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RESEARCH ARTICLE

Characterization of a landrace collection for *Tomàtiga de Ramellet* (*Solanum lycopersicum* L.) from the Balearic Islands

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Abstract *Tomàtiga de Ramellet* (*Ramellet*) is a traditional long-storage tomato from the Mediterranean Islands of Mallorca, Menorca, Eivissa (Ibiza), and Formentera (Balearic Islands). To investigate the observed phenotypic and shelf life variability within local varieties, we studied 142 accessions characterized as *Ramellet* varieties, and 29 accessions representing other local varieties. The 171 accessions constitute a new germplasm collection at the University of Balearic Islands (UIB). The evaluation of the collection demonstrated wide variability in most agronomic, morphological and quality traits of *Ramellet* accessions and clearly differentiates them from other local varieties with respect to fruit shape, size, fruit composition, and fruit storage. All accessions of *Ramellet* evaluated have the ripening mutation *alcobaça* (*alc*), suggesting an important role for this variant in controlling shelf life. Results of evaluation also showed wide variability between the *Ramellet* accessions for storage time, suggesting that other traits like fruit size and composition may play a role in fruit conservation. Observed variation within the *Ramellet* accessions is consistent with a genetically variable population or landrace group. The establishment of this collection will help prevent genetic erosion of this local landrace group and constitute a resource for future investigation and breeding.

Keywords Collection characterization · Genetic diversity · Genetic resources · Mediterranean tomato landraces · *Solanum lycopersicum*

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Introduction

The increased awareness of traditional crop varieties for specific market uses is driving interest in describing regionally adapted crop varieties, or landraces. Landraces are characterized by genetic diversity, local adaptation and specific cultural uses, which may be subject to selection. Domestication follows a multi-step process in which initial domestication is followed by dispersal and subsequent selection for adaptation to

new conditions of cultivation and environment (Gepts 2002). The history of tomato (*Solanum lycopersicum* L.) domestication and early breeding has been extensively reviewed (Boswell 1937; Jenkins 1948; Walter 1967; Rick 1995; Peralta and Spooner 2007; Sim et al. 2009, 2012; Blanca et al. 2012), and there is an agreement that the center of origin for tomato is Andean South America. In this region, there are several sympatric species, including the red-fruited *Solanum pimpinellifolium* L. and *S. lycopersicum* var. *cerasiforme* (Alef.) D. M. Spooner, G. J. Anderson and R. K. Jansen the most likely progenitors of the first domesticated varieties (Ranc et al. 2008). The sequence of tomato domestication remains an unresolved issue, though recent analysis suggests that pre-domestication events may have occurred in Andean South America, and the process was completed in the Yucatan peninsula of Mexico (Peralta and Spooner 2007; Blanca et al. 2012). Large-fruited landraces were in existence when Europeans first explored Central and South America (Jenkins 1948). The first tomatoes travelled to Europe with Spanish explorers and quickly spread throughout the Iberian Peninsula and to Italy. Regional differences in the form of fruit and specific uses indicate that selection continued following dispersal in Europe (Rodríguez et al. 2011).

Tomàtiga de Ramellet (*Ramellet*) is a traditional long-storage tomato from the Balearic Islands in the Mediterranean including Mallorca, Menorca, Eivissa (Ibiza), and Formentera. The *Ramellet* tomatoes were traditionally cultivated by families in small plots for personal consumption. The origin of its name appears to be related to the occurrence of fruits in a short, dense, sympodical bunch-like inflorescence (Alcover and de Borja-Moll 1983). Since *Ramellet* tomatoes are typically tied in bunches and stored hanging throughout the fall, winter and spring, sometimes they are also referred to as *Tomàtiga de Penjar* ("tomato for hanging"), which is a name also used for similar cultivars existing in the eastern Iberian Peninsula (Alcover and de Borja-Moll 1983). *Ramellet* tomatoes are well adapted to calcareous soils and summer droughts that are typical in the Balearic Islands (Espallardo et al. 2006). Specific adaptations to drought have been described, that permits cultivation under dry conditions (Galmés et al. 2011, 2013). The long shelf life property of the *Ramellet* is of cultural significance in local cuisine and may have played a historically important dietary role when other vegetables were limiting in the winter months. In general, the *Ramellet* is consumed fresh, usually rubbed on bread, thus the anthropomorphic selection process was presumably based in part on quality aspects in addition to storage durability. Traditional tomato varieties from the region possess cultural significance, traits of interest to contemporary plant breeding, and morphological variation.

The written record concerning the introduction of tomato to the Balearic archipelago is incomplete. Ludwig Salvator (Syn. Archduke Luis Salvador of Habsburg-Lorena, Austria) describes six types of tomato in use in Menorca: Tomàtiga d'Alger, de Bolla, de Carabasseta, de Pera, de Pruna and Catalans (Habsburg-Lorena 1989). The *Bolla*, *Carabasseta*, and *Pruna* types are described as tomatoes that are hung for later use, suggesting that morphological diversity existed in the *Ramellet* varieties at that time. Ludwig Salvator's description lags the known introduction of tomato to Europe by nearly 300 years. Works of art provide evidence that tomato was in cultivation in the Balearic Islands many years earlier than the written record suggests. Several "still life" paintings from the period *circa* 1630–1650 clearly show tomato fruit and leaves which are consistent with the *Ramellet* currently cultivated (Centre cultural *Sa Nostra* 1994). These paintings provide an historical record demonstrating that *Ramellet* has been cultivated in the Balearic Islands for at least three and a half centuries.

The extended fruit shelf life of the *Ramellet* variety is an important distinguishing feature. There are several naturally occurring mutations that affect the ripening and storage properties of tomato fruit. These include ripening inhibitor (*rin*), non-ripening (*nor*), and Never Ripe (*Nr*). These genes have been cloned and studied at the molecular level. The *Nr* is a mutation in the ethylene receptor that affects ethylene perception (Lanahan et al. 1994; Wilkinson et al. 1995). The *rin* mutation affects a MADS box transcription factor, resulting in fruits that fail to ripen (Vrebalov et al. 2002). Mutations in *nor* are of particular interest as distinct mutations have been selected for use in agriculture (Vrebalov et al. 2004). The gene *nor* encodes for a transcription factor of the NAC protein family, and plays a role in the control of ripening (Vrebalov et al. 2009). The *alcobaça* (*alc*) mutation appears to be the cause of the long shelf life properties in *Ramellet* tomato based on segregation

and sequence data (Vrebalov et al. 2004). Tomatoes with *alc* have less than 25 % of the ethylene production of wild type plants, increased fruit firmness and increased resistance to bacterial disease (Lobo et al. 1984; Mutschler 1984). The presence of *alc* has also been attributed to extended fruit shelf life in tomato cultivars, *Penjar* tomato, in eastern Spain (Casals et al. 2011). Tomatoes homozygous for *alcobaça* (*alc/alc*) are firmer and display longer storage than wild type (alc^{+}/alc^{+}) varieties (Mutschler et al. 1992). Tomatoes with the *alc* mutation appear synonymous with the DFD phenotype (Saladié et al. 2007).

The local importance of *Ramellet* in the Balearic Islands inspired the collection and characterization of locally saved varieties described here. In order to assess and quantify this phenotypic diversity, we collected grower-saved seed accessions across the archipelago, including inbred accessions both from local gardens, commercial growers and from regional germplasm collections. Data were collected for fruit, leaf and plant morphology, production characteristics and fruit quality in order to describe the variation existing among the Balearic accessions of *Ramellet.* The presence of the *alc* mutation was investigated in the collection of landrace tomatoes, and this mutation appears to be one of the defining characteristics of the *Ramellet.* The observed variation in plant phenotypes suggests that the *Ramellet* is a population or landrace group (Zeven 1998) consisting of diverse, inbreeding lines and conservation of the inherent variability may provide a reservoir for future breeding programs.

Materials and methods

Establishment of the UIB seed collection

The accessions were collected over a 2-year period (2007–2008), with the assistance and collaboration of the staff of Universitat de les Illes Baleares (UIB, Palma de Mallorca, Spain), from 40 municipalities of Mallorca, Menorca, Eivissa (Ibiza) and Formentera (Fig. 1). In total, seeds were obtained from about 120 small growers who save their own seed, large grower producers (commercial selections) or established collections. Seed from established collections included 22 accessions from the Botanical Garden of *Sóller*, and 4 accessions from the Seed Bank of Local Varieties from Menorca (APAEM-Consell Insular de Menorca) and the Conselleria d'Agricultura, Medi Ambient i Territori (local government). A total of 198 accessions were obtained from the Balearic Islands. From those, 171 accessions provided sufficient seed and germination suitable to be included in the present study, with 142 accessions characterized as *Ramellet* varieties, and 29 accessions representing other local varieties which we included in the seed collection as reference (Table 1).

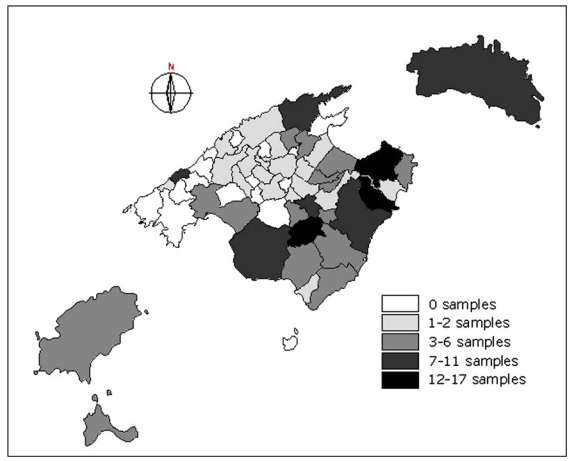


Fig. 1 Geographic locations of tomato accessions collected from the Balearic Islands

Most accessions were collected as fruit in February 2007 and February 2008, and seed extraction followed a standard protocol of treatment in 3 N HCl for 20 min. Following acid treatment, seed were washed with water several times and dried on absorbent paper. In some instances seed was obtained directly from organizations or individual donors.

Plant growth and plot design

Plants were grown at the experimental farm on the main campus of UIB. Seedlings were established in flats with potting mix (Prohumin Sustrato Klasmann-Deilmann, Comercial Projar S.A., Valencia, Spain) and 25 % perlite. Plants were grown for 1 month in a greenhouse (15–25 °C, natural light) and 1 month outdoors to harden prior to transplanting into the field.

Planting consisted of a randomized complete block design, with each accession replicated in two blocks, one replicate in each. The experimental field was flat and homogenous for texture and nutrients. Plants were irrigated through drip lines to each plot with manual

Table 1 Accession codes, collection location (provenance) and common names for the tomato germplasm collection

Code	Provenance	Common name	Code	Provenance	Common name	Code	Provenance	Common name
UIB0001*	Pollença	*Ramellet*	UIB0062*	Artà	*Ramellet*	UIB0124*	Capdepera	*Ramellet*
UIB0002*	Pollença	*Ramellet*	UIB0063*	Sant Llorenç	*Ramellet*	UIB0125*	Santanyí	*Ramellet*
UIB0003*	Sa Pobla	*Ramellet*	UIB0064*	Sant Llorenç	*Ramellet de ferro*	UIB0126*	Capdepera	*Ramellet*
UIB0004*	Pollença	*Ramellet*	UIB0065*	Palma	*Ramellet*	UIB0127*	Barcelona	*Ramellet*
UIB0005*	Sa Pobla	*Ramellet*	UIB0066*	Alaró	*Ramellet*	UIB0128*	Artà	*Ramellet*
UIB0006*	Pollença	*Ramellet*	UIB0067*	Manacor	*Ramellet*	UIB0129*	Artà	*Ramellet*
UIB0007*	Porreres	*Ramellet*	UIB0070*	Vilafranca	*Ramellet*	UIB0130*	Artà	*Ramellet*
UIB0008*	Santa Maria	*Ramellet*	UIB0071*	Campanet	*Ramellet*	UIB0131*	Artà	*Ramellet*
UIB0010*	Selva	*Ramellet*	UIB0072*	Sa Pobla	*Ramellet*	UIB0132*	Sant Joan	*Ramellet*
UIB0011*	Manacor	*Ramellet*	UIB0073*	Porreres	*Ramellet*	UIB0133*	Sant Llorenç	*Ramellet*
UIB0013*	Banyalbufar	*Ramellet*	UIB0074*	Muro	*Ramellet*	UIB0134*	Escorca	*Ramellet*
UIB0014*	Llucmajor	*Ramellet*	UIB0075*	Felanitx	*Ramellet*	UIB0135*	Escorca	*Ramellet*
UIB0015*	Felanitx	*Ramellet*	UIB0076*	Sant Joan	*Ramellet*	UIB0136*	Campanet	*Ramellet*
UIB0016*	Felanitx	*Ramellet*	UIB0078*	Felanitx	*Ramellet*	UIB0137*	Manacor	*Ramellet*
UIB0017*	Llubí	*Ramellet*	UIB0079*	Sant Joan	*Ramellet*	UIB0138*	Manacor	*Ramellet*
UIB0018*	Llucmajor	*Ramellet*	UIB0080*	Maria de la Salut	*Ramellet*	UIB0139*	Eivissa	*Ramellet*
UIB0019*	Soller	*Ramellet*	UIB0081*	Sant Llorenç	*Ramellet*	UIB0141*	Sant Llorenç	*Ramellet*
UIB0020*	Inca	*Ramellet*	UIB0082*	Sant Llorenç	*Ramellet*	UIB0146*	Ariany	*Ramellet* comercial selection
UIB0021*	Ariany	*Ramellet branca curta*	UIB0083*	Sant Llorenç	*Ramellet*	UIB0147*	Campos	*Ramellet* comercial selection
UIB0023*	Ariany	*Ramellet branca llarga*	UIB0084*	Sant Llorenç	*Ramellet*	UIB0148*	Porreres	*Ramellet* comercial selection very ancient line
UIB0024*	Ariany	Long term *Ramellet*	UIB0085*	Sant Llorenç	*Ramellet*	UIB0149	Porreres	Pepper tomato
UIB0025*	Felanitx	*Ramellet*	UIB0086*	Sant Llorenç	*Ramellet*	UIB0150*	Porreres	*Ramellet* comercial selection *branca curta*
UIB0026*	Sencelles	*Ramellet*	UIB0087*	Sant Llorenç	*Ramellet*	UIB0151*	Porreres	*Ramellet* big comercial selection
UIB0027*	Felanitx	*Ramellet*	UIB0088*	Sant Llorenç	*Ramellet*	UIB0152*	Eivissa	*Ramellet* comercial selection *de ferro*
UIB0028*	Pollença	*Ramellet*	UIB0089*	Sant Llorenç	*Ramellet*	UIB0154	Menorca	Rosadet tomato
UIB0029*	Unknown	*Ramellet*	UIB0090*	Sant Llorenç	*Ramellet*	UIB0155	Menorca	Big tomato

Table 1 continued

Code	Provenance	Common name	Code	Provenance	Common name	Code	Provenance	Common name
UIB0030*	Estallencs	*Ramellet*	UIB0091*	Sant Llorenç	*Ramellet*	UIB0157*	Menorca	*Ramellet Algendar*
UIB0031*	Banyalbufar	*Ramellet*	UIB0092*	Santa Margalida	*Ramellet*	UIB0159	Menorca	Elongated cherry
UIB0032*	Manacor	*Ramellet*	UIB0093*	Santa Margalida	*Ramellet*	UIB0162*	Formentera	*Ramellet de penjar*
UIB0033*	Manacor	*Ramellet*	UIB0094*	Pollença	*Ramellet*	UIB0163*	Llucmajor	Long *Ramellet de penjar*
UIB0034*	Santanyi	*Ramellet*	UIB0095*	Pollença	*Ramellet*	UIB0164*	Llucmajor	*Ramellet*
UIB0035*	Sa Pobla	*Ramellet*	UIB0098*	Santa Margalida	*Ramellet*	UIB0166*	Unknown	*Ramellet rampiz*
UIB0036*	Bunyola	*Ramellet*	UIB0099*	Palma	*Ramellet*	UIB0169*	Menorca	Cent flors
UIB0037*	Manacor	*Ramellet*	UIB0100*	Vilafranca	*Ramellet*	UIB0170*	Eivissa	Unknown
UIB0038*	Sa Pobla	*Ramellet*	UIB0101*	Sant Joan	*Ramellet*	UIB0171*	Llucmajor	*Burot* tomato
UIB0039*	Maria de la Salut	*Ramellet*	UIB0102*	Campos	*Ramellet*	UIB0172	Sóller	Plum tomato
UIB0040*	Vilafranca	*Ramellet*	UIB0103*	Porreres	*Ramellet*	UIB0173	Montuïri	Unknown
UIB0041*	Pollença	*Ramellet*	UIB0104*	Montuïri	*Ramellet*	UIB0174	Sineu	Pepper tomato
UIB0042	Menorca	*Cirereta* Menorca	UIB0105*	Porreres	*Ramellet*	UIB0176	Menorca	Early tomato
UIB0044*	Menorca	*Ramellet de ferro*	UIB0106*	Campos	*Ramellet*	UIB0177	Sant Joan	Creeping tomato
UIB0045	Menorca	*Cirereta* Menorca	UIB0107*	Montuïri	*Ramellet*	UIB0178	Banyalbufar	*Ramellet Banyalbufar Antiga*
UIB0046*	Unknown	*Ramellet*	UIB0108*	Porreres	*Ramellet*	UIB0179*	Banyalbufar	*Ramellet brot vermell*
UIB0047*	Unknown	*Ramellet*	UIB0109	Banyalbufar	*Ramellet Banyalbufar Antiga*	UIB0180*	Sant Joan	*Ramellet de penjar*
UIB0048*	Mallorca	Commercial *Ramellet* (Batlle)	UIB0110	Banyalbufar	*Ramellet Banyalbufar Antiga*	UIB0181*	Unknown	White *Ramellet de penjar*
UIB0049*	Palma	*Ramellet*	UIB0111*	Banyalbufar	*Ramellet*	UIB0182*	Unknown	*Ramellet de ferro*
UIB0050*	Palma	*Ramellet*	UIB0112*	Banyalbufar	*Ramellet*	UIB0185*	Unknown	*Ramellet de mamelló*
UIB0051*	Artà	*Ramellet*	UIB0113*	Lloret	*Ramellet*	UIB0186	Unknown	Morada
UIB0052*	Artà	*Ramellet*	UIB0114*	Sineu	*Ramellet*	UIB0187	Unknown	Plum-pear tomato
UIB0053*	Son Servera	White *Ramellet*	UIB0115*	Petra	*Ramellet*	UIB0189*	Palma	Cor de bou
UIB0054*	Manacor	Red *Ramellet*	UIB0116*	Vilafranca	*Ramellet*	UIB0190	Lloseta	Unknown
UIB0055*	Sant Llorenç	*Ramellet*	UIB0117*	Vilafranca	*Ramellet*	UIB0191*	Unknown	Cor de bou
UIB0056*	Manacor	*Ramellet*	UIB0118*	Petra	*Ramellet*	UIB0192	Formentera	Cor de bou
UIB0057*	Porreres	*Ramellet*	UIB0119*	Pollença	*Ramellet*	UIB0193	Porreres	Cor de bou

Table 1 continued

Code	Provenance	Common name	Code	Provenance	Common name	Code	Provenance	Common name
UIB0058*	Artà	*Ramellet*	UIB0120*	Maria de la Salut	*Ramellet*	UIB0194	Unknown	Valldemossa
UIB0059*	Artà	*Ramellet*	UIB0121*	Campanet	*Ramellet*	UIB0195	Porreres	Muchamiel
UIB0060*	Artà	*Ramellet*	UIB0122*	Capdepera	*Ramellet*	UIB0197*	Formentera	Tres cantos
UIB0061*	Artà	*Ramellet*	UIB0123*	Capdepera	*Ramellet*	UIB0198*	Unknown	Cor de bou

*Verified *alc* mutation by molecular maker

control based on potential evaptranspiration (PET) measurements monitored in a nearby weather recording station. Precipitation was monitored and subtracted from the corresponding PET irrigation dose. In general, no more than 2 irrigations per week were performed and were based on the PET coefficient (K_c) of 0.8 l/m^2 (estimated from Castilla and Fereres 1990; Gianquinto et al. 1990 data for fresh market tomato). Plants were grown without support and were otherwise maintained according to recommended practices (Castilla and Fereres 1990).

Leaf and plant morphology

We examined leaf morphology with respect to the number of leaflets and the appearance of the leaflet margin. The collection contained plants with five or more leaflets and deeply indented to lobulated margins (wild-type) and plants with a reduced number of leaflets and reduced number of lobes characteristic of the *potato leaf* (*c*) mutation (Kessler et al. 2001). Plants were scored qualitatively for leaf type based on leaflet number and margins as wild-type when there were 4–9 leaflets with divided margins or as *potato leaf* when there were <4 leaflets and smooth margins.

Other aspects of plant morphology were measured, based on two plants per accession. Plant size was estimated by measuring in three dimensions (x, y, z) which correspond, respectively, to the direction parallel to irrigation line, perpendicular to the irrigation line, and height. Plant size was then expressed as a volume (m^3) based on multiplying measurements across the three dimensions (x, y, z) or as the occupied area (m^2) based on multiplying the "x" and "y" dimensions. Finally the number of main branches per plant was counted for two plants per accession at the end of the vegetative growth period.

Production measurements

Harvest was initiated with the first ripe fruits, and continued once or twice a week, depending on production. All fruits per plant were counted and weighed. The sum of all harvested fruits (weight and number) from each plot was calculated and data from both blocks was combined to estimate the total production for each accession, expressed on a per plant basis.

Fruit shelf life was estimated for each accession according to the randomized complete block design. At peak production (15 July–15 August), 20 healthy representative fruits were collected and stored on paper sheets in plastic produce storage boxes which permitted air flow. Fruits were dispersed in the boxes to prevent contact with each other. Boxes with fruit were placed in a well-ventilated storage area. Rotten or damaged fruits were scored weekly, and shelf life was reported as a % of marketable fruit following 5 months of storage.

Fruit quality analyses were performed three times during the shelf life studies: at harvest time, after 55 days, and 170 days from harvest. In each analysis, four basic parameters were measured: sugar content (°Brix), pH, titratable acidity, and firmness. An aliquot of 10 ml of fresh fruit juice puree derived from 2 to 10 random healthy fruits was used for measurements. The sugar content was measured as soluble solids using a portable refractometer (Zuzi Series 300, model 50301130, Auxilab s.l., Beriain, Navarra, Spain), and expressed in °Brix. The pH was measured using a pH meter (Hanna Instruments, S.L, model 221, Eibar, Spain) and titratable acidity was measured by titrating the sample with 0.1 N NaOH to pH 8.1. Results were expressed as g of citric acid per 100 ml of tomato juice (correction factor 0.64 for a 1/10 dilution). Firmness

was measured with a PCE-PTR 200 penetrometer (Albacete, Spain), using a 6 mm probe and measuring at the point of maximum perimeter, after removing about 1 cm^2 of cuticle with a scalpel. Firmness measurements represent the force (in kg cm^{-2}) needed to puncture the flesh following peel removal.

Fruit morphology

Images were obtained for lateral and longitudinal sections by cutting 4–10 fruit per plant so that 8–20 fruit were scanned per accession. One half of the fruit was placed directly on a standard flat-bed scanner (Epson Stylus CX3600, Seiko Epson Corp., Japan). Following scanning, images were saved as JPG files at 300 dpi. Analysis with the Tomato Analyzer Software (TA, version 2.2) was used to generate quantitative values for 32 fruit attributes (Gonzalo and van der Knaap 2008; Brewer et al. 2007). These attributes correspond to specific nodes in the Solanaceae phenotype ontology (http://solgenomics.net/search/phenotypes/traits) specifically, traits nested under ontology terms "fruit" (SP:0000037) and fruit morphology (SP:0000011) and were used to provide an objective description of fruit shape.

To resolve differences between the UPOV and IPGRI classification systems, and to reconcile these descriptors with phenotype ontology terms developed to support Solanaceae genomics efforts, categories were adopted based on the objective measurements obtained with TA (Rodríguez et al. 2011). In this system, the "flattened" and "slightly flattened" classes have been merged into a single "flat" category distinguished by a length/width ratio <0.85. The term "round" is reserved for fruit with a spherical shape in which the length/width ratio approaches 1. The categories "ellipsoid", "obovoid" and "rectangular" describe fruits with oval shape (length/width >1.1), and rectangular fruit are distinguished by a flattened proximal end. The term "obovoid" replaces "pear-shaped" or "pyriform"; with the shape characterized by a length/width ratio >1.2 and <1.7, and a restriction of the proximal end. The "heart-shaped" category in UPOV and IPGRI has been renamed "oxheart", referring to large and tapered fruit with prominent shoulders. The term "heart" is used to describe fruit that are smaller than the "oxheart", wider toward the proximal end than the distal end, with less prominent shoulders than oxheart, and with a distinct tip at the distal end. The category "long" includes varieties that produce very elongated, cylindrical, tapered and often slightly curved fruit. The term "rectangular" remains the same. The eight categories, as proposed by Rodríguez et al. (2011), are "flat", "round", "rectangular", "ellipsoid", "heart", "long", "obovoid" and "oxheart" were used to assign a shape classification to each accession.

Marker-assay for "*alcobaça*"

The "*alcobaça*" (*alc*) allele of *NAC–NOR* was assayed as a Cut Amplified Polymorphism (CAP) using the BsrF1 restriction enzyme to detect the T/A mutation at position 1110 (relative to Genbank accession AY573803), corresponding to the second exon. A 1,300 pb fragment was amplified with primers F: Nor-F: ACTCATCGTCCACTACCTCA, and R: Nor1556R: TGTCGAACCTCTTTCGGCTA, and the amplicon was digested at 37 °C. Digestion produces fragments of 36, 825 and 447 bp corresponding to the "wild-type" allele while digested fragments of 36 and 1,272 correspond to "*alcobaça*". The 36 bp fragment is rarely visible under assay conditions. Ambiguous CAP assays were confirmed through direct sequencing of the Nor-F/Nor1556 amplicon.

Seed storage

Seeds from all accessions were extracted following the standard HCl protocol, described above. Seeds were placed on paper towels and room temperature air-dried, then placed in standard coin envelopes. An aliquot of 2.5–3 ml of seeds were dehydrated to 5 % by placing the open envelops into sealed containers containing silica-gel for 7 days. Following drying, seeds were placed in glass tubes with a silica–gel pill containing a color indicator, and labeled with the accession number. Tubes were fire sealed and stored at −20 °C (for more details see Ellis et al. 1993).

Statistical analysis

Analysis of variance (One-way ANOVA) was carried out using SPSS software (SPSS 16.0 for windows). Mean comparison of measured parameters were done using Tukey HSD test ($\alpha = 0.05$). To estimate the genetic variability for the different characters, we used the coefficient of variation. The correlation test was

performed with the analytical bivariate of a Pearson test. The effect of the *alc* mutation on shelf life was estimated based on a linear regression $y = alc + \varepsilon$ where y was the average shelf life per accession, *alc* was treated as a factor for the allele state of the *NAC–NOR* gene, and ε was the residual error.

Quantitative traits contributing to variation and the relationship between classifications were analysed using principle coordinates analysis (PCA) as implemented in R (R Core Team 2013). Analysis was conducted using the data available in Table S1 (Supplementary data). PCA was conducted for all quantitative data, including production characteristics, vine attributes, quality data at harvest, and the fruit morphology data. The analysis was repeated in the second phase using only fruit morphology data collected with the Tomato Analyser Software, as described above. Missing data were imputed based on the mean and standard deviation for the entire collection. The relationship between accessions was visualized by plotting scores for PCs. Trait contributions to the loadings of each PC were extracted in order to determine which of the phenotypic characteristics contributed most to the observed diversity in the collection. Significant differences between classifications were tested via analysis of variance (ANOVA) for the given values of the first three principal components.

Results and discussion

Collection and classification

Over a two-year period, we assembled a collection of 198 tomato accessions from the Balearic Islands including accessions from *Mallorca*, *Menorca*, *Eivissa* (Ibiza) and *Formentera*. 171 accessions, a representative part of the collection with adequate seed numbers and germination, were evaluated in order to describe the phenotypic variation (Table 1). Describing the phenotypic diversity among accessions required the establishment of appropriate criteria, based on the historical record and objective data, to determine which accessions represented the *Ramellet* tomato. Ludwig Salvator described three distinct shapes for storage tomatoes, *Tomàtiga de Bolla* (round), *de Carabasseta* (flattened and lobed) and *de Pruna* (ellipsoid) (Habsburg-Lorena 1989). We based our criteria for scoring (i.e. *Ramellet* or not) primarily on storage, determined in the common garden experiment, or based on the donation of fruit for seed extraction which were received in good condition at least 5 months post-harvest. In addition, historical background provided by the donors and local landrace names facilitated the classification. The presence of the *alc* (*alc*/*alc*) mutation of *NAC–NOR* was considered an essential character for classification as *Ramellet* (Table 1). Of the 171 accessions evaluated in detail, 142 accessions fit the criteria for *Ramellet* or synonyms (e.g. *Ramellet de Ferro*, *Ramellet de Penjar*). Three additional accessions were identified as *Banyalbufar Antiga* (old). The remaining 26 accessions are representative of other cultivars commonly grown in the Balearic Islands for different culinary uses.

The *Ramellet* collection contained significant diversity in plant and leaf morphology, and in fruit size and shape. Based on the Analysis of Variance, most of this diversity was explained by differences between accessions; whereas within accessions variation was minimal. During replicated evaluation, only 4.08 % of the accessions, UIB0025, UIB0040, UIB0059, UIB0078, UIB0095, UIB0118, UI0137 and UIB0173, appeared to be either segregating or a mixture of two distinct varieties. This observation is consistent with the high degree of autogamy in cultivated tomato leading to inbreeding and therefore homozygosity within accessions.

Plant and leaf morphology

Visual assessment of the plants revealed that most accessions had leaves with five or more leaflets with indented or lobulated margins (wild-type leaf). Eleven, 6.4 % of the accessions, had only 3–5 leaflets per leaf and straight margins, morphology characteristic of the *potato leaf* (*c*) mutation (Kessler et al. 2001). In the UIB collection, most accessions presented wild-type leaf phenotype, only 6.3 % of *Ramellet* and 6.8 % of the non-*Ramellet* varieties displayed this mutation (Fig. 2a).

The diversity with respect to number of principal branches appeared continuous (Fig. 2b), with a range of *Ramellet* accessions having <4 branches (5.55 %) to accessions between 8 and 10 branches (24.7 %), but 48.6 % of the accessions had between 6 and 8 branches. No *Ramellet* plant had more than 10 branches. Similar variability was observed among the other varieties, with 3.6 % of plants with <4 branches or more than 10 and

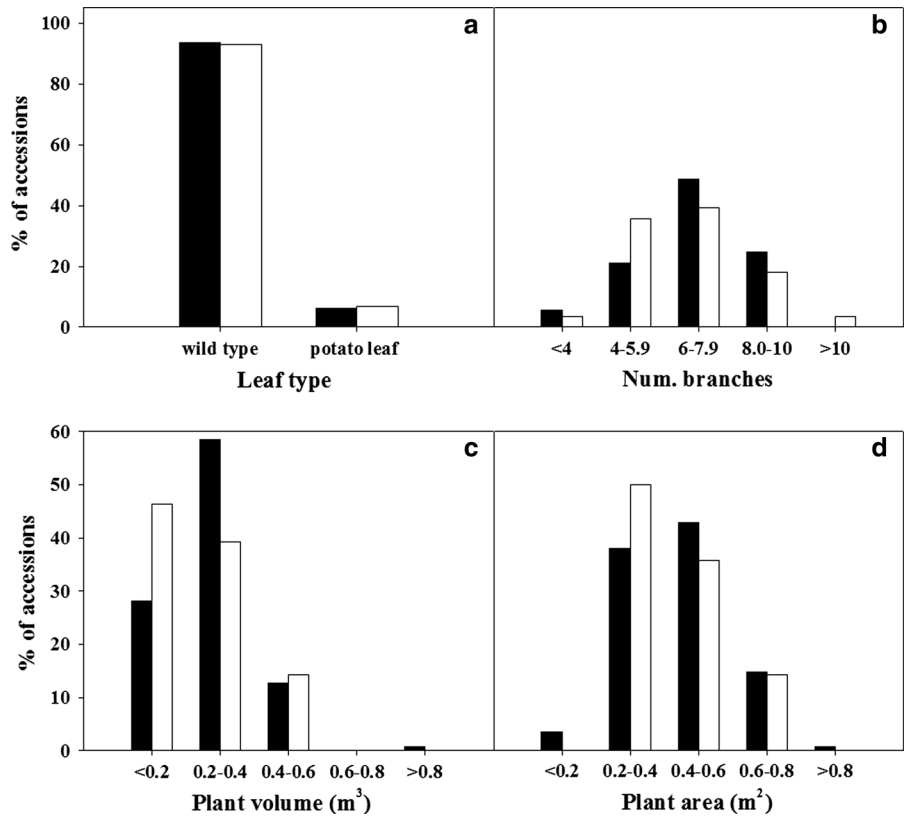


Fig. 2 Morphological traits related to plant and leaf form for 142 *Ramellet* accessions (*black bars*) and 29 non-*Ramellet* varieties (*white bars*)

35.7 and 39.3 % had between 4 and 6 branches or 6–8 branches, respectively (Fig. 2b).

Some diversity was also observed with respect to plant size (m^3) in *Ramellet* accessions (Fig. 2c). Most of the plants were between 0.2 and 0.4 m^3. Just a single *Ramellet* accession had large plants averaging nearly 0.8 m^3. An important trait to optimize for planting lay-out is the plant "foot print" in terms of the occupied area (m^2) (Fig. 2d). Again *Ramellet* accessions displayed more variability for this trait than the rest of the cultivars. The distribution of accessions was 38 % of the *Ramellet* and 50 % of non-*Ramellet* accessions occupied 0.2–0.4 m^2 (small) respectively, another 43 % of *Ramellet* and 35.7 % of non-*Ramellet* occupied 0.4–0.6 m^2 (medium), with the remainder in the large category. Again, just one *Ramellet* accession was considered very large (0.8 m^2).

Fruit morphology

The collection from the Balearic Islands displays continuous variation for attributes related to both shape and size of the fruit. TA attributes were important for classification of variety fruit shapes (Table 2). Occasionally, discrepancies arose between the visual classification and the objective classification based on TA attributes. The fruit shape index did not prove completely discriminatory for flat, round and ellipsoid tomatoes due to within plant variation and a pronounced shoulder and indentation on some otherwise round fruit. The pronounced indentation resulted in a low measurement for height such that some round fruit may have been classified as flat, based on TA. Generally, these discrepancies could be resolved using cross-sectional images; flat fruit tended to be lobed. Overlapping values for attributes in the ellipsoid, obovoid and rectangular shape categories also exist (Rodríguez et al. 2011). Within plant variation further complicated discrete assignment. Despite these issues, in general, the objective assessment using attributes from TA provided a robust classification of fruit shape categories consistent with subjective international descriptors.

The collection of 171 accessions contained eight fruit shape categories (Table 2). The most common shapes were flat and round, accounting for 50 and

Table 2 Morphological traits, production parameters and fruit conservation expressed in % of accessions per interval

Parameter	Interval	Accessions all collection (%)	*Ramellet* accessions[a] (%)	Non-*Ramellet* accessions[b] (%)
Fruit shape	Long	1.8	0.0	10.3
	Heart	4.1	4.9	0.0
	Ellipsoid	7.0	6.3	10.3
	Flat	50.3	56.3	20.7
	Round	31.0	30.3	37.9
	Obovoid	2.9	1.4	10.3
	Oxheart	2.3	0.7	10.3
	Rectangular	0.6	0.7	0.0
Width (mm)	<40	9.9	4.9	34.5
	40–59.9	84.2	93.0	41.4
	60–80	3.5	2.1	13.8
	>80	2.3	0.0	13.8
Height (mm)	<20	0.0	0.0	0.0
	20–39.9	46.8	50.7	27.6
	40–59.9	47.8	48.6	44.8
	60–80	2.9	0.0	17.2
	>80	2.3	0.7	10.3
Fruit weight (g)	<15	2.9	0.0	17.2
	15–49.9	52.0	56.3	31.0
	50–74.9	38.0	41.5	20.7
	75–100	2.3	1.4	6.9
	>100	4.7	0.7	27.6
Yield (kg/plant)	<0.5	5.3	4.9	3.4
	0.5–1	30.4	28.9	37.9
	1–1.5	32.7	33.1	31.0
	1.5–2	20.5	21.1	17.2
	>2	11.1	12.0	6.9
Fruit/plant	<20	30.4	26.8	48.3
	20–40	50.3	54.9	24.1
	40–60	15.2	17.6	3.4
	>60	4.1	0.7	13.8
Fruit at 5th month (%)	100	1.8	2.1	0.0
	99–75	15.2	17.6	3.4
	74–50	31.6	38.0	0.0
	49–25	19.9	24.6	13.0
	24–1	13.5	13.4	13.8
	0	15.2	4.2	69.0

[a] 142 Accessions of *Ramellet*

[b] 29 Local varieties. Supplementary material, Table S1, displays data for each accession separately

31 % of the accessions, respectively (Table 2). Tomatoes classified as *Ramellet* (142 accessions) were flat (56.3 %), round (30.3 %), ellipsoid (6.3 %), heart (4.9 %), obovoid (1.4 %), whereas oxheart and rectangular shapes were found in a single *Ramellet* accession each. No long *Ramellet* tomatoes were observed. The first three categories are consistent with the description of Ludwig Salvator (1871): *Bolla* (round), *Carabasseta* (flattened and lobed) and *Pruna* (ellipsoid), accounting for 93 % of the *Ramellet* accessions. Most of the heart shaped tomatoes derived from Banyalbufar, having a distinct history of cultivation and selection. In this region, tomato production was expanded after *Phyloxera* destroyed the wine-

Table 3 Means for different fruit quality parameters for the accessions according to type

Parameter value	Non-*Ramellet* mean	*Ramellet* mean	CV *Ramellet*	*P*
Width (mm)	50.14 ± 3.97	47.70 ± 0.52	12.97	0.250
Height (mm)	50.41 ± 3.19	40.94 ± 0.52	15.08	<0.001
Fruit weight (g)	66.70 ± 11.07	47.43 ± 1.38	34.04	0.001
Yield (kg/plant)	1.13 ± 0.09	1.30 ± 0.05	41.87	0.120
Number of fruits (fruit/plant)	36.47 ± 7.47	29.02 ± 1.02	42.71	0.067
Fruits at 5th month (%)	9.59 ± 3.47	51.01 ± 2.12	50.96	<0.001

grape industry. Shortly after the arrival of *Phyloxera* in 1891, tomato cultivation was widespread in Banyalbufar. A variety originally cultivated in Sòller locality was introduced to Banyalbufar locality by Francesc Albertí i Coll and Joan Albertí i Albertí (Albertí and Albertí 1998). Three accessions with a Banyalbufar origin were treated as a separate group because their appearance was clearly different from the rest of the accessions classified as *Ramellet*. We hypothesize that these accessions were a varietal type cultivated before the introduction of *Ramellet* to Banyalbufar. We classified these types as *Banyalbufar Antiga* (old). All of the *Banyalbufar Antiga* accessions were in the "round" category. Currently, grower-saved tomato varieties grown in Banyalbufar differ from the *Antiga* type in the shape of the fruit. Among accessions from Banyalbufar not classified as *Antiga*, three of those considered *Ramellet* were heart shaped and a fourth was round. The other local varieties included in the collection (i.e. non-*Ramellet* varieties) were diverse with respect to fruit shape. Among this group, flat and round were again the most common shapes, with 20.7 and 37.9 %, respectively (Table 2).

Phenotypic diversity of the collection was observed for fruit size, with most of the variation due to the non-*Ramellet* accessions. Among the *Ramellet* accessions, coefficients of variation (cv), determined from the analysis of variance (ANOVA), for maximum width and height were 12.97 and 15.08 %, respectively (Table 3). Most accessions displayed medium-sized fruits, also characteristic of the *Ramellet* accessions (Tables 2, 3). Only two *Ramellet* accessions displayed large fruit, one flat (>80 mm width) and one ellipsoid (>80 mm height). Fruits with extreme phenotypes (either large or small) within the collection corresponded to the non-*Ramellet* varieties. The *Banyalbufar Antiga* accessions also displayed medium-sized round fruits, ranging between 30 and 50 mm in width and height. Fruit size was also correlated to fruit weight (g/fruit). A 0.84 correlation ($p < 0.001$) was found between fruit weight and maximum width, lower but still significant correlation was found between fruit weight and maximum height (0.43, $p < 0.001$).

Fruit production and conservation

The traits with highest coefficient of variation within the *Ramellet* accessions were those related to fruit production (Table 3). Significant variation was observed for yield, ranging from very low production, below 0.5 kg/plant, to production over 2 kg/plant (Table 2). The highest yields corresponded to accessions not considered *Ramellet*. This observation is not surprising as many of the non-*Ramellet* accessions reflect the influence of modern breeding efforts for fresh-market varieties. However, 12 % of the *Ramellet* accessions yielded over 2 kg/plant. The number of fruit per plant was also a heterogeneous trait with high variation between accessions (cv 42.71 %).

Tomatoes considered *Ramellet* displayed variation in shelf life characteristics (Table 2), with a CV for this parameter close to 51 %. Although significant differences were observed in shelf life between *Ramellet* accessions and other local varieties (Table 3), some of the non-*Ramellet* accessions displayed moderate storage characteristics (Table 2). *Banyalbufar Antiga* accessions were also reputed to have long shelf life properties. However, our results showed that fruits from two of the three accessions included in the study could not be stored for 5 months. The third *Banyalbufar Antiga* accession had only 20 % marketable fruit after 5 months of storage. The dehydration level in non-*Ramellet* accessions was so high that none could be considered suitable for market following 5 months.

Alc *genotyping*

The presence of the *alc* mutation was investigated to explain differences in shelf life between accessions. The name of the *alcobaça* allele of *NAC–NOR* derives from an accession of tomato from the Portuguese city of the same name, located in the Leiria district (Leal and Tabim 1974). The similarity of fruit shape and storage properties between *Alcobaça* and *Ramellet* tomatoes, and the geographical location relative to the Iberian Peninsula, made the *alc* allele a strong candidate for the long-storage properties of the *Ramellet* tomatoes. Additionally, a recent study of *Penjar* tomato, a varietal type in northeast Spain, revealed strong similarities with *Ramellet* since it presents a wide variability in morphological traits and long shelf life, attributed to *alc* mutation based on sequence data (Casals et al. 2011; Vrebalov et al. 2004, 2009). Analysis of the "cut amplified polymorphism" (CAP) marker for *alc* provided an adequate method to predict storage properties. The analysis of *nor* sequence revealed that all *Ramellet* accessions presented the *alc* mutant of the *NAC–NOR* gene (Table 1).

The percentage of fruit conservation after 5 months was significantly different between accessions with the *alc* mutation *(alc)* and accessions with no mutation (alc^+). When used as a variable to predict storage in the collection, *alc* was significantly associated with fruit storage ($p < 0.001$) and explained 26 % of the phenotypic variance associated with shelf life. The wide variability of this trait has been already reported in other studies on germplasm carrying the *alc* mutation (Casals et al. 2011). There have been suggestions that storage capabilities of *alc* fruit are also affected by size with smaller fruit having longer storage (Leal and Tabim 1974; Mutschler et al. 1992; Casals et al. 2011). To explain differences in shelf life among *Ramellet* accessions, we evaluated the correlation between fruit conservation after 5 months and the fruit-related variables related to fruit size and quality. A weak but significant negative correlation was found between shelf life and fruit size (g) ($r = -0.21$; $p = 0.012$). Similarly, a positive correlation was found between fruit conservation and titratable acidity ($r = 0.23$; $p = 0.006$). These correlations suggest that not only fruit size, but also fruit composition may play a role in fruit conservation.

Fruit quality

Fruit quality parameters were analyzed at harvest time (Fig. 3) and through storage of *Ramellet* accessions (Table 4). Analysis of variance was conducted to determine whether differences existed between the two groups (*Ramellet* accessions and other local, non-*Ramellet,* varieties). No significant differences were found between the *Ramellet* accessions and other local varieties studied, relative to sugar content (°Brix) and pH. *Ramellet* accessions showed higher titratable acidity relative to the other varieties (Fig. 3). The higher acidity did not correlate with a significantly lower content of soluble sugars. Mean values for *Ramellet* varieties were

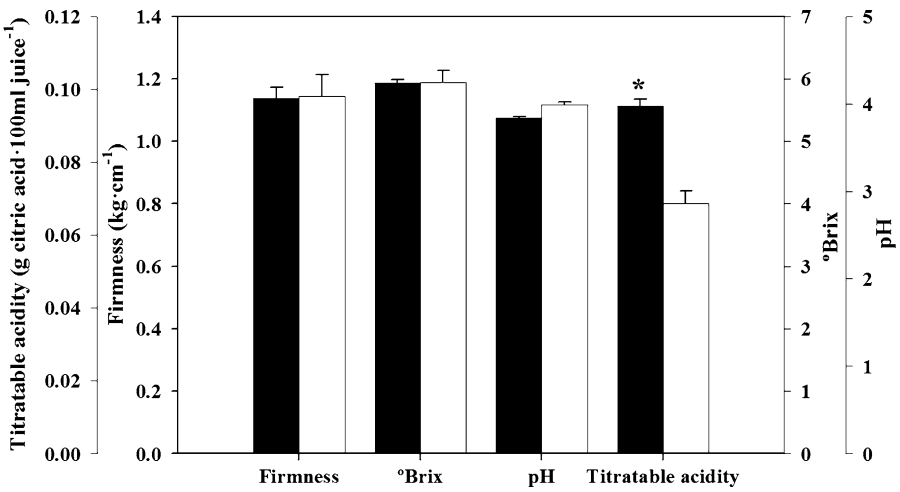


Fig. 3 Fruit quality assessed as firmness, soluble solids (°Brix), pH and titratable acidity for 142 *Ramellet* accessions (*black bars*) and 29 non-*Ramellet* varieties (*white bars*). Data are expressed as mean ± SE. *Statistically significant difference ($p < 0.05$)

Table 4 Means for different fruit quality parameters of *Ramellet* accessions and changes over storage time

	Firmness (kg/cm^2)	Soluble solids (°Brix)	pH	Tritable ac. (g citric ac./100 ml)
Harvest time	1.14 ± 0.04a	5.90 ± 0.06c	3.85 ± 0.01a	0.095 ± 0.001c
55 days storage	1.8 ± 0.03c	5.48 ± 0.07b	4.19 ± 0.01b	0.069 ± 0.001b
170 days storage	1.57 ± 0.04b	5.10 ± 0.08a	4.49 ± 0.02c	0.045 ± 0.001a
p value	<0.001	<0.001	<0.001	<0.001

close to 6 °Brix and the sugar/acid ratio was high, indicating that the fruit quality was within the accepted range for regional varieties (Cebolla-Cornejo et al. 2011). No significant differences were found in firmness between the two groups.

In the *Ramellet* accessions, the fruit quality parameters changed significantly over time (Table 4). An important increase in fruit firmness was observed 55 days after harvest (Table 4). We assume that this trend reflects water loss during storage. The firmness value decreased after 55 days and at 170 days after harvest was lower, but still significantly higher than values measured at harvest (Table 4). The °Brix values of *Ramellet* fruits showed significant reductions after 55 days of harvest. The results showed that the reduction during the last period of the experiment (55–170 days after harvest) was milder (Table 4) indicating maintenance of fruit quality. Reductions were also observed in titratable acidity during the storage period (Table 4), with the trend towards increased fruit sugar/acid ratio. These results indicate that fruit maintain their quality during the storage period, as the ratio between soluble solids and titratable acidity is considered an indicator of overall taste (Baldwin et al. 1998).

Morphologic and agronomic determinants of variation in the Ramellet collection

In order to examine the main sources of variation observed across all traits measured, principal components analysis (PCA) was conducted. PCA was used to analyse all quantitative data collected for plant habit, yield characteristics, quality and fruit attributes (Fig. 4a). When the entire data set was used, the first three principal components (PC) explained 58 % of the variation in the collection with PC1 accounting for 27.0 %; PC2, 20.5 %; and PC3, 10.4 %. Loadings for each PC were used to determine the most variable traits. For PC1, shape indices which distinguish flattened from elongated fruit were the most important features. PC2 was influenced by fruit size (area, perimeter, maximum width), symmetrical shape ("Circular" and "Ellipsoid"), fruit number, fruit conservation and titratable acidity. For PC3, asymmetrical fruit attributes ("Heart" and "Triangle"), and vine characteristics (e.g. branch number). The three general classifications of tomatoes in the collection (*Ramellet*, *Banyalbufar Antiga*, and other) were used as a fixed factor to assess whether these groups were different with respect to the first three PC. Although the classifications overlap, mean values for the three groups differ significantly for PC1 ($p < 0.0001$) and for PC2 ($p < 0.0001$), thought not for PC3 ($p = 0.373$). A similar approach was used with classification based on the allele of *NAC–NOR*. The two categories differed for PC1 ($p < 0.0001$), PC2 ($p < 0.0001$) and PC3 ($p = 0.008841$).

We repeated PCA using only fruit-shape data to determine which attributes collected with the tomato analyzer (TA) software were most variable in the collection (Fig. 4b). All fruit shape attributes measured using the TA software (Brewer et al. 2006) are summarized in Table S1 (supplementary data). The first three principle components explained 35.3, 24.9 and 12.0 % of the total variance respectively. Examination of the traits contributing high positive and high negative loadings suggest that PC1 was highly influenced by shape indices such as the external and internal length to width ratios. PC2 was influenced by size components (area and perimeter) as well as symmetrical shape attributes captured by the TA components "Ellipsoid" and "Circular". PC3 was influenced most by asymmetrical shape features such as how well the fruit fit a "Heart" or "Triangle" as well as distal features. The three general classifications of tomatoes in the collection (*Ramellet*, *Banyalbufar Antiga*, and other) also appear to demarcate overlapping but different groups based on shape (Fig. 4b). Despite the overlap, mean values differ significantly

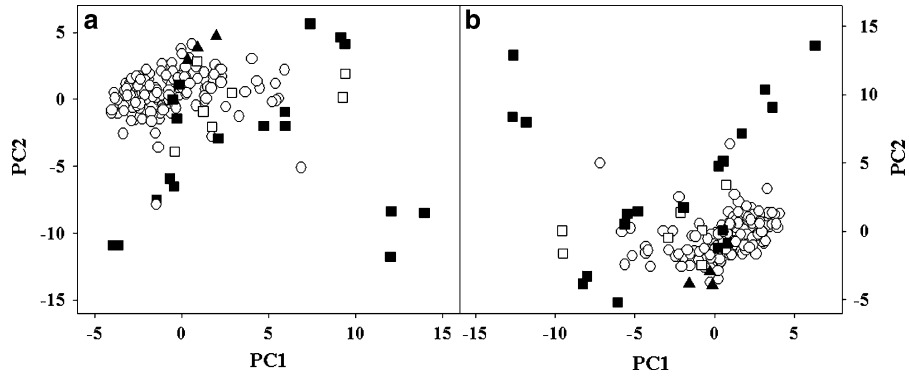


Fig. 4 Principal component analysis (PCA) based on agro-morphological traits. **a** PC1 (*X* axis) and PC2 (*Y* axis) based on data for all quantitative characters. **b** PC1 (*X* axis) and PC2 (*Y* axis) based on only fruit morphology data as collected from the Tomato Analyzer Software. Accessions are coded based on the allele of the *NAC–NOR* gene (*open* = *alc*; *filled* = alc^{+}) or the accession classification (*circle* = *Ramellet*; *triangle* = *Banyalbufar antiga*; *square* = all other)

for PC1 ($p < 0.0001$) and for PC2 ($p < 0.0001$), thought not for PC3 ($p = 0.603$). There was similar overlap in shape features based on classification using the allele of *NAC–NOR*, but as a group the *alc* and alc^{+} categories differed for PC1 ($p < 0.0001$), PC2 ($p < 0.0001$) and PC3 ($p = 0.0286$).

Collection and genetic resources conservation for the future

The culinary and cultural relevance of the *Ramellet* in the Balearic Islands, the importance of this tomato as a protected designation of origin type, and the history of selection makes it a primary target in local landrace preservation programs. The seed collection described here is, to our knowledge, the most complete collection of *Ramellet* and the first maintained by a public institution for investigation and preservation purposes. The term "landrace" generally refers to a local variety or breed that has been improved by traditional methods, and has been adapted to the natural and cultural environment in which it is cultivated. Landraces are generally understood to be more genetically and physically diverse than formal cultivars (Hawtin et al. 1997). The amount of phenotypic diversity associated with the cultivars characterized as *Ramellet* suggests that "landrace group" (Zeven 1998) may be a more appropriate term as the collection clearly represents a population with diversity for fruit and vine characteristics, yet distinct from other local varieties. The observed variation within *Ramellet* accessions for fruit morphology, fruit quality, shelf life, plant habit, and agronomic traits likely traces to the tradition of grower-saved seed with selection based on grower preferences. We hypothesize that this tradition may have led to particular adaptations to the variable soil, climate and local food preferences within the Balearic archipelago (Galmés et al. 2011, 2013). These traditional varieties are currently being replaced with commercial long shelf life hybrids, and therefore, this collection may be a first step in preventing genetic erosion of the *Ramellet*. This collection may help in future investigations, protected designation registration, and breeding programs in order to select the most suitable accessions. On a global scale, this collection represents a valuable source of alleles in a cultivated background related to adaptation to drought stress (Galmés et al. 2011, 2013) fruit extended shelf life, and fruit quality which are the principal traits defining the *Ramellet*.

Concluding remarks

A collection of tomato varieties was established and lead to a new germplasm collection at the University of Balearic Islands (UIB). The collection emphasized *Ramellet* varieties adapted to the region. The geographical boundaries of the Balearic archipelago and the diagnostic presence of the *alc* mutant of the *NAC–NOR* gene provide firm guidelines for establishing a collection of *Ramellet*. *Ramellet* tomato landraces showed phenotypic variation for all traits measured; this variation is consistent with historical records and

suggests that local selection may play a role in the maintenance of phenotypic diversity. Our results suggest that this varietal type is a landrace group with variability in most agronomic, morphological and quality traits. Despite the wide trait variability, the results show that the accessions identified as *Ramellet* are clearly different from other local varieties with respect to plant morphology, fruit size, fruit composition (titratable acidity), and especially in fruit storage. All accessions of *Ramellet* evaluated have the ripening mutation *alc*. Our results show that the shelf life is largely controlled by the *alc* mutation, but also may depend on other characteristics including fruit size and fruit titratable acidity. This collection represents a resource for the preservation of locally adapted germplasm, with cultural interest, and a source of alleles related to adaption to Mediterranean climatic and agronomic conditions, especially drought.

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