSatX11-1 (PIC = 0.49). Observed heterozygosity (Ho) at locus level varied from zero (for SLM6-11 and TomSatX2-2) to 0.20 (for LE20592), with an average value across the loci of 0.05. Similarly, expected heterozygosity (He) varied from 0.011 (for TomSatX2-2) to 0.178 (for SLM6-11), with a mean of 0.07. The corresponding values for unbiased expected heterozygosity (uHe) for these loci were 0.01 and 0.20, respectively. The SSR loci showed wide variation in their fixation index values, with minimum, maximum and mean values of −0.45, 1.0 and 0.4 for F_{IS}, 0.62, 1.0 and 0.87 for F_{IT}, and 0.72, 0.90 and 0.80 for F_{ST}. Estimated genetic differentiation (G_{ST}) at locus level varied from 0.67 (for SLR20) to 0.89 (for TomSatX11-1), with a mean of 0.77, and was highly significant at all loci (p = 0.001) (Table 2).

Table 2. Total number of alleles (TNA), number of different alleles (Na), effective number of alleles (Ne), polymorphism information content (PIC), observed heterozygosity (Ho), expected heterozygosity (He), unbiased expected heterozygosity (uHe), fixation indices (F_{IS}, F_{IT} and F_{ST}), and population differentiation (G_{ST}, an analogue of F_{ST} adjusted for bias) and its p-value ($P(G_{ST})$), for each SSR locus.

Locus	TNA	Na	Ne	PIC	Ho	He	uHe	F_{IS}	F_{IT}	F_{ST}	G_{ST}	$P(G_{ST})$
SSR22 [a]	3	1.21	1.18	0.27	0.117	0.080	0.087	−0.45	0.62	0.74	0.72	0.001
SLR20 [b]	3	1.18	1.09	0.19	0.020	0.056	0.063	0.65	0.90	0.72	0.67	0.001
SLM6-11 [c]	5	1.57	1.31	0.65	0.000	0.178	0.198	1.00	1.00	0.75	0.69	0.001
LE20592 [d]	4	1.43	1.28	0.51	0.198	0.150	0.165	−0.32	0.65	0.73	0.71	0.001
TomSatX2-2	2	1.04	1.02	0.05	0.000	0.011	0.013	1.00	1.00	0.79	0.75	0.001
TomSatX7-1	3	1.18	1.12	0.30	0.080	0.069	0.077	−0.16	0.76	0.79	0.77	0.001
TomSatX7-2	2	1.04	1.04	0.10	0.012	0.018	0.021	0.33	0.88	0.82	0.80	0.001
TomSatX8-1	2	1.29	1.14	0.33	0.042	0.090	0.102	0.53	0.90	0.79	0.75	0.001
TomSatX9-2a	2	1.07	1.02	0.13	0.007	0.015	0.017	0.53	0.95	0.89	0.87	0.001
TomSatX9-2b	2	1.07	1.02	0.13	0.007	0.015	0.017	0.53	0.95	0.89	0.87	0.001
TomSatX11-1	5	1.18	1.10	0.49	0.013	0.049	0.055	0.75	0.98	0.91	0.89	0.001
Mean		1.21	1.12	0.29	0.045	0.067	0.074	0.40	0.87	0.80	0.77	0.001
SE		0.025	0.017	0.19	0.010	0.009	0.010	0.15	0.04	0.02	0.03	

[a] Frary et al. [23], [b] Korir et al. [25], [c] Geethanjali et al. [24], [d] Smulders et al. [22].

3.2. Genetic Diversity of the Accessions

The genetic diversity of each accession was estimated using several parameters (Table 3). Accessions BOL-8223-HT, BOL-8281-HT, BOL-8284-HT, BOL-8328-HT, BOL-8330-HT and BOL-8340-HT were homozygous for a single allele at each of the 11 loci. Hence, they had observed Na and Ne values of one, and percentage of polymorphic loci (PPL), Shannon information index (I), Ho, He and uHe values of zero. The highest value of Na (1.64) and Ne (1.38) was recorded for accession 'HT-25' and BOL-8288-HT, respectively. Analysis of the number of private alleles (NPA) revealed that accessions BOL-8222-HT, BOL-8225-HT, BOL-8282-HT, BOL-8335-HT and BOL-8348-HT have a single private allele (NPA = 0.09) at locus SSR22, SLM6-11, LE20592, TomSatX2-2 and TomSatX11-1, respectively (Table 3). Of the 28 accessions, 79% had alleles shared by ≤25% of the accessions (NLCA ≤ 25%), whereas all accessions had alleles shared by ≤50% of the accessions (NLCA ≤ 50%). The highest NLCA ≤ 25% value (0.46) was recorded for accession BOL-8288-HT, while the high-

est NLCA ≤ 50% value (0.64) was recorded for two accessions ('HT-25' and BOL-8288-HT) (Table 3).

Table 3. Estimates of different population genetics parameters for the 28 tomato accessions studied.

Genotype	Na	Ne	NPA	NLCA ≤ 0.25	NLCA ≤ 0.50	PPL	I	Ho	He	uHe	F
'Lia'	1.18	1.18	0.00	0.09	0.27	0.18	0.13	0.18	0.09	0.10	−1.00
'Shanty'	1.27	1.27	0.00	0.18	0.27	0.27	0.19	0.27	0.14	0.15	−1.00
'Huichol'	1.27	1.21	0.00	0.09	0.27	0.27	0.16	0.18	0.11	0.12	−0.33
'Rio Grande'	1.36	1.05	0.00	0.27	0.46	0.36	0.08	0.03	0.04	0.04	0.21
'HT-36'	1.09	1.09	0.00	0.00	0.18	0.09	0.06	0.09	0.05	0.05	−1.00
'HT-37'	1.18	1.03	0.00	0.00	0.18	0.18	0.05	0.01	0.03	0.03	0.47
'HT-23'	1.18	1.12	0.00	0.27	0.36	0.09	0.09	0.00	0.05	0.06	1.00
'HT-25'	1.64	1.34	0.00	0.27	0.64	0.55	0.29	0.24	0.18	0.20	−0.17
BOL-8222-HT	1.36	1.24	0.09	0.18	0.36	0.27	0.18	0.05	0.11	0.12	0.52
BOL-8223-HT	1.00	1.00	0.00	0.09	0.18	0	0.00	0.00	0.00	0.00	na
BOL-8224-HT	1.09	1.03	0.00	0.18	0.36	0.09	0.03	0.02	0.02	0.02	−0.14
BOL-8225-HT	1.18	1.07	0.09	0.18	0.27	0.09	0.07	0.00	0.04	0.04	1.00
BOL-8226-HT	1.36	1.25	0.00	0.27	0.46	0.36	0.22	0.03	0.15	0.18	0.70
BOL-8281-HT	1.00	1.00	0.00	0.36	0.36	0	0.00	0.00	0.00	0.00	na
BOL-8282-HT	1.18	1.07	0.09	0.27	0.36	0.18	0.08	0.00	0.05	0.06	1.00
BOL-8284-HT	1.00	1.00	0.00	0.18	0.36	0	0.00	0.00	0.00	0.00	na
BOL-8288-HT	1.46	1.38	0.00	0.46	0.64	0.45	0.29	0.03	0.21	0.25	0.87
BOL-8290-HT	1.09	1.06	0.00	0.00	0.09	0.09	0.05	0.00	0.03	0.04	1.00
BOL-8292-HT	1.36	1.18	0.00	0.18	0.36	0.36	0.18	0.03	0.12	0.13	0.81
BOL-8295-HT	1.09	1.05	0.00	0.00	0.18	0.09	0.05	0.01	0.03	0.03	0.58
BOL-8316-HT	1.18	1.15	0.00	0.00	0.18	0.18	0.12	0.00	0.08	0.10	1.00
BOL-8322-HT	1.27	1.12	0.00	0.00	0.27	0.27	0.13	0.00	0.08	0.09	1.00
BOL-8328-HT	1.00	1.00	0.00	0.09	0.18	0	0.00	0.00	0.00	0.00	na
BOL-8330-HT	1.00	1.00	0.00	0.09	0.18	0	0.00	0.00	0.00	0.00	na
BOL-8335-HT	1.27	1.08	0.09	0.27	0.36	0.27	0.11	0.04	0.06	0.07	0.26
BOL-8340-HT	1.00	1.00	0.00	0.18	0.18	0	0.00	0.00	0.00	0.00	na
BOL-8348-HT	1.55	1.37	0.09	0.36	0.55	0.45	0.29	0.04	0.19	0.20	0.81
BOL-8349-HT	1.09	1.01	0.00	0.36	0.46	0.09	0.02	0.01	0.01	0.01	−0.07
	1.21	1.12	0.02	0.18	0.32	0.19	0.10	0.05	0.07	0.07	0.36
	0.03	0.02	0.02	0.10	0.15	0.03	0.01	0.01	0.01	0.01	0.04

Na = Observed number of alleles; Ne = Effective number of alleles; NPL = Number of private alleles (number of alleles unique to a single population); NLCA ≤ 0.25 = Number of locally common alleles found in 25% or fewer accessions; NLCA ≤ 0.50 = Number of locally common alleles found in 50% or fewer accessions; PPL = Percentage of polymorphic loci; I = Shannon's information index; He = Expected heterozygosity; Ho = Observed heterozygosity; uHe = Unbiased expected heterozygosity; F = Fixation index. Note: Loci with private alleles in populations BOL-8222-HT, BOL-8225-HT, BOL-8282-HT, BOL-8335-HT are SSR22, SLM6-11, LE20592, TomSatX2-2 and TomSatX11-1, respectively.

Among the 28 accessions, six did not have polymorphic loci (PPL = 0), as indicated above, whereas less than 10% of the loci were polymorphic in six other accessions (PPL < 0.1) (Table 3). The advanced breeding line 'HT-25' was the only accession with more than 50% polymorphic loci (PPL = 0.55). The second highest PPL value (0.45) was recorded for accessions BOL-8288-HT and BOL-8348-HT, both of which are wild. The mean PPL value for the accessions was 0.19, indicating that only 19% of the loci were polymorphic on average.

The genetic diversity of each accession was estimated by Shannon's I and He (gene diversity). In addition to the six accessions that did not show genetic variation (see above), 14 accessions had very low genetic variation, with I and He values below 0.14 and 0.10, respectively (Table 3). Four accessions, 'HT-25' (advanced breeding line), BOL-8226-HT (cultivated), BOL-8348-HT (wild) and BOL-8288-HT (wild), had relatively high genetic variation, with I and He values above 0.20 and 0.14, respectively (Table 3). For example, accession BOL-8288-HT (wild) had I, He and uHe values of 0.29, 0.21 and 0.25, respectively. The mean values of I, He and uHe for the 28 accessions were 0.10, 0.07 and 0.07, respectively. The vast majority of the accessions (86%) had observed heterozygosity (Ho) below 0.10, including those that were totally homozygous (Ho = 0). 'Shanty' (a commercial cultivar from Israel) was the most heterozygous accession, followed by Bolivian advanced breeding line 'HT-25', 'Lia' (a commercial cultivar from Israel) and 'Huichol' (a commercial cultivar from Thailand), with Ho values of 0.27, 0.24, 0.18 and 0.18, respectively (Table 3). The fixation index (F) of the accessions that have polymorphic loci varied from −1.00 (the lowest possible value) in accession 'Lia', Shanty and 'HT-36' to 1.0 (the highest possible value) in six Bolivian accessions, including one advanced breeding line, one wild and four cultivated accessions (Table 3).

3.3. Analysis of Molecular Variance (AMOVA)

AMOVA was performed using 1000 permutations at both the accession and higher hierarchical levels (Table 4). The results revealed that 77.3% of the total variation was attributable to variation between accessions (F_{ST} = 0.773, $p < 0.001$), while 22.7% was attributable to variation within accessions, of which 7.1% was accounted for by variation among individuals within accessions and 15.6% by variation within individuals.

Table 4. Results of analysis of molecular variance (AMOVA) based on 1000 permutations without grouping the accessions and on grouping them according to geographical region of origin, altitude, cultivation status, fruit shape, fruit colour, fruit size and growth type.

Grouping Factor	Source of Variation	Degrees of Freedom	Sum of Squares	Variance Components	Percentage of Variation	Fixation Indices	Probability (P) Value
	Among accessions	27	464.92	1.349 Va	77.29	F_{ST} = 0.77	Va & F_{ST} = 0.000
	AIWA *	146	75.86	0.123 Vb	7.07	F_{IS} = 0.31	Vb & F_{IS} = 0.000
	Within individuals	174	47.50	0.273 Vc	15.64	F_{IT} = 0.84	Vc & F_{IT} = 0.000
	Total	347	588.28	1.745			
Geographical region of origin	[a] Among groups	1	83.48	0.438 Va	21.71	F_{CT} = 0.22	Va & F_{CT} = 0.000
	AAWGr **	23	351.54	1.232 Vb	61.07	F_{SC} = 0.78	Vb & F_{SC} = 0.000
	Within accessions	281	97.61	0.347 Vc	17.22	F_{ST} = 0.82	Vc & F_{ST} = 0.000
	Total	305	532.63	2.018			
Altitude groups	[b] Among groups	3	54.43	−0.027 Va	−1.43	F_{CT} = −0.01	Va & F_{CT} = 0.566
	AAWGr	15	260.65	1.602 Vb	85.08	F_{SC} = 0.84	Vb & F_{SC} = 0.000
	Within accessions	189	58.19	0.308 Vc	16.35	F_{ST} = 0.84	Vc & F_{ST} = 0.000
	Total	207	373.27	1.883			
Cultivation status	[c] Among groups	1	61.93	0.361 Va	17.92	F_{CT} = 0.18	Va & F_{CT} = 0.005
	AAWGr	21	332.80	1.276 Vb	63.36	F_{SC} = 077	Vb & F_{SC} = 0.000
	Within accessions	259	97.61	0.377 Vc	18.72	F_{ST} = 0.81	Vc & F_{ST} = 0.000
	Total	281	492.34	2.013			
Fruit shape	[d] Among groups	2	71.59	0.282 Va	16.75	F_{CT} = 0.16	Va & F_{CT} = 0.000
	AAWGr	16	195.54	0.948 Vb	56.25	F_{SC} = 0.67	Vb & F_{SC} = 0.000
	Within accessions	221	100.56	0.455 Vc	26.99	F_{ST} = 0.73	Vc & F_{ST} = 0.000
	Total	239	367.69	1.686			
Fruit colour	[e] Among groups	2	12.73	−0.034 Va	−2.05	F_{CT} = −0.02	Va & F_{CT} = 0.533
	AAWGr	24	378.92	1.263 Vb	77.11	F_{SC} = 0.76	Vb & F_{SC} = 0.000
	Within accessions	292	119.28	0.408 Vc	24.94	F_{ST} = 0.75	Vc & F_{ST} = 0.000
	Total	317	510.93	1.634			
Fruit size	[f] Among groups	1	70.74	0.440 Va	22.53	F_{CT} = 0.23	Va & F_{CT} = 0.000
	AAWGr	19	252.18	1.076 Vb	55.08	F_{SC} = 0.71	Vb & F_{SC} = 0.000
	Within accessions	233	101.97	0.438 Vc	22.39	F_{ST} = 0.77	Vc & F_{ST} = 0.000
	Total	253	424.89	1.954			
Growth type	[g] Among groups	2	111.77	0.420 Va	21.98	F_{CT} = 0.22	Va & F_{CT} = 0.000
	AAWGr	25	353.13	1.106 Vb	57.85	F_{SC} = 0.74	Vb & F_{SC} = 0.000
	Within accessions	320	123.36	0.385 Vc	20.16	F_{ST} = 0.79	Vc & F_{ST} = 0.000
	Total	347	588.28	1.912			

* AIWA = among individuals within accessions; ** AAWGr = among accessions within groups. [a] The 25 Bolivian accessions were divided into two groups based on their geographical region of origin (La Paz vs. Cochabamba + Chuquisaca + Santa Cruz + Beni); [b] Nineteen Bolivian accessions with known altitude of collecting site were divided into four altitude groups (<500 masl, 950–1200 masl, 1450–1750 masl and 1850–2250 masl); [c] Twenty-three Bolivian accessions with known cultivation status were divided into two groups (cultivated and wild); [d] Nineteen accessions were divided into three groups according to fruit shape (cylindrical, round and slightly flattened); [e] Twenty-six accessions were divided into two groups according to fruit colour (red vs. yellow); [f] Twenty-one accessions were grouped into two groups according to fruit size (intermediate vs. very small); [g] Twenty-eight accessions were divided into three groups according to growth type (determinate, semi-determinate and indeterminate).

The AMOVA analysis at higher hierarchical level was performed by grouping the accessions using seven different criteria: geographical region of origin (La Paz and other regions (Cochabamba, Sucre, Santa Cruz and Beni)); altitude (<500 m above sea level (masl), 950–1200 masl, 1450–1750 masl, and 1850—2250 masl); cultivation status (cultivated and wild); fruit shape (cylindrical, round, slightly flattened); fruit colour (red and yellow); fruit size (intermediate and very small); and growth type (determinate, semi-determinate and indeterminate). Accessions from La Paz differed significantly from those of the other regions in Bolivia, and region of origin accounted for 21.7% of the total variation revealed by the markers used ($p < 0.001$). Cultivated and wild accessions also differed significantly, with cultivation status accounting for 17.9% of the total variation ($p < 0.001$) (Table 4).

In addition, there were significant genetic differentiations into fruit shape groups, fruit size groups and growth type groups, accounting for 16.8%, 22.5% and 22.0% of the total variation, respectively. However, no significant differences were recorded for altitude groups and fruit colour groups (Table 4).

Pairwise F_{ST} analysis of the 28 accessions revealed significant differentiation ($p < 0.05$) among the vast majority (94%) of the pairs of accessions (Figure 3). The three non-Bolivian accessions ('Lia', 'Shanty' and 'Huichol') showed significant differentiation from the Bolivian accessions. The differentiation among the four Bolivian advanced breeding lines was also significant ($p < 0.05$) (Figure 3). 'Rio Grande', a widely cultivated variety in Bolivia, showed significant differentiation from all other accessions except 'HT-23' and BOL-8224-HT. 'Rio Grande' and BOL-8224-HT showed no significant differentiation from each other, having an F_{ST} value close to zero (marked with a purple asterisk in Figure 3). Other pairs of accessions that were not significantly differentiated included BOL-8328-HT versus BOL-8330-HT (semi-determinate wild accessions from La Paz with very small red fruits), and BOL-8316-HT (a cultivated accession from La Paz) versus 'HT-36' and 'HT-37' (advanced breeding lines) (Figure 3). Accessions BOL-8281-HT, BOL-8335-HT, and BOL-8349-HT (wild accessions from Beni, La Paz and La Paz, respectively) showed high differentiation from most accessions studied, as revealed by pairwise F_{ST} (Figure 3).

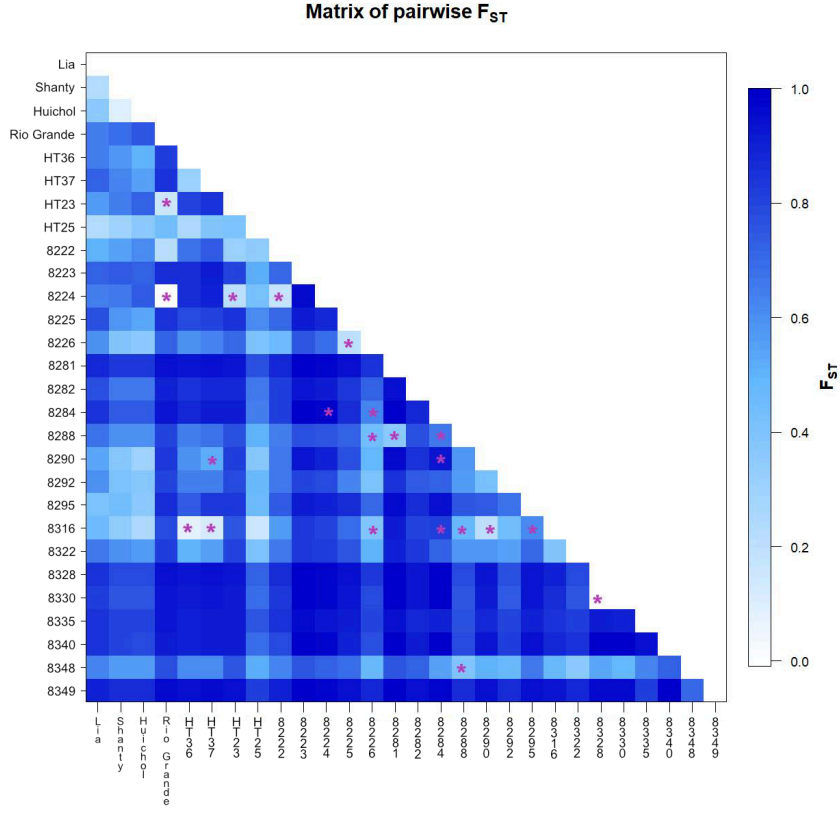

Figure 3. Heatmap of pairwise fixation index F_{ST} of the 28 tomato accessions, calculated using the number of different alleles as a distance method. The differentiation between each pair of accessions was significant ($p < 0.05$) except in the case of pairs marked with a purple asterisk.

A heatmap of pairwise Nei's distance (below diagonal in Figure 4) corroborated the low differentiation between most accessions except BOL-8281-HT, BOL-8284-HT, BOL-

8335-HT and BOL-8349-HT. These accessions had higher genetic distance to most other accessions, as can also be observed from the heatmap of pairwise F_{ST} (Figure 3). In line with the Shannon's I and He values (Table 3), the within-accession variation was zero for accessions BOL-8223-HT, BOL-8281-HT, BOL-8284-HT, BOL-8328-HT, BOL-8330-HT and BOL-8340-HT (Figure 4). In addition, extremely low variation was recorded within accession BOL-8349-HT. Accessions 'HT-25', BOL-8288-HT and BOL-8348-HT were the most diverse (Figure 4), again in agreement with the Shannon's I and He values.

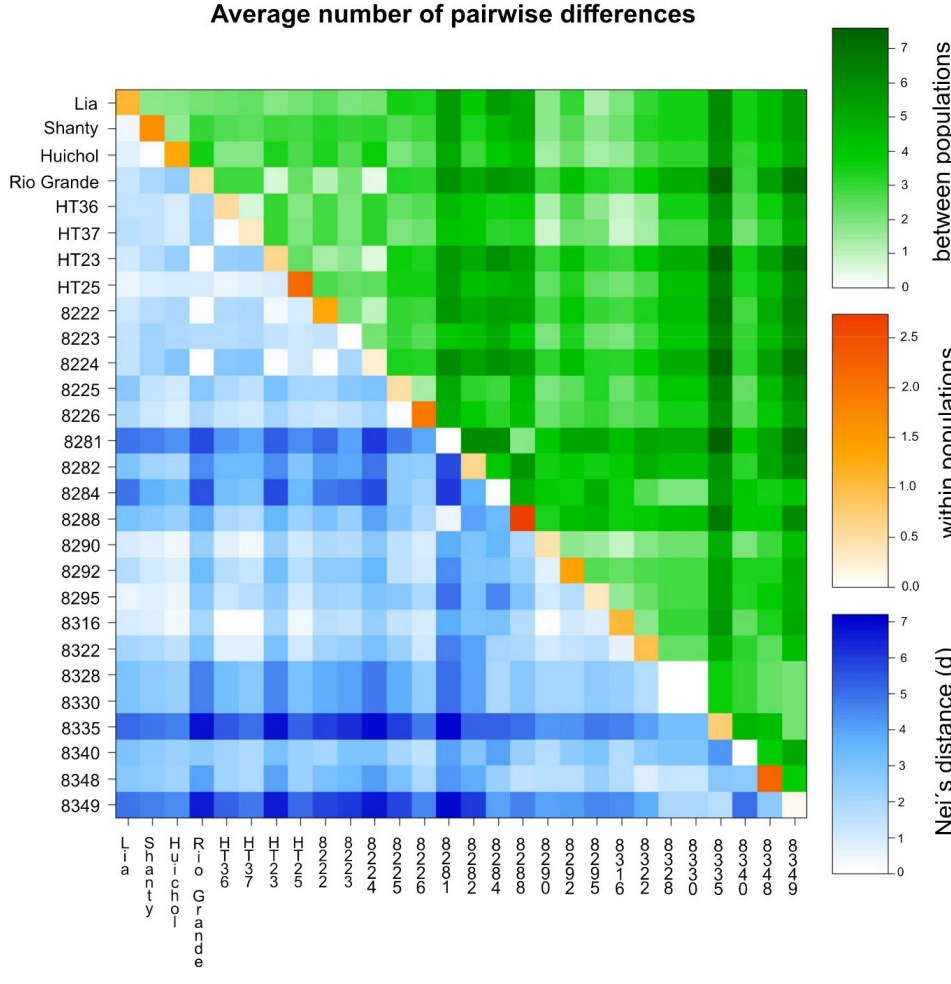

Figure 4. Heatmap displaying average number of pairwise differences of the 28 accessions, estimated using a number of different alleles as a distance method: average number of pairwise differences between the accessions (PiXY; above diagonal), average number of pairwise differences within the corresponding accession (PiX; diagonal); and corrected average pairwise difference (PiXY − (PiX + PiY)/2; below diagonal), also referred to as Nei's distance (d).

3.4. Cluster Analysis and Principal Coordinate Analysis

The UPGMA cluster analysis based on Nei's unbiased genetic distance revealed various genetic relationships among the 28 tomato accessions, including four clusters and two solitary accessions (Figure 5). Cluster 1 comprised five wild accessions with semi-

determinate growth habit from La Paz (BOL-8328-HT, BOL-8330-HT, BOL-8348-HT, BOL-8349-HT and BOL-8335-HT) and one cultivated accession with indeterminate growth habit from Beni (BOL-8284-HT). Cluster 2 comprised two phenotypically similar wild accessions (BOL-8288-HT from La Paz and BOLTH-0119-HT from Beni). A wild accession from Beni (BOL-8282-HT) with indeterminate growth habit and a cultivated accession from La Paz (BOL-8340-HT) with semi-determinate growth habit remained solitary, forming clusters 3 and 4, respectively. Cluster 5 comprised five cultivated accessions, four of which have a determinate growth habit (BOL-8222-HT, 'HT-23' and 'Rio Grande' from Cochabamba and BOL-8224-HT from Santa Cruz), and one semi-determinate type from Sucre (BOL-8223-HT). Cluster 6 was the largest cluster, comprising 13 accessions that were further divided into three sub-clusters. The first sub-cluster comprised three semi-determinate accessions, two of which were from Sucre (BOL-8225-HT and BOL-8226-HT; both cultivated) and one from La Paz (BOL-8292-HT; wild). The second sub-cluster comprised four accessions with determinate growth habit that included a cultivated accession from La Paz (BOL-8295-HT) and the three foreign commercial cultivars ('Lia', 'Shanty' and 'Huichol'). The third sub-cluster comprised three advanced breeding lines with determinate growth habit from Cochabamba ('HT-25', 'HT-36' and 'HT-37') and three semi-determinate accessions from La Paz (two cultivated: BOL-8316-HT and BOL-8322-HT, and one wild: BOL-8290-HT).

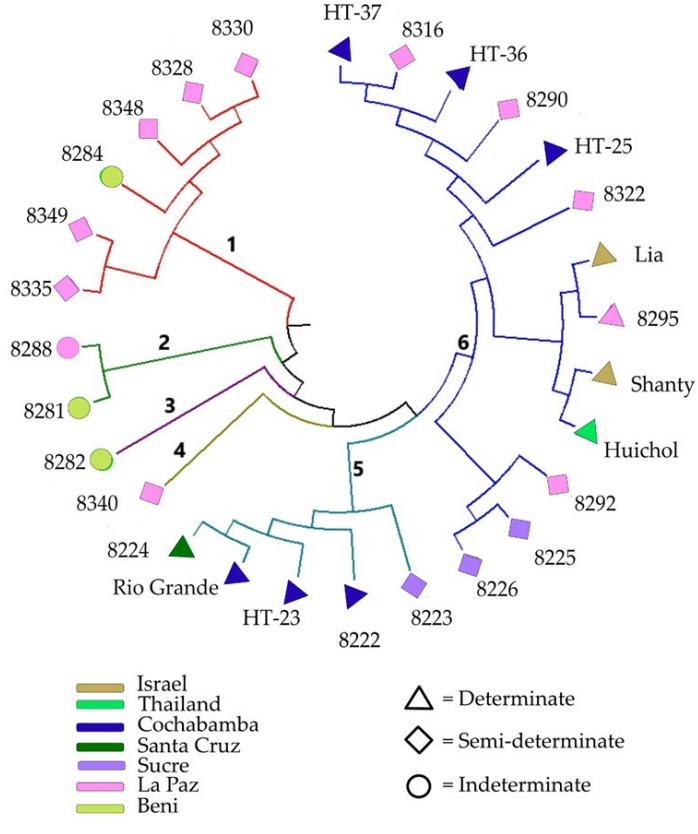

Figure 5. Unweighted pair group method with arithmetic mean (UPGMA) tree showing the genetic relationship between the 28 accessions analysed in the present study. NOTE: Label colour indicates geographical origin of the accessions (regions within Bolivia or other countries), while label shape indicates growth habit (determinate, semi-determinate, indeterminate).

The Nei's unbiased genetic distance-based principal coordinate analysis (PCoA) further revealed the genetic relationship between the 28 tomato accessions (Figure 6). The first two principal coordinates (PCoA) together explained 70% of the total variation, with PCoA1 explaining 52.3% and PCoA2 17.2%. The six accessions in cluster 1 of the UPGMA tree (Figure 5) formed two small clusters (highlighted in sky blue and green in Figure 6). The two accessions in cluster 2 of the UPGMA tree (BOL-8281-HT and BOL-8288-HT) were separated along PCoA2. Three of the five accessions in cluster 5 of the UPGMA tree ('HT-23', 'Rio Grande' and BOL-8224-HT) were separated along PCoA1, forming a group highlighted in pink in Figure 6. The remaining two accessions in cluster 5 (BOL-8222-HT and BOL-8223-HT), the solitary accessions BOL-8282-HT and BOL-8340-HT, and all accessions in cluster 6 of the UPGMA tree formed the group highlighted in yellow in Figure 6.

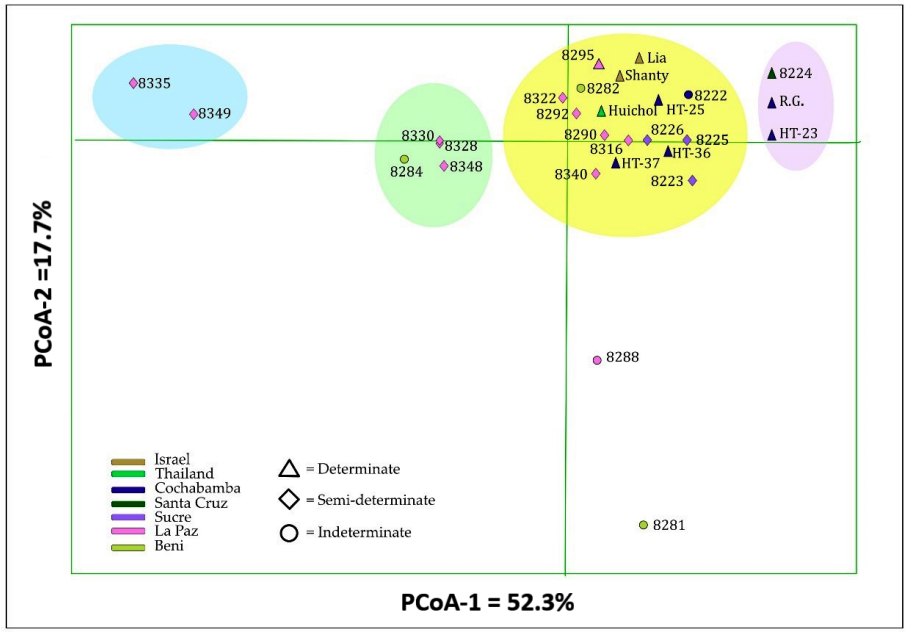

Figure 6. Principal coordinate analysis (PCoA) bi-plot, generated based on Nei's unbiased genetic distance, demonstrating the relationship between the 28 tomato accessions, with the first two principal coordinates (PCoA1 and PCoA2) explaining 70% of the total variation. Accessions with the same label colour belong to the same region within Bolivia, or to the same country.

3.5. Population Structure Analysis

Analysis of admixture model-based population structure using the STRUCTURE and STRUCTURESELECTOR programs revealed that three genetic clusters (K) was the optimal number, according to the method of Evanno et al. [38] (Supplementary Figure S1). This suggests that the 279 individuals from the 28 accessions analysed in this study originated from three genetic populations. The graphical illustration of the population structure of the 28 accessions clearly showed that most were admixed, albeit to varying degrees. BOL-8335-HT and BOL-8249-HT were the only accessions that were not admixed (Figure 7). All accessions that formed cluster 1 in the UPGMA tree (Figure 5) were represented by deep-purple-dominated bars except BOL-8348-HT, which appeared to show high admixture. Similarly, all accessions that formed cluster 6 in the UPGMA tree were represented by blue-dominated bars, except accessions BOL-8225-HT and BOL-8226-HT (Figure 7). Other highly admixed accessions were BOL-8225-HT, BOL-8282-HT and BOL-8288-HT. Among

highly admixed accessions, BOL-8282-HT was the only one with significant proportions of alleles from the three genetic clusters represented by the three different colours in Figure 7. In general, the results of cluster, PCoA and population structure analyses were in good agreement regarding the genetic relationships between the accessions studied.

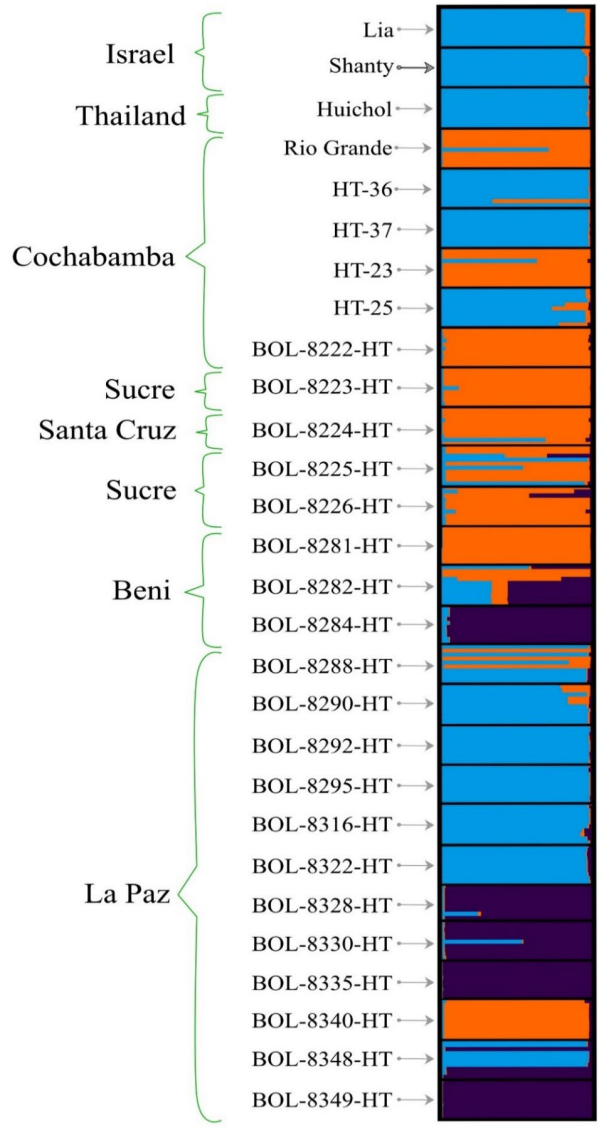

Figure 7. Graphical display of optimal genetic structure of the 279 individual genotypes representing the 28 tomato accessions. Light blue, yellow and deep purple represent the three clusters (K) identified in population structure analysis. In each accession, the proportion of each colour represents the average proportion of alleles that placed each accession in one or more cluster. A black rectangular border delimits each accession.

4. Discussion

Our analysis of 28 tomato accessions revealed a low level of diversity persisting in exanimated Bolivian tomatoes, despite the fact that Bolivia is part of the centre of diversity [40] or origin [1,41] of tomatoes. The diversity identified in the analysis was mainly between accessions, with factors such as geographical region of origin, cultivation status, fruit shape, fruit size and growth type clearly dividing the tomatoes into groups that were genotypically differentiable by the markers used. Thus, the markers developed within this study and their relationship to the various parameters considered provide opportunities to select parents for crossbreeding in tomato breeding programmes, which is an important measure to generate new Bolivian cultivars [42]. The present study also contributed novel knowledge on the genetic diversity and population structure of Bolivian tomatoes, as previous studies have only included Bolivian accessions of cherry tomatoes [43] and its wild relatives *S. lycopersicum* var. *ceraciforme* and *S. neorickii* [9]. This novel knowledge increases understanding of genetic relationships among Bolivian tomato germplasm and the domestication processes of tomatoes.

4.1. The SSR Markers in Revealing Tomato Genetic Diversity

Use of SSR loci previously employed to study the genetic diversity of tomatoes revealed a considerably lower number of alleles for the Bolivian tomatoes investigated here than reported for tomatoes from other countries. For instance, only three alleles were detected at the SLR20 locus among the 28 tomato varieties studied here, whereas Korir et al. (2014) reported five alleles among 42 tomato varieties from China and Kenya at this locus [25]. Further, Gonias et al. (2019) reported eight alleles at this locus in their study of 107 tomato accessions, including cultivars and landraces from Greece and international hybrids [44]. At the SLM6-11 locus, five alleles were observed in this study, whereas six alleles were reported by Geethanjali et al. (2010) for 16 tomato accessions [24]. Smulders et al. (1997) identified seven alleles at the LE20592 locus for 10 tomato accessions encompassing seven *S. lycopersicum* cultivars and three wild *Solanum* species [22], but in the present study only four alleles were recorded at this locus. Some of these previous studies analysed a higher number of accessions than in the present study, while other studies analysed fewer accessions, but a higher number of alleles was reported in all cases. In light of this, Bolivian tomatoes can be considered to have low allelic diversity.

Among the 11 SSR loci studied, SLM6-11 was the most informative, with a PIC value of 0.65, corresponding to the value reported in a previous study [24]. Hence, for population genetics analysis of tomato genetic resources, this locus should be prioritised, along with LE20592 (PIC = 0.55) and TomSatX11-1 (PIC = 0.49), the latter developed in the present study. As in a previous study [45], which reported PIC values ranging from 0.06 to 0.60 (mean 0.31) for inbred tomato lines from different countries, the PIC value in this study ranged from 0.05 to 0.65, with a mean of 0.29. However, higher PIC value ranges and mean values have been reported in other studies, e.g., 0.62–0.85 (mean 0.74) for diverse tomato varieties of modern, landrace and hybrid type using SSR markers [44], 0.42–0.87 (mean 0.69) for different tomato species using SSR markers and 0.17–0.74 (mean) 0.45 for tomato varieties from different countries [25,46]. Despite the fact that the PIC values are directly related to the choice of SSRs, the results of the present study indicate that the genetic diversity of Bolivian tomatoes is relatively low. However, the genetic differentiation between the accessions was highly significant, as shown by the high F_{ST} and G_{ST} values at each SSR locus. Highly significant differentiation between tomato accessions has been reported previously for local landraces from southern Italy and contemporary tomato varieties [47], and for tomato landraces from Cyprus, France and Greece [48].

Tomato is predominantly a self-pollinating species [49,50], and for such species observed heterozygosity (Ho) should normally be lower than expected heterozygosity (He) at a neutral polymorphic locus. In line with expectations, the He values were higher than the Ho values for most of the SSR loci analysed in this study. However, Ho exceeded He for the SSR22, LE20592 and TomSatX7-1 loci, indicating that these loci might be linked

to genes under balancing selection, which favours heterozygosity [51]. The He values for the different accessions in the present study were generally low (mean 0.07), whereas higher values (0.17–0.71) have been reported in previous studies [2,52,53]. The significant number of open- and self-pollinated accessions included in the present study might be the reason for the low average He values found, as a higher level of heterozygosity can be expected for hybrids [54] than for open- or self-pollinated accessions [55]. Based on available information, only seven ('Lia', 'Shanty', 'Huichol', and the four advanced breeding lines) of the 28 accessions were hybrids, while the others were open- or self-pollinated. Correspondingly, the commercial hybrid cultivars 'Lia', 'Shanty', and 'Huichol' had higher Ho values (0.27, 0.18, and 0.18, respectively) than the other accessions (Ho < 0.1), while the open/self-pollinated group had Ho values that ranged from zero to 0.05. As expected, Ho was lower or equal to He for the open/self-pollinated group.

For the hybrid group, Ho exceeded He except for 'HT-23' and 'HT-37', with both having higher He than Ho and high positive fixation index (F). Steps taken following hybridisation could explain the differences seen in F and He/Ho ratio. One possibility is that 'HT-23' and 'HT-37' have undergone a series of self-pollination events after the hybridisation event, resulting in homozygosity at the majority of their loci and positive F values. In contrast, the two Israeli commercial cultivars ('Lia' and 'Shanty') and the Bolivian hybrid 'HT-36' are most likely F1 hybrids, since they possess maximum levels of heterozygosity (F = −1), while 'Huichol', a cultivar from Thailand, and the Bolivian hybrid 'HT-25' may have been reproduced through open pollination after the hybridisation event took place. The 20 Bolivian accessions can be classified into three subgroups based on their expected heterozygosity, which measures the genetic diversity within the accessions: those with He = 0 (inbred), those with He = 0.01–0.08 (extremely low diversity), and those with He = 0.11 = 0.21 (low to medium diversity). Shannon's I values can also be used to discern these subgroups. However, in terms of phenotypic characteristics and geographical region of origin, each of these groups was found to be generally diverse. For example, the six inbred accessions differ in fruit colour and shape, as well as altitude and geographical region of origin of the germplasm. That group also comprised both cultivated and wild types. Additionally, some of those with very small fruits appeared to be inbred, while others had He values as high as 0.21. Hence, neither geography nor phenotypic characteristics sufficiently explained the genetic variation within the accessions.

The results of the present analysis indicated differences in reproductive mechanisms among both cultivated and wild Bolivian tomatoes. The accessions BOL-8223-HT, BOL-8281-HT, BOL-8284-HT, BOL-8328-HT, BOL-8330-HT and BOL-8340-HT are genetically uniform, and are characterised by very small red fruits except for BOL-8223-HT (which has very small yellow fruits). Their lack of within-accession genetic variation may indicate that they have cleistogamous flowers that prevent pollen movement, resulting in strict inbreeding. Crossbreeding such inbred accessions can be advantageous, since they will produce genetically suitable F1 hybrids that may be superior to their parents in terms of desirable traits. It is possible, for example, to crossbreed BOL-8223-HT (cultivated) with BOL-8330-HT (wild), since they are genetically distinct, as revealed by our cluster, PCoA and pairwise F_{ST} analyses, while they show some differences in their phenotypic characteristics.

The accessions BOL-8225-HT, BOL-8282-HT, BOL-8290-HT, BOL-8316-HT, BOL-8322-HT) and 'HT-23' have similar characteristics to the above group except that they have genetic variation within accessions. However, the high fixation index values obtained here (F = 1) indicate that these accessions are strictly self-pollinating types, a reproductive mechanism determined in previous studies to be dominant in tomatoes [2,49]. In view of the fact that they are cultivated types with the exception of BOL-8290-HT, the low level of genetic variation within these accessions might be due to unintentional gene flow in the form of seeds. The accessions with high He and PPL values, i.e., BOL-8226-HT, BOL-8288-HT and BOL-8348-HT, are most likely open-pollinating types with a high rate of outcrossing.

The two most genetically diverse Bolivian accessions, BOL-8288-HT (He = 0.21; PPL = 0.45) and BOL-8348-HT (He = 0.19; PPL = 0.45), are both wild accessions bear-

ing small round fruits. They differ in fruit colour (red and yellow, respectively) and flowering habit (indeterminate and semi-determinate, respectively) and in the altitude and geographical location of the sampling site. Further characterisation may lead to identification of genotypes with desirable characteristics that can be incorporated into elite cultivars through crossbreeding. The present study clearly indicated that none of the specific geographical regions or altitude ranges within Bolivia can be considered a hotspot for the genetic diversity of Bolivian tomatoes.

4.2. AMOVA

The analysis of molecular variance (AMOVA) results for the 28 *S. lycopersicum* accessions revealed significant variation both between accessions (77.3%) and within accessions (22.7%), which is in line with the characteristics of species that are predominantly self-pollinating. A previous study [56] that evaluated two wild tomato species from the Galapagos Islands (*S. cheesmaniae* and *S. galapagense*) also revealed much higher variation between accessions (>90% of the total genetic variation) than within accessions. In fact, these two wild species are strict inbreeders, unlike some open-pollinating *S. lycopersicum* accessions analysed in the present study. However, higher variation within accessions (accounting for 29% and 36% of the total variation, respectively) has previously been reported for *S. lycopersicum* and *S. pimpinellifolium*, both being self-compatible [50]. In contrast, in the outcrossing *S. peruvianum*, only 32.2% of the total variation is between accessions [50]. A possible conclusion from the above discussion is that the reproductive mechanism of a species can have a profound impact on population differentiation.

Based on the highly significant genetic variation found between the accessions and the significant differentiation between over 90% of accession pairs, crossbreeding between genotypes bearing desirable agronomic and fruit characteristics may prove to be the most effective approach for cultivar development. It should be noted that in the present study, hierarchical AMOVA revealed significant differences between groups based on a variety of factors, such as region of origin, domestication/breeding status, fruit shape, fruit size and flowering habit. Around 20% of the total genetic variation differentiated accession groups from La Paz versus other regions in Bolivia, pointing to the importance of isolation by distance and geographical barriers in population differentiation. This significant divergence can also be explained partly by the fact that most of the accessions from La Paz are wild populations with small fruits and semi-determinate flowers, while most of the accessions from other regions are cultivated types. This differentiation level is comparable with that of *S. chesmaniae*, but four times higher than that of *S. galapagense* from different regions of the Galapagos Islands [56]. On the other hand, the lack of genetic differentiation among altitude groups despite the wide range of altitude of their sampling sites (227–2858 masl) indicates that tomatoes can adapt to altitude. The AMOVA analysis did not significantly differentiate tomatoes with red and yellow skin colour.

Although cultivated Bolivian tomatoes differed significantly from their wild counterpart, that differentiation explained only 18% of the total variation. The majority of the cultivated tomatoes still bear small fruits, similar to wild types, indicating that little has been accomplished in terms of selection-based improvement in fruit size. Significant variations were observed between fruit shape groups, fruit size groups and flowering habit groups, accounting for 16.8%, 22.5% and 22.0% of the total variation, respectively. These results are not surprising, since the commercial cultivars and advanced breeding lines have predominantly cylindrical and round fruits, while the majority of the wild accessions have somewhat flattened fruits. Additionally, commercial cultivars and advanced breeding lines have larger fruits and determinate flowers, while wild and landrace accessions have small fruits and indeterminate/semi-determinate flowers.

Since determinate growth habit and larger fruits are desired characteristics in tomato cultivars, resulting in synchronous maturity that facilitates harvesting and higher fruit yields, these traits have been the target of domestication and breeding programmes [57,58]. Therefore, the significant differences observed here between flowering habit groups and

fruit size groups relate to domestication status. The lack of significant differentiation between the fruit colour groups can be explained by the fact that both cultivated and wild accessions possess a large proportion of red fruits, but yellow fruits are also found in both cultivated and wild groups. Tomato skin colour is a phenotypic quality trait that is known to be regulated by several genes, including phytoene synthase 1 (PSY1), phytoene desaturase (PDS), 15-cis-zeta-carotene isomerase (ZISO) and DE-ETIOLATED 1 (DET1) [59]. Due to the fact that the SSRs used in this study are not linked to genes that regulate fruit colour, which is needed to differentiate this trait [60], the results indicate that cultivated tomatoes with different fruit colours have rather similar genetic backgrounds, as is also likely to be the case for wild tomatoes.

4.3. Cluster, Principal Coordinate and Population Structure Analyses

There was significant genetic differentiation between cultivated and wild accessions, with the exception of BOL-8282-HT and BOL-8284-HT, as visualised by the heatmaps of pairwise comparisons, UPGMA tree, PCoA scatter plot and STRUCTURE graph. Thus, use of wild accessions in crossbreeding with cultivated accessions of tomatoes provides the potential to develop cultivars that have favourable fruit characteristics and are well-adapted to Bolivian agroecosystems. It should be noted, however, that there are higher genetic similarities between the Bolivian cultivated tomatoes and the foreign commercial cultivars evaluated here than between Bolivian cultivated and wild tomatoes. This is an excellent example of how domestication has shaped crop evolution through the selection of germplasm for multiple desirable traits that are generally categorised as "domestication syndrome" traits [61]. Furthermore, the determinate and indeterminate types of tomatoes were found to be clearly separated from each other regardless of their cultivation status, unlike the semi-determinate types. Thus, indeterminate cultivated accessions clustered with indeterminate wild accessions, whereas determinate wild accessions clustered with determinate cultivated accessions. This suggests that the substantial genetic differentiation between determinate and indeterminate varieties predates tomato domestication.

Among the cultivated accessions, BOL-8284-HT was the most genetically distinct, exhibiting an indeterminate flowering habit and very small, slightly flattened red fruits. BOL-8335-HT and BOL-8349-HT were the most genetically distinct wild accessions, both exhibiting a semi-determinate flowering habit and producing very small red fruits. Consequently, these accessions could be valuable for crossbreeding to facilitate the development of superior genotypes through genetic recombination for further breeding.

It is noteworthy that some closely clustered accessions, e.g., BOL-8328-HT vs. BOL-8330-HT, 'Rio Grande' vs. 'HT-23' and 'HT-36' vs. 'HT-37', have similar fruit characteristics and flowering habits, suggesting the presence of genetically similar accessions that could be considered "duplicates" in the Bolivian ex situ conserved tomato germplasm. In contrast, some closely clustered accessions (high genetic similarity) such as BOL-8316-HT and 'HT-37' exhibited different fruit characteristics and flowering habit. Thus, grouping the accessions based on their phenotypic characteristics, followed by genotypic characterisation based upon single nucleotide polymorphisms (SNPs) and SSRs, would allow the creation of a core collection comprising distinct accessions.

In previous population genetics studies, different approaches have been used to determine how populations are genetically structured [36,62–64]. The Bayesian model-based population structure analysis assumes that populations are defined by the frequencies of alleles at multiple loci [36]. Using this method, each genotype within a predefined population is assigned to a cluster or, if the genotype is found to be admixed, to more than one cluster. Using this approach, we found that the majority of the 28 accessions studied and their individual genotypes exhibited varying degrees of genetic admixture, suggesting significant gene flow between the different groups. Such genetic admixture may result from natural gene flow in wild habitats and agroecosystems and from intentional crossbreeding to produce cultivars that meet desired characteristics such as fruit quality [57].

The commercial cultivars and most of the Bolivian advanced breeding lines displayed highly similar population structures to wild accessions collected from La Paz region, implying that the core germplasm collected in La Paz plays a significant role in tomato improvement programmes in Bolivia. To our knowledge, only one previous study has examined the genetic diversity of Bolivian core germplasm, using 31 accessions of *S. neorickii*, *S. chmielews*, *S. lycopersicum* ceraciforme and S. *lycopersicum* spp. [9]. That study reported genetic distance in collections between La Paz and other regions. In the present study, not all wild and landraces accessions from Bolivian core germplasm were used, as only accessions classified as *S lycopersicum* L. were included. The best-represented region was La Paz, which had a significant number of accessions in the core collection. Nonetheless, this study provides valuable novel information on genetic diversity and genetic structure for potential use in breeding programmes. Most of the alleles found in the cultivated accessions derived from two of the three genetic populations identified. Alleles of these genetic populations appear to be widely distributed in non-Bolivian and Bolivian accessions, since they are well represented in these accessions. The accessions that formed cluster 1 in the UPGMA tree, shown as sky blue and green clusters in the PCoA plot in Figure 6 (clearly differentiated along PCoA1) were represented by deep purple bars in the optimal genetic structure plot in Figure 7, except for BOL-8348-HT which apparently had high genetic admixture. The results presented here are partly consistent with those of another study in which tomato wild relatives and landraces showed less admixture than market-oriented cultivars [65]. As a whole, the results of the cluster, PCoA and population structure analyses agreed very well, clearly demonstrating genetic relationships between the accessions studied and the pattern of their genetic variation.

5. Conclusions

The number of alleles detected in this SSR-based study on Bolivian tomato accessions ranged from two to five, indicating low allelic diversity of examined Bolivian accessions. TomSatX11-1 proved to be the most informative of the newly developed SSR markers in this study and should be prioritised for population genetics analysis of tomatoes, together with other highly informative markers such as SLM6-11 and LE20592. While Bolivia lies within the centre of diversity and origin of tomatoes, explored Bolivian tomatoes have generally low genetic variation within each accession. However, there is highly significant genetic differentiation between the accessions, explaining approximately 75% of the total genetic variation. There is also significant genetic variation between wild and cultivated tomatoes and between tomatoes with different geographical origin, fruit shape, fruit size and flowering habits. However, there is no significant genetic difference between tomatoes from different altitude ranges or tomatoes with different fruit colours. The genetic differentiation between tomatoes with determinate and indeterminate flowers may predate tomato domestication. Tomatoes have genetically determined mechanisms that contribute to either cleistogamous flowers, generating genetically uniform genotypes, here represented by six accessions (BOL-8223-HT, BOL-8281-HT, BOL-8284-HT, BOL-8328-HT, BOL-8330-HT and BOL-8340-HT), or to flowers that allow open pollination, here represented by three accessions (BOL-8226-HT, BOL-8288-HT and BOL-8348-HT). The two most genetically diverse Bolivian accessions in the present study were BOL-8288-HT (He = 0.21; PPL = 0.45) and BOL-8348-HT (He = 0.19; PPL = 0.45). There is limited gene flow both within and between cultivated types and wild populations, resulting in genetic admixture. Crossbreeding of genotypes of genetically distinct cultivated accessions, such as BOL-8284-HT and BOL-8316-HT, could lead to the development of superior cultivars through genetic recombination.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13091505/s1 Table S1. Simple sequences repeat microsatellites (SSRs) developed in the present study. Table S2. Final selection of simple sequences repeat microsatellites (SSRs) for the present analysis. Figure S1. Delta-K plot with a maximum value at 3 ($\Delta K = 3$).

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Genotype and Maturity Stage Affect the Content and Composition of Polyamines in Tomato—Possible Relations to Plant and Human Health

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Abstract: Polyamines (*PAs*) are molecules affecting several physiological characteristics in all living organisms with cell protective effects, thereby impacting plant and human health. Here, we used HPLC-DAD-ESI-MS to evaluate the content and composition of *PAs* in eight tomato genotypes over their maturation period, and related the content and composition to other quality traits and possible implications for plant and human health. The tomato genotype, maturity stage and their interactions, significantly affected the content and composition of *PAs*. Two of the genotypes, 'Huichol' and 'Rio Grande' showed consistently lower levels of *PAs* than the other evaluated genotypes. The variation in content and composition of *PAs* among genotypes was found to vary inconsistently over the maturation period. Putrescine content in the different genotypes either did not vary significantly, increased, or showed the lowest level in the middle of the maturation period, while spermidine content decreased or did not show significant variation. The genotypes 'HT36' and 'HT25' showed high levels of *PAs* during red and green maturity stages, respectively, and can thereby be seen as suitable health promoting red and green candidate tomatoes. Depiction of variation of the *PAs* creates opportunities for breeding and production of health promoting tomato as a food or food additive.

Keywords: putrescine; spermidine; spermine; ascorbic acid; titratable acidity; total soluble solids

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1. Introduction

Polyamines (*PAs*) are low molecular weight aliphatic (non-aromatic), nitrogen rich, hydrocarbon molecules that form polymers containing one or more amino group (NH_2) [1,2]. *PAs* are universally present in all living organisms, including all types of plants, and the naturally abundant *PAs* (putrescine (*Put*), spermidine (*Spd*) and spermine (*Spm*)) are the most common [3,4]. *PAs* are involved in functions such as cell growth, gene regulation, and differentiation [5]. In plants, biosynthesis or catalysis of *PAs* have been related to growth, flowering, and stress signaling [6]. During fruit development in tomato, transcripts of *PAs* biosynthesis genes have been localized in fast growing tissues, and it has been suggested that the seed is a site of intense PA synthesis [7]. *Put* is the precursor of *Spd*, which in turn is the precursor of *Spm* [6]. Several studies have indicated the content of free and conjugated *PAs* to increase at biotic and abiotic stress conditions [6,8], suggesting that *PAs* exert a positive effect on the antioxidant system, resulting in a reduction in cellular damage by the capturing of free reactive oxygen species (*ROS*) [8]. *PAs* interact with macromolecules, act as osmolytes, and may play a role in biotic stress as regulators of gene expression [9].

In tomato plants, *PAs* play an active role during stress conditions, e.g., *PAs* content (especially *Spm*) correlated negatively to the amount of *ROS* and damage tissue under drought conditions [8]. Transgenic tomato plants have been used to verify the positive

correlation of increased levels of *PAs* with the content of lycopene, vine life, and juice quality [10]. *PAs* have also been shown to enhance shelf life in, e.g., apricot and tomato, when externally applied, by suppressing ethylene production [11], thereby acting as a senescence delay compound [10].

In humans, significant amounts of *PAs* are supplied by foods and absorbed in the small intestine, while microbiota are considered to affect the content of *PAs* in the large bowel [12]. At present, no daily intake recommendations of *PAs* are available, but it is well known that the majority of *Put* and *Spd* consumed by humans originate from plant-based products while *Spm* comes from animal-derived food [13].

Furthermore, *PAs* are suggested to have an impact on human health [3]. Potential health effects from *Put*, *Spd*, and *Spm* as dietary sources have been investigated, although such relationships require further elucidation. A negative association between dietary polyamines and cardiovascular disease has been found [14]. Recent studies have shown a cardio-protecting effect from supplementation with *Spd*, which has been explained by its stimulation of mitochondrial respiration, autophagy and mitophagy [15]. In addition, intake of *PAs* have been associated with inhibiting processes related to aging. *Spd* has been found to increase the life span of multi-cellular organisms, such as nematodes, flies and mice [16]. A study with increased intake of *Spm* and *Spd* in aged mice found reduced levels of pro-inflammatory markers, age-associated DNA methylation, and mortality [17]. Further, in a 20 year study of a cohort of 829 human participants, spermidine showed the strongest inverse relation with mortality among 146 nutrients investigated [18]. In contrast to these positive effects, *PAs* have been suggested to have procarcinogenic properties in cancer patients, since polyamine concentrations caused by enhanced biosynthesis have been found in several cancers [16,18–21]. In cancer diagnosed patients, consumption of *PAs* increased the risk of cancer cell proliferation [22,23] or increased the malignant cell growth rate in established tumors [22]. The connection between *PAs* and cancer therapy and treatments has resulted in an increased interest in understanding how the polyamine metabolism can be of use in anticancer strategies [24]. However, for healthy individuals, a recent review concluded that a consensus is being reached that polyamine intake does not induce cancer [19]. Some investigations point to an inverse relationship between colorectal cancer and polyamine intake, though this was not found in postmenopausal women with BMIs above 25 [25,26]. Several publications analyze the pros and cons of promoting *PAs* intake to increase longevity as well as prevention of new tumor formation in healthy subjects [20,27,28].

Horticultural crops are a good general source of vitamins, minerals and fibers among other compounds [28,29]. Many of these vegetables and fruits constitute substantial sources of *PAs* [30–32]. The effect of biotic or abiotic stressors on *PAs* composition has been addressed within the same species [33–35] or between spices [36–38]. However, reports on literature of *PAs* composition during different maturation stages within the same species are scarce. Tomato (*Solanum lycopersicum* spp.) is a major horticultural crop cultivated across the world, both in open fields and in green-houses to satisfy the increasing demand worldwide [39]. Tomato is highly appreciated by humans, consumed either as fresh produce or as an ingredient [40], and it holds a relatively high content of nutrients, e.g., amino acids, vitamins and minerals [41]. A few previous studies have evaluated the content of *PAs* in tomatoes, indicating that *PAs* [19], and specifically *Spm* [24], increase during early maturation and thereafter decrease. The varying content of *PAs* over the maturation period in tomato, together with the positive relationships reported for *PAs* impact on human health [3], indicates opportunities to tailor nutritive tomatoes for improved public health. Furthermore, genotype variation in the content of *PAs* has been shown [42], which might also have an impact on the variation of content over the tomato maturation period. To our knowledge, studies on interrelationships of *PAs* in various genotypes and over the full maturation time in non-stressed plants are scarce.

Several studies have shown relationships between the content of *PAs* with the content of ascorbic acid [36], as well as with lycopene content and ethylene production [43]. In-

creases in ethylene content is a physiological signal to promote fruit maturity in tomato [42]. A full comparison of *PAs* content and quality traits in tomatoes [44–46], such as firmness, freshness, shape, ascorbic acid, total soluble solid contents, titratable acidity and color, and effects of maturation has not been carried out. Based on the impact of tomato consumption and opportunities to tailor nutrition and quality in tomato for different purposes, a better understanding of variation in *PAs* over genotypes and maturation stages, and relation to other quality traits, is of outmost importance.

Thus, the aim of the present study was to evaluate the effect of tomato genotype and maturity stage on the content and composition of *PAs* in tomatoes. A second aim was to compare levels and composition of *PAs* with levels of other quality traits in tomatoes. Furthermore, the possible impact of *PAs* in various tomato genotypes at various maturity stages as biomolecules affecting plant development and health, and their effect on human health, is discussed.

2. Materials and Methods

2.1. Plant Material, Growing Conditions and Sampling

The tomato genotypes evaluated in the present study comprised four commercial genotypes; 'Lia' (seed producer; Hazera—Seeds of growth), 'Shanty' (Hazera—Seeds of growth), 'Huichol' (Seminis), and 'Rio Grande' (reproduced from the Bolivian National Center of Horticultural Seed Production CNPSH), and four genotypes developed by CNPSH; 'HT23', 'HT25', 'HT36', 'HT37'. The four commercial genotypes were selected as they are the preferred genotypes chosen by Bolivian tomato growers, recognizing them as holding favorable traits, e.g., size, elongated shape, diseases resistance, and suitability for local production conditions or market demand. The HT genotypes are advanced lines of which 'HT23' and 'HT25' meet the quality standards required from Bolivian growers, and 'HT36' and 'HT37' have a higher yield than the other genotypes in the present study. A total of thirty seeds from each genotype were germinated and thereafter five healthy plants were selected from each genotype for further cultivation to sample 3–4 replicates at each of six maturity stages. The plants were cultivated separately in 7.5 L pots in a greenhouse at ±24 °C/19 °C day/night temperature, 16/8 h (light/dark conditions) and 60% RH. A combination of natural light and HPS lamps with 100 to 150 μmol/m^2/s were used, and plants were fertigated and irrigated using an automatic daily ferti-irrigation system.

Tomatoes of each genotype were sampled at different maturity stages; green (*g*), breaker (*b*), turning (*t*), pink (*p*), light red (*lr*), and red (*r*) (Figure S1), based on the visual aid TM-L1 developed by the U.S. Department of Agriculture (USDA) [47]. A total of three to four tomatoes were sampled from each genotype at each maturity stage, and these were evaluated for their shape, fresh weight, and firmness immediately after harvest. The shape was determined according to IPGRI and OPOV [48]. Then, the tomatoes were chopped and for each genotype the tomatoes were divided into four equal portions. One portion was used immediately to process samples to analyze ascorbic acid and another portion was stored at −20 °C and was later used for the analyses of pH, color, titratable acidity (*Ta*), and total soluble solids (*Tss*). The remaining portions were stored at −80 °C for further analysis of *PAs*.

2.2. Reagents

Putrescine (*Put*; ref no 51799-100MG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Spermidine trihydrochloride was brought from Thermo Fisher Acreos Organic (NJ USA). Spermine tetrahydrochloride was purchased from ICN Biomedics inc (Morrow, OH, USA). Dansyl chloride and 1,7-diaminoheptane were purchased from ThermoFisher (Schnelldorf, BY, Germany). Acetonitrile for HPLC LC-MS grade, L(+)-ascorbic acid, sodium bicarbonate ($NaHCO_3$), and dithiothreitol were bought from VWR Chemicals (Leuven, VLG, Belgium, Radnor, PA, USA, and Lutterworth, Leics, England). Ammonium acetate, ethyl acetate 99.8%, hydrochloric acid, methanol, meta-phosphoric and sodium hydroxide were provided from Merck (Darmstadt, HE, Germany). Sodium carbonate was

obtained from PanReac Applichem (Darmstadt, HE, Germany). Dipotassium hydrogen phosphate, and potassium dihydrogen phosphate were brought from Duchefa (Haarlem, NH, The Netherlands).

2.3. Fruit Weight and Firmness

Each fresh tomato fruit was separately weighed on a digital balance and the firmness was measured by a fruit pressure tester (FT 327, Effegi, Italy). Then, the average fresh weight and the average firmness of the three to four tomatoes from each genotype and maturity stage were calculated.

2.4. Ascorbic Acid (Aa) Analysis

The *Aa* is one of the most well studied compounds in most fruits and vegetables, including tomatoes, and the content is known to correlate negatively with maturation in tomato [23], which justifies the analysis of its content in the present study. Here, the *Aa* content of the tomatoes was analyzed according to Bergquist et al. [49] with some modifications. Thus, extraction was carried out in a dim green light dark room. A total of 5 g of chopped tomatoes and 25 mL of 1.5% meta-phosphoric acid were added to a 50 mL brown conical flask. Samples were homogenized for 60 s using an Ultra-turrax IKA TP 18/10 (Werke GmbH & Co. KG Staufen, Germany), and thereafter they were kept cold (4 °C) and dark for 60 min before centrifugation (Eppendorf 5427 R; Hamburg, Germany) at 12,900× *g* for 10 min. Then, 1.7 mL of the supernatant aliquot of each sample was stored at −80 °C until HPLC analysis. For the HPLC analysis, samples were thawed and centrifuged, and an aliquot of 500 µL of the supernatant was mixed with 500 µL of dithiothreitol (11 µg-µL) to reduce dehydroascorbic acid to ascorbic acid. The samples were then centrifuged at 8944× *g* for 2 min at room temperature, and placed in amber vials. *Aa* stock solution (50 µg-mL) was treated the same way as the samples. HPLC analyses were carried out using an Agilent Technologies 1260 Infinity HPLC system (CA, USA). Vials were placed in a thermostated autosampler and an aliquot of 10 µL was injected on a Phenomenex (Torrence, CA, USA) Synergi Polar-RP 80 Å, LC column (4.6 × 50 mm, 4 µm). The eluent buffer consisted of methanol (4%) and KH_2PO_4 solution (20 mM) at pH 2.3. Standards and samples were run in an isocratic mode with a flow rate of 1 mL/min for 14 min and with a wavelength of 248 nm. The amount of ascorbic acid was quantified from comparisons with the standard curve.

2.5. Color Measurement, Total Soluble Solids (Tss), pH, and Titratable Acidity (TA)

Skin pieces of tomatoes were thawed and color at each maturity stage was evaluated as L* for value for lightness, Chroma for brightness, a* value, and b* value for red/green color and yellow/blue color, respectively [50,51], with a Chromameter Apparatus Konica Minolta CR-400 (Osaka, Japan). A representative sample of each tomato genotype at each maturity stage was used to produce pure tomato juice, from which *Tss* was evaluated in four replicated samples by a digital refractometer RFM80 (Wells, UK). Each juice sample was then diluted with distilled water (1:4) for measurement of pH and *Ta* by a digital titrator (Titroline Schott Instruments apparatus, Germany). For *Ta* measurements, a working solution of 50 mM NaOH and endpoint of the reaction of pH 8.3 was used. The *Tss*/*Ta* ratio is a comprehensive predictor of flavor [52] and this ratio was calculated by dividing % *Tss* with % *Ta* [53].

2.6. Polyamines Analysis

In plants, the majority of polyamines are present in their free form [54]. This study isolated and quantified free *PAs*, a combination of methods developed previously was used [55–58] and with modifications as follows: standards of *Put*, *Spd*, and *Spm* were prepared separately, by diluting each of them in millipore ultrapure water, storing them at −20 °C, and thereafter mixing 25 µL of each diluted polyamine with 925 µL of a premixed solution made of HCl (1 M) and the internal standard 1,7-diaminoheptane (30 µg/mL).

Before and during analysis, the standard solution (100 µg/mL) was treated the same way as the tomato samples extracted for polyamine analysis. Tomato samples of different genotypes and maturity stages were lyophilized at −105 °C (CoolSafe TM SCANVAC) until a steady weight was achieved. Lyophilized samples were homogenized to a powder in an Ultra centrifugal mill (ZM 200 RETSCH GmBH; Haan, Germany) equipped with trapezoid sieve holes of 0.5 mm pore size, at 2016 g. The powder was then kept at −80 °C until polyamine extraction.

The extraction of free polyamines was carried out according to Buranaphalin [59] with some modifications. Thus, 50 mg of a representative powder sample of each tomato sample (genotype and maturity stages) was placed in a Eppendorf tube and thereafter 1000 µL of 1 M HCl and 30 µg-mL of 1,7-diaminoheptane (internal standard) was added. Samples were homogenized with a vortex (Combi-spin FVL-2400, Biosan, Latvia) for 30 s and ruptured by a sonication-shear bath (Bandelin Sonorex digitec DT 100 H, Bandelin, Germany) for 5 min. Thereafter, a cold extraction was carried out using an incubation period of 1 h followed by centrifugation at 4 °C for 10 min at 12,900× *g* (Eppendorf 5427 R Hamburg, Germany).

Dansylation of the polyamines was performed as follows: In an aliquot of 200 µL of supernatant from the hydrochloric extraction performed previously, 300 µL of saturated $NaHCO_3$, 100 µL of 2 M NaOH, and 600 µL of dansyl chloride (5 mM) were added. Treated samples were then mixed thoroughly with a vortex for 30 s, thereafter heated for 45 min at 60 °C in a heating bath with circulator (MS/2 Lauda, Königshofen, Germany), and then placed immediately in cold conditions until the liquid–liquid extraction was carried out.

Liquid–liquid extraction (LLE) was performed by adding 500 µL of pure ethylacetate to the dansylated sample, followed by homogenization with a vortex for 30 s and centrifugation for 1 min at room temperature. Thereafter, the supernatant was transferred to a new 2 mL Eppendorf tube. The same procedure was repeated twice on the same sample, and the two additional supernatants were pooled to the first one in the same Eppendorf tube. The pooled samples were evaporated using a water bath evaporator (TurboVap LV Biotage Charlotte, USA). The dry samples were thereafter resolved in 100 µL of pure acetonitrile, the samples were homogenized in a vortex for 30 s and centrifuged for 5 min at 12,900× *g* at room temperature. An aliquot of each sample was transferred to an amber vial for HPLC-DAD-MS analysis.

An HPLC-DAD-MS 1260 infinity system from Agilent Technologies (CA, USA) equipped with a diode array detector (DAD) G4212, and a single Quadrupole 6120 b run at 100 °C, equipped with an electrospray ion source with drying gas N_2 (12 L-min) at a gas temperature of 300 °C, and nebulizer 30 psig run in positive mode, was used for the analyses. The *PAs* were separated using a reverse-phase column 2.1 × 50 mm, 1.8 µm Agilent Zorbax SP (Santa Clara, USA) operated at 40 °C with an injection volume of 10 µL per sample.

The mobile phase used consisted of Eluent A: ammonium acetate 100 mM and Eluent B: pure acetonitrile, with the gradient as follows: 0–13 min 80–14% A, 13–14 min 14–80% A, 14–17 min 80% A, and a constant flow rate of 500 µL/min. HPLC-DAD-MS analysis: DAD was carried out using a wavelength of 221 nm± 4 nm for quantification of dansylated *PAs*. MS was run in SIM mode $(M + H)^+$. Identification of *PAs* was as follows: *Put*: 556.60, internal standard (*Is*): 597.80, *Spd*: 846.90, and *Spm*: 1138.30 in all samples. For data acquisition, the Chemstation software (B04.03-SP1, Ver87, Agilent Technologies, Waldbronn, Germany) was used. Table S1 shows the mass calculation used for *Put*, *Spd* and *Spm* bound to dansyl chloride. Total polyamines (*Tpa*) was calculated as the sum of *Put*, *Spd*, and *Spm*. A chromatogram of the standards (Figure S2a) and a representative chromatogram (Figure S2b) of the analyzed tomato samples is included as Figure S2.

2.7. Statistical Analysis

Statistical analysis was performed using R 3.5.2 with the packages, emmeans, factoextra, and ggplot2. Data were evaluated with the Shapiro normality test before statistical analysis. All study sample data were subject to analysis of variance (ANOVA) in a general

linear model including the factor genotypes, maturity stages, and interaction between genotype and maturity stage. Factors were considered statistically significant if $p \leq 0.01$. Differences between means were evaluated using Tukey posthoc test with a significance level of 0.05. Principal component analysis (PCA) was used to find relationships between *PAs* and conventional quality parameters in different genotypes, and at different stages.

3. Results and Discussion

The eight genotypes evaluated showed significant differences in type, form and color, as visualized in Figure 1.

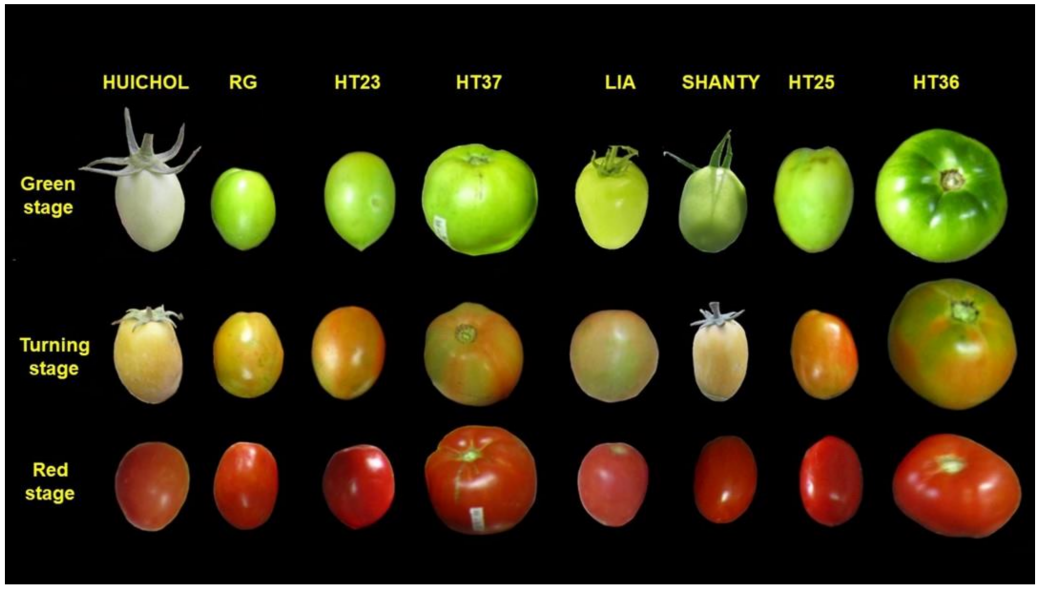

Figure 1. Tomato fruits of eight genotypes (From left to right: 'Huichol', 'Rio Grande' (RG), 'HT23', 'HT37', 'Lia', 'Shanty', 'HT25' and 'HT36') at three maturity stages (green, turning and red).

The ANOVA analysis (Table S2) showed a significant impact of tomato genotype, maturity stages, and their interactions on practically all analyzed parameters. Thus, although studies on genotypic and environmental effects on content and composition of *PAs* in tomatoes are scarce, our results correspond with those of other studies on other components and other crops [60–66]. Genotype differences are known to have an impact on a large number of quality parameters and components in almost any crop [60,61]. The level of the impact from the genotype is related to the type of quality characteristic evaluated and how broad a genetic variation is included in the selection of the plant material analyzed [62]. Additionally, the maturity stage has been verified as having a high impact on a range of quality parameters in a vast array of crops [63,64]. Furthermore, interactions between genotype and maturation stages are commonly seen in other studies [65,66].

Principal component analyses (PCA) revealed differentiation of components related to maturity along the first principal component axes (PC1—explaining 34% of the variation). Chroma, a* and b* values, total soluble solids *(Tss)* and titratable acidity *(Ta)* were all found with positive PC1 values and all of these components are known from previous studies with a positive correlation to maturity in tomatoes [67–70]. Furthermore, firmness and L* values, found with negative PC1 values, are known to correlate negatively with maturity in tomatoes [67,71]. Both the total polyamines content (*Tpa*) and amount of the different polyamines (putrescine (*Put*), spermidine (*Spd*) and spermine (*Spm*)) were found

to have positive values for the second principal component (PC2—explaining 20.3% of the variation), indicating variation in PC2 to be in principal determined by the *PAs* content and composition. However, values of PC1 were positive for *Tpa* and *Put* while negative for *Spd* and *Spm*. Thus, maturity of the tomatoes might influence the content of *PAs*, with tomato maturity being positively correlated with *Tpa* and *Put* and negatively correlated with *Spm* and *Spd*. The relationships between *PAs* and tomato maturity indicated by our PCA correspond well with previous results in transgenic tomatoes, where increased levels of *Spd* in the fruits delayed maturity [72].

The PCA loading plot (Figures 2B and S3) further verified the differentiation of tomato maturity along the PC1, with green and break stages of the tomatoes having negative PC1 values; turning and pink stages with neutral and light red and red stages with positive PC1 values. Thus, a comparison of the score and loading plot results in high levels of firmness and L* values in green and break stages of tomatoes and high Chroma, a* and b* values, *Tss* and *Ta* in light red and red stages of tomatoes, which also verifies previous results [45,69,73]. The genotypes in the present study showed a differentiation along the PC2 direction, indicating differences in the content of *PAs* (compare Figure 2B with Figure 2A), with high levels in the genotype 'HT37' through all maturation stages. Studies on *PAs* content and composition in tomatoes of different maturation stages are rare and mostly reported over a narrow range of tomatoes [36,72,74], although genotype variation has been reported in some studies on mature tomatoes [75–77]. The tomatoes evaluated in the present study generally showed high levels of polyamines, especially of *Put* and *Spd*, as compared to values reported in most other studies (Table S3). However, as genotype, cultivation conditions as well as experimental conditions are known to effect the content of *PAs* reported in tomatoes, such factors may also impact on the differences from various studies reported here.

Previous studies [74], have indicated a role of *PAs* for the determination of the fruit architecture, i.e., expression of yeast spermidine synthase (*Spds*) under tomato maturation, resulted in increased levels of obovoid fruits. In this study, tomato genotypes of various shapes and with different content and composition of *PAs* were included, although the limited number of genotypes of various shapes hindered a more thorough comparison of such relationships.

Comparison of mean values showed the effect of tomato genotype and maturation period on the content and composition of *Tpa, Put*, *Spd* and *Spm* (Figure 3 and Table S4). Thus, a y low level of *Tpa, Put* and *Spd* were found in genotypes such as 'Huichol' and 'Rio Grande', while a generally higher amount was found in the other evaluated genotypes. Changes in *PAs* over the maturation time varied inconsistently for the different genotypes evaluated here (Figure 3A). The content of *Put* was either showing no significant differences in the tomatoes along the maturation period or showing a significant increase over the maturation period, e.g., in 'Huichol', 'Rio Grande' and 'HT36' to a final content of 155, 133 and 1855 μg/g/DW, respectively (Figure 3B). Indications of a decrease in *Put* content in the middle of the maturation time was also found in 'Lia' and 'Shanty' with high values at the *g* and *r* stages (Figure 3B). The content of *Spd* showed either no significant variation over the maturation period or a significant decrease over the maturation period, e.g., in 'Huichol', 'HT23', 'Lia', 'Shanty' and 'HT25' (Figure 3C). *Spm* showed either no variation or a decrease ('HT23', 'Lia', 'Shanty' and 'HT36') over the maturation period. Thus, for *Tpa* over the maturation period, the different changes found in the individual polyamines led to no significant changes ('Huichol', 'Rio Grande', 'HT23', and 'HT37'), a decrease in the middle of the maturation period ('Lia'), or increases ('HT36') and decreases ('Shanty' and 'HT25') over the maturation period (Figure 3 and Table S4).

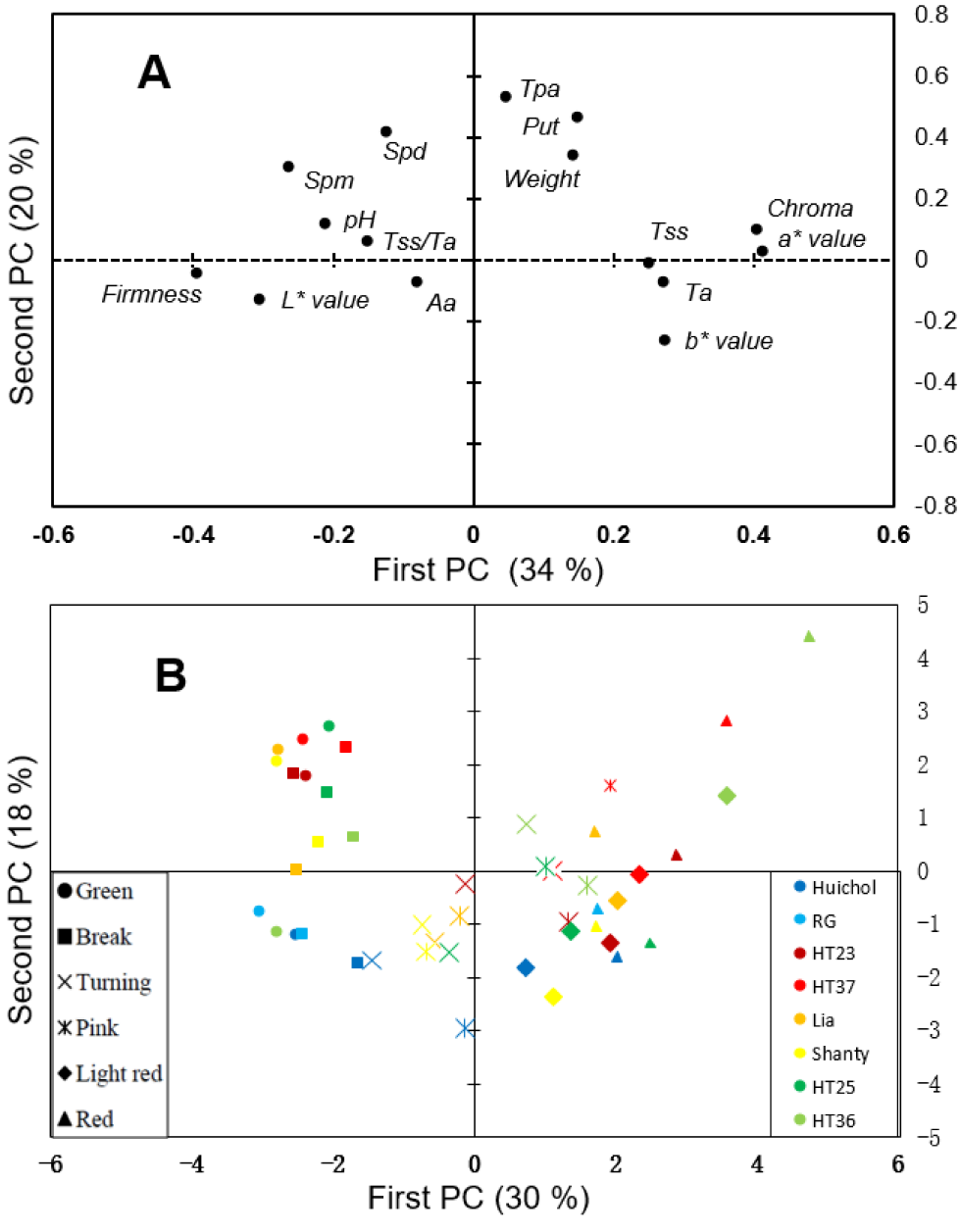

Figure 2. Score (**A**) and loading (**B**) plots generated from PCA on all and mean values, respectively, of quality traits (Chroma (brightness), a* value (redness), b* value (blueness), L* value (lightness), *TSS* (total soluble sugar), *Ta* (titratable acidity), *TSS*/*Ta* (total soluble sugar and titratable acidity ratio), *Aa* (ascorbic acid), Firmness, pH and Weight) and polyamines contents (*Tpa* (total content of polyamines), *Put* (putrescine), *Spd* (spermidine), *Spm* (spermine)) of eight genotypes 'Huichol', 'Rio Grande (RG)', 'HT23', 'HT37', 'Lia', 'Shanty', 'HT25', and 'HT36' (marked with different colors) at six maturity stages (marked with different symbols). Loading plot on data from each tomato evaluated are shown in Figure S3.

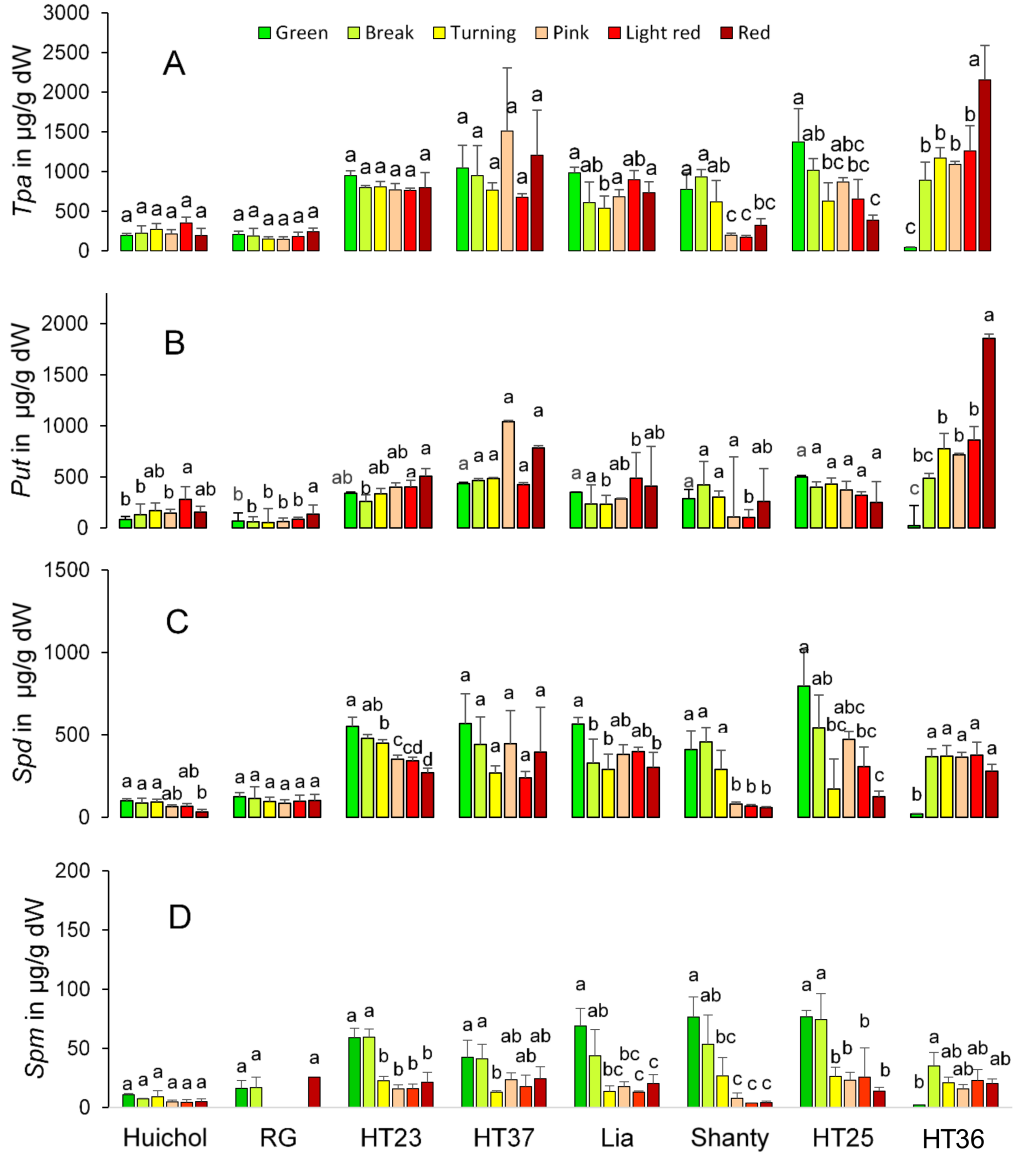

Figure 3. Content of Tpa (total polyamines (**A**)), *Put* (putrescine (**B**)), *Spd* (spermidine (**C**)) and *Spm* (spermine (**D**)) in µg/g dW of eight tomato genotypes ('Huichol', 'Rio Grande' (RG), 'HT23', 'HT37', 'Lia', 'Shanty', 'HT25' and 'HT36') at six maturity stages (green, break, turning, pink, light red and red). Values are the average of three to four samples. Bars indicate standard deviations. Letters above the staples that differ indicate significant differences ($p < 0.05$ using Tukey post hoc test) between tomatoes of different maturity stages within the same genotype. A comparison of statistical differences among mean values of all genotypes and stages is shown in Table S4.

A possible developmental inter-relationship between *PAs* and ethylene has frequently been discussed as they share a common substrate, S-adenosylmethionine (SAM), for their biosynthesis. The possible competition for the substrate SAM, as well as their suggested potential opposite functions as pro-senescence/pro-ripening for ethylene and pro-growth

for *PAs*, make these compounds interesting in relation to understanding fruit ripening and senescence processes [78]. Early studies indicated that polyamines inhibit ethylene biosynthesis in a variety of fruit tissues [79,80], though later investigations further concluded that the effects of *PAs* are also dependent on the developmental stage, which part of a plant that investigated and *PAs* concentrations [78]. It has been suggested that the SAM levels might not be generally rate limiting for the biosynthesis of *PAs* and ethylene, and their interrelationship rather is developmentally regulated in a tissue- and cell-specific manner [78]. In addition, the different *PAs* have been suggested to have different functions. The levels of *Put* are suggested to increase during conditions of low activity, while *Spd* and *Spm* act as ripening and growth stimulators, and the ratio *Spd/Put* could to some extent control ripening, senescence, and quality in climacteric fruits [81]. Further, *Put* has been indicated to act as a negative regulator while *Spd–Spm* are positive regulators of cellular amino acid metabolism [82]. In tomato, *PAs* have been found to influence the expression of genes related to ethylene synthesis, while 1-aminocyclopropane-l-carboxylic acid synthase (ACC) expression was repressed after the exogenous application of *PAs* [83]. In this study, the decrease in the concentrations of *Spd* with advancing tomato ripening in five of the cultivars and in *Spm* in four of the cultivars could be in accordance with these compounds acting as stimulators of growth and ripening. Previous investigations reported that the ethylene concentration peaked at the pink stage of the tomato [84], which in this investigation in some of the cultivars coincided with a drop in the concentration of *Spm*. In the later ripening stage in tomato, senescence-related genes are activated [85], and the onset of senescence processes could in this investigation be indicated by the lower levels of *Spd* and *Spm* in some cultivars, leading to downregulated amino acid metabolism.

Previous studies have reported similar variations in *PAs* effect of tomato genotype and maturation to the present study. Thus, a steady content of *Put* during all maturity stages was reported for *Lycopersicum esculentum* c.v. Indalo [86]. Another study on cherry tomato cv 'Chiou', reported a constant decrease of *Put* from *g* to *b* stage followed by a constant increase from *b* to *r* stage [7]. Decreasing levels of *Spd* over the maturation time has previously been reported [86]. Most previous studies that have evaluated changes in *PAs* over the tomato maturation time have focused on only one genotype meaning that the genotypic variation in *PAs* over maturation time, as the present study is depicting, has not previously been reported.

For the plant, *PAs* content and composition is reported to effect a range of cellular functions, impacting characteristics such as plant development and response to stresses and extreme environments, plant longevity, content of lycopene, physiological memory, and carbon/nitrogen allocation and signaling [3]. The present study clearly showed differences in the content of *PAs* between different tomato genotypes and also in patterns of accumulation over the tomato maturation period. Based on the knowledge from the literature, that transgenic tobacco, rice and tomato plants with high accumulation of *PAs* all showed increased tolerance to a range of stresses including salt and heat [87,88], indicate a possibility that the high *PAs* content genotypes (all except 'Huichol' and 'Rio Grande') in the present study may have higher tolerance to stresses than low *PAs* genotypes ('Huichol' and 'Rio Grande'). However, the present study did not evaluate such relationships, although such relationships might be of relevance in the development of stress tolerant tomatoes. Furthermore, studies on transgenic *Arabidopsis*, pear and potato with overexpression of *Spd* have shown a positive correlation with tolerance to stresses [88–92]. In the present study, the genotype variation in *Spd* followed the same pattern as *Tpa*. An interesting feature is that both *Spd* and *Tpa* changed in the tomatoes during the maturation period, and the impact of these differences needs to be further elaborated on when it comes to relationships to cellular functions. Previous studies have indicated that *PAs* metabolism can be upregulated, downregulated, or moderately expressed, and affect other quality traits depending on the particular maturity stage, genotypes, or even fruit type [93].

Due to the fact that the contents of *PAs* are known to be effected by external factors and stressors, such environmental conditions can be used to increase or decrease the

content of *PAs* in tomatoes. In the present study, all genotypes were grown under the same experimental conditions in a well-controlled environment, providing the same amount of nutrient solutions, light regime, humidity, and temperature. Thus, the environmental conditions are expected to have had a limited effect on the outcomes of the present study. However, environmental conditions can be used to interplay with genetic and maturation parameters to produce tomatoes with certain levels of specific *PAs*. The present study does, however, show the need for a thorough study into such relationships due to the fact that the different tomato genotypes reacted differently during the maturation period.

The variation in *PAs* content and composition in various tomato genotypes and over maturation time, depicted in the present study, is also of relevance when it comes to the effects of tomato consumption on human health. A number of studies have indicated positive effects of intake of food with a sufficient amount and composition of *PAs*, as reviewed by Handa et al. [3]. *PAs* have been shown to positively affect physiological processes connected to normal growth, cardio protection, aging, oxidative stress and loss of memory, as well as a negative correlation with diseases such as cancer, Alzheimer's disease and Parkinson's disease, has been reported [24,94–98]. Based on the many positive health perspectives gained rom the intake of *PAs*, nutritionally high value *PAs* tomatoes might in the future be of potential interest, either as health food or as food additives. The present study shows the genotype 'HT36' to be an interesting candidate as a health promoting red tomato, while 'HT25' is interesting as a health promoting green tomato, and 'Lia' shows high and stable *Spd* levels throughout the maturation period. However, to be able to declare a tomato health promoting based on *PAs* content and composition, additional studies are needed, not least connecting animal/human models with knowledge on tomato genotypes and maturation. Furthermore, the procarcinogenic properties of *PAs* in cancer patients [16–21] have to be further studied to secure a positive and not a negative effect from the intake of certain tomato genotypes rich in *PAs*.

4. Conclusions

Content and composition of *PAs* vary significantly among tomato genotypes. Thus, high content genotypes of both *Tpa* and of the different types of *PAs* can be differentiated and used for breeding and production of health promoting tomatoes for direct consumption or for use as food additives. Variation over the maturation period, both in *Tpa* content and in content of specific *PAs*, was inconsistent among genotypes, and needs to be characterized separately for every genotype. However, this variation creates opportunities to select and produce both health promoting green and red tomatoes as well as those that are stable over the entire production period. The found variation in tomato genotypes and over the maturation period calls for extended studies to understand these implications for human health. The fact that the *PA* content and composition vary over the tomato maturation period and do so differently for different genotypes most likely affects the plant and fruit development as well as physiological characteristics, including tolerance to stresses and diseases. However, such relationships need further evaluation, as do the connections of specific tomatoes at specific maturity stages on human health.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7090300/s1; Table S1: Mass Calculation for Putrescine, Spermidine and Spermine bound with Dansyl Chloride; Table S2: Mean Square Value from Analysis of Variance (ANOVA) of Polyamines Contents+ and Quality Traits++ in Tomato Samples as Affected by Genotype and Maturity Stage; Table S3: Mean values (μg/g DW) of Polyamines in Tomato at Different Maturity Stages from Various Studies; Table S4: Mean values (μg/g DW) of Polyamines in Tomato at Different Maturity Stages.; Figure S1: Representative samples showing the different maturation stages of tomatoes from various genotypes; Figure S2: Polyamines chromatogram; Figure S3: Loading plot generated from PCA on values of each tomato evaluated, from quality traits.

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Tomato is the most common vegetable worldwide and Bolivia holds a germplasm collection of wild relatives of tomato. This thesis provides new information on genetic diversity, population structure, quality traits and bioactive compounds in Bolivian wild relatives, cultivars and advanced lines. Bioactive compounds include polyamines and carotenoids, which are important for human health and plant survival in challenging climates. Analysis of the tomato value chain identified constraints affecting quality in developing country conditions.

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