Application of high-throughput phenotyping tool Tomato Analyzer to characterize Balkan *Capsicum* fruit diversity

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**ABSTRACT**

For years, the main emphasis of industrial pepper production has been the creation of large-fruited high-yielding varieties. This has led to the exploitation of pepper (*Capsicum annuum* L.) varieties with a significantly narrow genetic background. The Balkan region has a range of valuable local landraces that are noticeably decreasing in prevalence and may be lost. In this study, we utilized Tomato Analyzer (TA) to characterize the intra- and inter-varietal type fruit diversity using 50 different fruit shape, size, and color descriptors of a Balkan pepper collection. The collection comprised of 168 diverse accessions and collected from 62 locations and 31 districts in six different Balkan countries. Fruit shape was the main trait used for visual accession grouping into 5 different varietal groupings of elongated, round, conical, bell, and pumpkin shape, respectively. Beyond visual grouping, hierarchical cluster analysis grouped the evaluated accessions into eight distinct clusters based on fruit shape, size, surface, and color. In total, 47 TA descriptors were identified to contribute to the total variation, with first two components explaining 54% of variation, and 90% of variation contained in 12 components. Among TA descriptors, fruit size and proximal/distal fruit end shape contributed to component 1 variation whereas fruit perimeter, area and color contributed to component 2. We hope that this research will assist pepper breeding and genetic resources communities to better understand Balkan pepper fruit diversity and develop pepper varieties with desirable fruit traits. Quantification of fruit diversity could be crucial for further investigation into the genetic determinants of fruit shape and size by a genome-wide association study.

1. Introduction

Pepper (*Capsicums* pp.) is an important vegetable crop due to its enhanced nutritional composition and “nutraceutical bioactive” substances (Reifschneider and Ribeiro, 2008; Nadeem et al., 2011; Kim et al., 2014). The *Capsicum* genus includes more than 36 species (Moscone et al., 2007; Eshbaugh, 2012), but *C. annuum, chinense, pubescens, frutescens,* and *baccatum* are domesticated (IBPGR, 1983) and predominantly cultivated (Pickersgill et al., 1979; Pickersgill, 1997). Peppers are known for their varied uses in food, spice, medicine and even as an ornamental plant (Hayman and Kam, 2008; Bosland and Votava, 2012). They also possess a wide diversity in terms of growth habit, maturity, and fruit traits (shape, size, color, and pungency). Cultivated *Capsicums* species are distributed all over the world due to their resilient adaptability across different environmental conditions and their socio-cultural significance in cross-cultural cuisines (Hayman and Kam, 2008; Bosland and Votava, 2012).

In the Balkan Peninsula, vegetable production has been a major source of subsistence and income for smallholder farming communities (Lampietti et al., 2009) and pepper is an important vegetable across Balkans (Andrews, 1993; Nasto et al., 2009). Bulgaria, due to its unique climate conditions and breeding traditions, appears to be a secondary region of pepper diversity (Masheva et al., 2005) and significant acreage is utilized for pepper production. Well-adapted local forms can be found mainly in small farms with specific characteristics for taste, nutritional value, shape, and color-variable nuances before and at botanical maturity (Masheva, 2014). These unique local forms have been utilized in different breeding programs to develop nutritionally

**Abbreviations:** TA, tomato analyzer; HCA, hierarchical cluster analysis; CD, conventional descriptor; FL, fruit length; FW, fruit width; FWT, fruit wall thickness; MH, maximum height; MW, maximum width; PT, pericarp thickness; ANOVA, analysis of variance; PCA, principal component analysis; OB, obovoid; DEC, distal eccentricity; OV, ovoid

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enhanced and high-yield pepper varieties (Todorov and Todorova, 2002; Tsonev et al., 2017).

Pepper diversity depends on morphometric and colorimetric trait variation, which is critical for varietal identification and selection (Naegele et al., 2016). Traditionally morphological, agronomic, biochemical, and molecular pepper descriptors (Dias et al., 2013; Tripodi and Greco, 2018) have been used to characterize genetic diversity. Growth-stage-specific agronomic and horticultural evaluation of morphological diversity is being utilized for varietal characterization across different pepper producing regions (Pickersgill, 1988; Yayeh and Zeven, 1997; Geleta et al., 2005; Sharma et al., 2010). Genetic resources and plant breeding communities still rely on conventional descriptors to characterize agronomic performance of peppers (Gepts, 2006; Upadhyaya et al., 2008). However, conventional phenotyping limits the investigations on detailed internal and external fruit features. Hence application of a high-throughput phenotyping tool is important to fill this gap, although its use is still limited to just a few crops and traits (Cabrera-Bosquet et al., 2012).

New high-throughput phenotyping tools are being continuously developed and adapted to characterize seed quality (Gagliardi and Marcos-Filho, 2011), whole plant phenotyping (Song et al., 2011; Dieleman et al., 2012; Van der Heijden et al., 2012), and fruit diversity (Tripodi and Greco, 2018) of Capsicum. Phenotypic diversity assessment and trait characterization using high-throughput phenotyping has been seen to be more sensitive and cost-effective than conventional phenotyping (Furbank and Tester, 2011; Cobb et al., 2013). In Solanaceous vegetables, phenomic tools such as Tomato Analyzer (TA) have proven to be very useful to characterize the morphometric and colorimetric traits of fruits and have been used in tomato (Gonzalo and van der Knaap, 2008; Gonzalo et al., 2009; Mazzucato et al., 2010; Rodríguez et al., 2011), eggplant (Hurtado et al., 2013), and pepper (Tripodi and Greco, 2018). In this study we characterized the variability of fruit morphometric and colorimetric traits of Balkan pepper accessions using TA and a conventional descriptor (CD). The starting hypothesis was that the accessions would be similar within and between varietal types. It was tested by identifying intra- and inter-varietal accession differences.

2. Materials and methods

2.1. Experimental material

A total of 168 diverse pepper accessions included in this study were collected from Bulgaria (110), Serbia (24), Macedonia (14), Romania (9), Albania (7), Greece (3), and one accession of unknown origin (Fig. 1). The majority of accessions were landraces, though some local varieties and breeding lines were also included. Each accession was represented with 30 plants in three replications (10 plants per replication) in field trials at MVCRI in Plovdiv, which is located in Southcentral Bulgaria (42°10'35.3″N 24°45'50.5″E). Some accessions collected from different regions of Bulgaria, Serbia, and Macedonia are known locally by the same name but appear to differ in their morphological characteristics and have been labeled differently (Table S1). Based on fruit shape, the tested pepper accessions were divided into five varietal groups with conical, elongate, pumpkin shape, bell or blocky, and round fruit types.

2.2. Conventional descriptor measurement, sample collection, and data processing

After second harvest, fruit length (FL), fruit width (FW), and fruit wall thickness (FWT) was measured according to IPGRI Capsicum descriptors (IPGRI et al., 1995). Conventional descriptors (CD) data was collected from randomly selected eight fruits in each of the three biological replicates grown in field. Similarly, eight fruit samples for each replication were prepared for TA descriptor characterization. Collected fruit samples were further cleaned and cut into longitudinal and latitudinal sections and both fruit sections were analyzed for fruit shape, size, and color diversity using TA in Fruit Quality Laboratory.

2.3. Tomato Analyzer descriptor characterisation

Eight fruits per replication were sampled and four were cut longitudinally and four transversally and scanned with an Epson Perfection V19 J371A photo scanner (Epson, Amsterdam, The Netherlands) at a resolution of 300 dpi. Image data was subjected to morphometric and colorimetric analysis using TA version 3 software (Rodríguez et al., 2010a; Strecker et al., 2010). A total of 47 different fruit morphometric and colorimetric descriptors were studied in longitudinal and latitudinal fruit sections analyzed by TA. In the longitudinal section, the traits were measured across nine different categories and were related to: fruit size basic measurements (7), fruit shape index (3), blockiness (3), homogeneity (3), proximal/distal fruit end (4/4), asymmetry (6), internal eccentricity (5), and fruit color traits (9). Latitudinal section traits (3) were limited to pericarp, placenta, and septum. Fruit morphometric descriptors were assessed based on parameters explained by Brewer et al. (2006) and colorimetric descriptors were assessed based on parameters explained by Darrigues et al. (2008). Specific characterization and more a detailed explanation about each descriptor is provided by (Gonzalo and van der Knaap, 2008; Rodríguez et al., 2010a; Hurtado et al., 2013; Colonna et al., 2019).

2.4. Correlation between conventional and tomato analyzer fruit descriptors

Manually measured fruit length (FL), width (FW), and fruit wall thickness (FWT) were compared with TA descriptors of maximum height (MH), maximum width (MW), and pericarp thickness (PT), and respective correlations were estimated.

2.5. Statistical analysis

Data derived from processed images were statistically analyzed using SAS, XLSTAT, and R programs. A cluster analysis based on 47 fruit TA descriptors was estimated by agglomerative hierarchical cluster analysis using Ward’s coefficient. Analysis of variance (ANOVA) was estimated for each accession to detect differences among accessions, between varietal types, and within each varietal type. Significant differences between accessions were detected using Student’s t test. Descriptive Statistics and ANOVA was calculated using SAS v.9.3 (SAS Institute, 2002). Scattergrams for different TA descriptor groups were built using XLSTAT software version 15. The correlations between TA and CD descriptors were estimated using R functions cor and rcorr for correlation. To understand pepper germplasm diversity and to identify potential within and between varietal types, TA fruit descriptors were comprehensively examined through a multivariate analysis using principal component analysis (PCA). The data analyzed through PCA was projected into first and second principal components (PC1 and PC2) to illustrate intra and inter-accession variation. Diverse PCA parameters were estimated using different R packages including ggplot2, FactoMineR, Factoextra, and missMDA. Data visualization was displayed by ggplot2 and missing data was imputed by missMDA. A correlation matrix between different TA descriptors was estimated using corrcor function, and a correlation coefficient heatmap was produced using ggecorrplot. Correlation between different TA descriptors was estimated using the correlation coefficient heatmap and a correlation network was built using qgraph. The correlation coefficient heatmap and network was estimated to understand which TA descriptor features are correlated with each other and how they contribute to fruit morphometric and colorimetric diversity.
3. Results

3.1. Visual assessment

Considerable diversity was observed among accessions concerning fruit shape, size, and color. Representative fruit shape diversity of these accessions is shown in Fig. 2. The fruit shape was the main trait used for visual grouping and based on this trait the whole tested plant material was divided in five varietal groups: conical (54.2%), elongated (18.4%), pumpkin (12.5%), bell or blocky (10.7%), and round (4.2%). The fruit color varied before maturity from pale yellow to green (light to dark), whereas at the botanical maturity stage color ranged from yellow, orange, brown, and light to dark red.

3.2. Fruit trait variation between accessions and within varietal groups

Image analysis of pepper accessions brought forth detailed information about the external features including fruit size, shape, and color from the longitudinal section (Fig. 3A-I) and internal fruit features including pericarp, septum, and placenta from the latitudinal fruit section (Fig. 3J). External features including fruit size (Fig. 3A), shape (Fig. 3B-H) and color (Fig. 3I) showed distinctive variations among accessions, except distal eccentricity-DEC (Table 1). Among all descriptors, the coefficient of variation was less than 25% for 35 descriptors. Traits related to fruit size basic measurements, homogeneity, fruit shape index, blockiness, and color were the least variable as compared to the fruit asymmetry and proximal/distal fruit end (Table 1). The sum of squares for accessions was higher or equal than the residual sum of squares for all conventional and TA descriptors, except for the circular trait, which was related to the homogeneity. Within each varietal group, variation for more than 42 TA descriptors was observed highly significant for elongated, conical, bell or blocky, and pumpkin varietal groups, except for round shape which displayed differences for 18 fruit descriptors only. Among all TA descriptors, only Obovoid or OB, except for significance in pumpkin shape, horizontal asymmetry or Asob, except for bell and pumpkin shape, and DEC, except in conical type were consistently non-significant among all fruit types.

Distribution-wise, most of the fruit size, blockiness, homogeneity, and color related TA descriptors displayed unimodal symmetrical distribution (Fig. S1). Fruit shape descriptors of fruit shape index, asymmetry, distal fruit end shape and internal eccentricity features were left skewed except symmetrically distributed proximal eccentricity (Fig. S1). However, proximal fruit end features were skewed to the right except proximal indentation area and shoulder height seen to distribute left skewed. In latitudinal fruit section lobedness degree, pericarp area, and pericarp thickness were respectively distributed symmetrically, right and left skewed (Fig. S1). The CIELAB color analysis demonstrated that all color parameters displayed distinctive variations among accessions (Fig. 3I). Highly significant ranges of ‘a’ and ‘b’ indicate larger
greenness and yellowness components. Additionally, 96% luminosity and 35% chroma were suggestive of a significant amount of lightness and moderate saturation of colors. The wide range of hue angle suggests that Balkan pepper collections range from red to green. The obtained results clearly demonstrate the existence of significant differences between Balkan pepper accessions concerning their fruit shape, size, and color.

3.3. Correlation between conventional and TA fruit descriptors

Fruit size correlation between conventional and TA descriptors were used to detect differences between respective measurement techniques. Manually measured FL (9.80 cm) and FW (4.13 cm) were different than TA measured MH (9.54 cm) and MW (5.63 cm). Both techniques appear to measure respective traits differently; therefore, correlation coefficients of these traits were estimated for each varietal type (Table 2). A significantly strong positive correlation (> 0.85) between FL and MH was seen across all varietal types, except bell and pumpkin shape in which it was < 0.75. The FW and MW were strongly correlated with conical (0.84), pumpkin (0.83), and round (0.75) shapes but moderate in elongated (0.58) and bell (0.63) types and none of the fruit types displayed negative correlation. The elongated (-0.02) and bell or blocky (-0.07) varietal types showed a lack of correlation for fruit wall thickness (FWT) and pericarp thickness (PT). When there was a strong to moderate correlation between FW and MW, it was reflected as weak to negative between FWT and PT. Though the traits of fruit length and width measured by manual measurement and TA are highly correlated, differences observed in the range of these traits suggest that both phenotyping methods measure respective fruit traits differently and are not repetitive or redundant.

3.4. Hierarchical cluster analysis

Hierarchical cluster analysis (HCA) based on 47 TA descriptors was performed. The HCA clearly divided studied accessions into eight distinct clusters based on their fruit shape, size, color, and surface (Fig. 4). A moderate eophenic correlation coefficient of 0.573 was reported for compared TA descriptors. In each cluster, the majority of accessions were mainly represented from Bulgaria followed by Serbia, Macedonia, Romania and Albania. The variance between and within the cluster was reported as 65.5% and 34.5%, respectively.

Among all 8 distinct clusters, cluster 7 was populated with the maximum (i.e., 57) accessions representing kapia (89.47%) and paprika or ground pepper (10.53%) types (Table S3) and around 94.7% of accessions possessed conical fruit shape also known as kapia type. Cluster 1 exhibited the largest diversity among accessions by fruit shape (i.e., round, conical, and pumpkin), whereas lowest diversity was reported for fruit size (i.e., long, large, and small). Around 88.9 % of accessions in this cluster were observed to be small-fruited with smooth or slightly corrugated surfaces. In cluster 2, accessions were predominantly from the conical varietal group (66.7%) followed by elongated varietal group (33.3%) and 83.3% of accessions were long fruited with a smooth or slightly corrugated surface. In cluster 3, elongated (53.9%) and conical (46.1%) fruits were mostly populated in this group and 92.3% of accessions exhibited corrugated fruit surfaces. Cluster 4 (Chorbadzhiyski_K422, Dracula, and Chorbadzhiyski_B1E0301 accessions) and cluster 8 (Kapia_B1E0061) were seen to be the least numerous and exhibited elongated fruit shape (Table S2). Generally, accessions belonging to these clusters were longest among all tested pepper accessions. In cluster 5, all accessions were large-fruited for green pepper production, and 87.5% of accessions were bell-shaped except Kaloyan and Artim which were conical with wider fruits and a small fruit shape index (Table S2). Cluster 6 included accessions with a
Fig. 3. Scattergrams displaying distribution of different fruit morphometric and colorimetric parameters measured using TA. The external fruit features from the longitudinal section were measured by basic measurements of fruit size (A), shape (B–H), and color (I) descriptors. Internal fruit features were measured using latitudinal sections (J). The variables with the superscripts P and S are plotted with reference to the primary axis (y− axis left side) and secondary axis (y− axis right side), respectively.
pumpkin shape, regardless of their length, width, size, and surface. Based on HCA grouping, closely related accessions appear to be populated within most of the clusters, except cluster 3. Predominantly, this similarity was populated by accessions belonging to similar shape and size. However, there were accessions in each cluster displaying dissimilarity, and it appears to be due to variability contributed by fruit color and surface.

Table 1
Descriptive statistics and analysis of variance (ANOVA) for all pepper accessions evaluated by conventional and TA descriptors. The level of significance expressed is NSP > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations or code used for TA descriptors are adapted from Tripodi and Greco (2018).

| Descriptor (Unit) | °Code | Across Varietal Types | ANOVA |
|------------------|-------|-----------------------|-------|
|                  |       | Mean | Range | LSD_{0.05} | CV   | R     | Accession | Residual |
| Mean of squares (%) |       |      |       |            |      |       |           |          |
| Conventional Descriptors: |
| Fruit Length (cm) FL | 9.80 | 0.85-21.3 | 1.86 | 11.79 | 0.96 | 122.2*** | 4.76 |
| Fruit Width (cm) FW | 4.13 | 1.02-8.07 | 0.77 | 11.61 | 0.96 | 19.95*** | 0.008 |
| Fruit Wall Thickness (mm) FWT | 3.42 | 0.52-7.44 | 0.84 | 15.29 | 0.91 | 10.17*** | 0.004 |
| Tomato Analyzer Descriptors: |
| Basic Measurements: |
| Perimeter (mm) P | 271.4 | 39.6-533.2 | 49.4 | 9.98 | 0.94 | 37460.2*** |
| Area (mm²) A | 3336.9 | 142.7-8026.8 | 908.8 | 14.92 | 0.95 | 14117619.3*** |
| Width mid-height (mm) WMH | 44.6 | 11.6-93.4 | 9.12 | 11.22 | 0.96 | 1669.9*** |
| Maximum width (mm) MW | 50.63 | 13.0-94.5 | 9.09 | 8.83 | 0.96 | 1699.9*** |
| Height mid-width (mm) HMW | 83.06 | 12.8-192.7 | 22.8 | 15.02 | 0.94 | 7524.9*** |
| Maximum height (mm) MH | 95.4 | 13.7-220.6 | 23.6 | 13.56 | 0.94 | 8389.9*** |
| Curved height (mm) CH | 98.4 | 15.0-234.6 | 23.7 | 13.21 | 0.94 | 8638.5*** |
| Fruit Shape Index: |
| Fruit shape index external I FSEI | 2.15 | 0.50-5.50 | 0.74 | 18.89 | 0.92 | 6.1*** |
| Fruit shape index external II FSEII | 20.7 | 0.30-12.4 | 0.93 | 2.47 | 0.96 | 17.4*** |
| Curved fruit shape index FSC | 2.93 | 0.53-15.5 | 1.11 | 20.83 | 0.96 |
| Blockiness: |
| Proximal fruit blockiness PFB | 0.95 | 0.43-1.60 | 0.19 | 10.75 | 0.82 | 0.15*** |
| Distal fruit blockiness DFB | 0.52 | 0.30-1.0 | 0.16 | 16.34 | 0.73 | 0.06*** |
| Fruit shape triangle FST | 1.99 | 0.77-3.80 | 0.70 | 19.31 | 0.78 | 1.6*** |
| Homogeneity: |
| Ellipsoid E | 0.10 | 0.05-0.20 | 0.03 | 14.95 | 0.73 | 0.002*** |
| Circular C | 0.26 | 0.10-0.47 | 0.07 | 14.67 | 0.91 | 0.042*** |
| Rectangular R | 0.47 | 0.20-0.63 | 0.09 | 10.25 | 0.81 | 0.029*** |
| Proximal Fruit End Shape: |
| Shoulder height SH | 0.05 | 0.0-20.0 | 0.06 | 76.36 | 0.75 | 0.011*** |
| Proximal angle micro (Degrees) PMI | 199.9 | 43.1-287.8 | 64.8 | 17.88 | 0.80 | 15378*** |
| Proximal angle macro (Degrees) PMA | 144.1 | 5.4-287 | 71.30 | 27.10 | 0.84 | 24596*** |
| Proximal indentation area PIA | 0.18 | 0.0-0.63 | 0.13 | 39.07 | 0.83 | 0.073*** |
| Distal Fruit End Shape: |
| Distal angle micro (Degrees) DMI | 100.3 | 4.0-294.3 | 61.6 | 33.61 | 0.82 | 15033*** |
| Distal angle macro (Degrees) DMA | 78.87 | 9.28-218.1 | 41.8 | 29.03 | 0.88 | 11845*** |
| Distal indentation area DIA | 0.02 | 0.0-0.23 | 0.06 | 165 | 0.74 | 0.006*** |
| Distal end protrusion DEP | 0.06 | 0.0-0.40 | 0.15 | 132.8 | 0.52 | 0.021*** |
| Asymmetry: |
| Obovoid OB | 0.01 | 0.0-0.17 | 0.05 | 287.1 | 0.62 | 0.004*** |
| Ovoid OV | 0.35 | 0.0-0.70 | 0.14 | 22.92 | 0.82 | 0.086*** |
| V. Asymmetry Asv | 0.31 | 0.01-1.70 | 0.12 | 38.20 | 0.82 | 0.20*** |
| H. Asymmetry Ob Asov | 1.02 | 0.0-0.44 | 0.55 | 29.67 | 0.83 | 0.018*** |
| H. Asymmetry Ov Asov | 1.02 | 0.0-4.4 | 0.55 | 29.67 | 0.83 | 0.018*** |
| Width widest pos WWP | 0.06 | 0.0-0.40 | 0.15 | 132.8 | 0.52 | 0.021*** |
| Internal Eccentricity: |
| Eccentricity EC | 0.68 | 0.40-0.80 | 0.08 | 6.07 | 0.84 | 0.028*** |
| Proximal eccentricity PEC | 0.90 | 0.83-1.07 | 0.04 | 2.47 | 0.56 | 0.002*** |
| Distal eccentricity DEC | 0.95 | 0.63-7.53 | 2.33 | 134.4 | 0.21 | 1.32*** |
| Fruit shape index internal FSI | 2.48 | 0.30-12.47 | 0.95 | 20.86 | 0.96 | 17.69*** |
| Average Color Values: |
| Red Red | 131.9 | 89.3-183.2 | 15.4 | 6.37 | 0.87 | 1412.3*** |
| Green Green | 99.4 | 44.4-171.8 | 20.1 | 11.01 | 0.95 | 6587.5*** |
| Blue Blue | 70.7 | 36.8-122.3 | 12.8 | 9.83 | 0.94 | 2416.5*** |
| Luminosity AL | 96.3 | 59.9-142 | 7.40 | 8.39 | 0.93 | 667.2*** |
| a a | 9.90 | -15.5-38.3 | 7.80 | 42.84 | 0.97 | 1852.9*** |
| b b | 26.6 | 15.0-41.4 | 4.52 | 9.27 | 0.83 | 91.8*** |
| Hue AH | 74.7 | 33.6-115.9 | 15.1 | 11.0 | 0.97 | 5598.9*** |
| Chroma AC | 34.5 | 18.6-51.3 | 4.38 | 6.93 | 0.92 | 188.9*** |
| Lattitudinal Section: |
| Lobedness Degree LD | 3.25 | 0.89-7.56 | 1.44 | 24.13 | 0.81 | 8.20*** |
| Pericarp Area PA | 0.68 | 0.50-1.20 | 0.14 | 11.21 | 0.67 | 0.04*** |
| Pericarp Thickness PF | 0.19 | 0.10-0.30 | 0.05 | 15.35 | 0.79 | 0.01*** |
Gene diversity, proximal/distal fruit end, internal eccentricity, color, and lateral were identified accordingly (Table S4). Fruit size, shape, homogeneity, proximal/distal end shapes were positively correlated with traits contributed approximately 90% of the total variation. First component (PC1) determined 33.3% of the total variation, while the first two components explained 54% of the total variation, and the first 12 components contributed approximately 90% of the total variation. 

Table 2

Fruit-shape-based correlation between conventional descriptors (CD) and tomato analyzer (TA) descriptors for different fruit shape types. Manually measured Fruit Length (FL), Fruit Width (FW), and Fruit Wall Thickness (FWT) was equivalent to Tomato Analyzer measured Maximum Height (MH), Maximum Widths, and Pericarp Thickness (PT). The level of significance expressed is NSP > 0.05, P < 0.05, **P < 0.01; ***P < 0.001.

| Varietal Type | FL/MH     | FW/MW     | FWT/PT     |
|---------------|-----------|-----------|------------|
|               | CD        | TA        | CD         | TA        | CD         | TA         |
| Across Varietal Types: |           |           |            |           |            |            |
| CD            | 1         | 0.96***   | 1          | 0.88***   | 1          | 0.29***    |
| TA            | 0.96***   | 1         | 0.88***    | 1         | 0.29***    | 1          |
| Elongated:    |           |           |            |           |            |            |
| CD            | 1         | 0.90***   | 1          | 0.58***   | 1          | -0.02 NS   |
| TA            | 0.90***   | 1         | 0.58***    | 1         | -0.02 NS   | 1          |
| Round:        |           |           |            |           |            |            |
| CD            | 1         | 0.89**    | 1          | 0.75*     | 1          | 0.59 NS    |
| TA            | 0.89**    | 1         | 0.75*      | 1         | 0.59 NS    | 1          |
| Conical:      |           |           |            |           |            |            |
| CD            | 1         | 0.93***   | 1          | 0.84***   | 1          | 0.18 NS    |
| TA            | 0.93***   | 1         | 0.84***    | 1         | 0.18 NS    | 1          |
| Bell or Blocky: |         |           |            |           |            |            |
| CD            | 1         | 0.73***   | 1          | 0.63**    | 1          | -0.07 NS   |
| TA            | 0.73***   | 1         | 0.63**     | 1         | -0.07 NS   | 1          |
| Pumpkin Shape: |          |           |            |           |            |            |
| CD            | 1         | 0.72***   | 1          | 0.83***   | 1          | 0.11 NS    |
| TA            | 0.72***   | 1         | 0.83***    | 1         | 0.11 NS    | 1          |

3.5. Multivariate Analysis

Multivariate PCA was used to identify potential trait combinations that contributed to variation in fruit shape, size, and color traits. In PCA, a total of 47 principal components were identified that contributed to the total variation (Fig. 5). The PCA biplot results showed that all pepper accessions were spread in all four quadrants (Fig. 6A). The first two components explained 54% of the total variation, and the first 12 components contributed approximately 90% of the total variation. The PCA biplot results showed that all pepper accessions were spread in all four quadrants (Fig. 6A). The first two components explained 54% of the total variation, and the first 12 components contributed approximately 90% of the total variation. The PCA biplot results showed that all pepper accessions were spread in all four quadrants (Fig. 6A). The first two components explained 54% of the total variation, and the first 12 components contributed approximately 90% of the total variation. The PCA biplot results showed that all pepper accessions were spread in all four quadrants (Fig. 6A).

4. Discussion

Significant morphological differences are recognized within evaluated pepper accessions suggesting that there is abundant diversity in Balkan pepper-producing regions. In the Balkans, fruit shape diversity has been mainly characterized using conventional descriptors, but TA has not been utilized so far; thus, we envisaged characterizing Balkan pepper collection using predefined TA morphometric and colorimetric descriptors. We have utilized a total of 50 different fruit shape, size, and color descriptors to study fruit diversity in intra- and inter-varietal types. Longitudinal sections analyzed by TA were found to be helpful to obtain information about external boundary-based fruit shape, size, and color measurements but were unsuitable to measure internal features. Therefore, latitudinal or cross-fruit sections were used as an alternative way to measure internal features of pericarp area, thickness, placenta, and septum locules related to fruit mass. A similar approach has been used in previous studies, and observations have been made in tomato (Gonzalo et al., 2009; Rodríguez et al., 2010b) and pepper (Tripodi and Greco, 2018) fruit diversity analyses.

Across the evaluated accessions and between varietal types, all...
descriptors were significantly different except DEC. This result suggests that these accessions possess valuable diversity for fruit shape, size, and color and were in agreement with the pepper fruit shape investigation of Tripodi and Greco (2018), except for non-significant differences of eccentricity and proximal eccentricity. The coefficient of variation for fruit asymmetry, fruit shape index, and proximal/distal end were highly variable and non-significant, as demonstrated in eggplant (Hurtado et al., 2013; Hurtado et al., 2014), tomato (Rodríguez et al., 2013; Figuera et al., 2015) and pepper (Tripodi and Greco, 2018). Significant differences among accessions were found to be higher across different varietal types but were least or nonsignificant within each varietal type. This suggests that different fruit shape, size, and color descriptors were specific to a given varietal type. Overall, within each varietal type, 20 of the TA descriptors were non-significant for round-shaped fruits. This may be likely due to the small fruit size since round fruits were very small, which may have confounded with other TA descriptors.

Basic measurements related to fruit length (or height) and widths are important for classifying pepper fruits into small, medium, and large categories (Zhigila et al., 2014). The relationship between these traits or techniques measuring those traits is important to understand fruit morphology. Manually measured FL was slightly higher than TA-measured MH, and this observation was in agreement with observations made in eggplant fruit shape study (Hurtado et al., 2013). It appears that this was due to differences in the measurement technique used: FL is measured from the end of pedicel, and MH is measured where the internal pedicel ends. Manually measured FW was also slightly lower than TA-measured MW due to the fact that the width as measured by TA was taken where fruits are wider and can vary based on fruit shape. The non-significant correlation between FWT and PT might be associated with how FWT or PT is measured. TA measures the PT from both sides of the fruit wall, whereas manually measured FWT was measured from only one side of the fruit wall. The direct positive relationship between FW and FWT suggests that wider fruit widths would result in thicker fruit walls. This is due to the fact that as the cell expands, the fruit width and fruit wall thickness increases steadily during anthesis and then levels off during fruit maturity (Cheniclet et al., 2005).

Multivariate PCA displayed the fruit morphometric and colorimetric
diversity of the Balkan pepper collection captured by TA and how it was influenced by fruit shape, size, and color related traits. Danojevic et al. (2017) reported that the most important positively correlated traits in the first PC were fruit index, fruit shape in the longitudinal section, and capsaicin in the placenta, while the negatively correlated traits included fruit diameter, fruit weight, pericarp thickness, and number of apexes.

Fig. 5. PCA pairwise variance plot to display percent variation explained by each principle component. The blue line indicates the cumulative variation of 1–47 components, and the red line indicates variation explained by an individual component (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 6. A. PCA biplot displaying clusters of accessions categorized by fruit shape. Each cluster represents accessions with specific fruit shape, and different symbols and colors are assigned to display different fruit shape groupings. B. PCA biplot displaying fruit morphometric and colorimetric descriptors.
Bozokalfa et al. (2009) Turkish pepper diversity study found that in the first two PCs, the greatest variation was described with fruit traits (i.e., fruit length, diameter, weight, volume and fruit wall thickness, and pedicel length). Tsonev et al. (2017), in a study of 19 Bulgarian cultivars, established significant differences for all 13 phenotypic traits. Fruit width, weight, and pericarp thickness were the most strongly correlated traits (in the negative direction) with the first axis explaining a total of 55.6% variance. The most discriminative traits for the second axis were fruit length (in the positive direction) and explained a total variance of 22.6%. Our results confirmed that the first component was negatively correlated (-0.58 and -0.53) with fruit width (MW), and the second component was positively correlated (0.76 and 0.53) with fruit length (MH) but did not show a negative relation between first component and pericarp thickness.

Apart from fruit shape characterization, TA has also been used to identify fruit-shape-related QTLs from tomato (Gonzalo and van der Knaap, 2008; Gonzalo et al., 2009). In peppers, TA has been utilized to identify fruit-shape-related SNPs from a pepper collection (Chaim et al., 2001; Xue-jun et al., 2012; Colonna et al., 2019; Pereira-Dias et al., 2019). Genetic linkage between fruit shape and size descriptors has been investigated for their role in potential disease resistance (Naegle et al., 2016) and breeding thick-fruited peppers for disease resistance appears to be an arduous task. However, the pepper diversity study of Tripodi and Greco (2018) was a global pepper collection with nine Capsicum species representing 48 world regions, and the capsicum diversity included in our study was limited to Capsicum annuum accessions and in six Balkan countries.

5. Conclusions

A comprehensive analysis of a Balkan pepper collection confirms that Bulgaria and other Balkan countries possess appreciable diversity for different fruit shapes, sizes, and colors. This unique pepper diversity would be a useful resource to identify traits of interest that are relevant for the Balkan region. We demonstrate the ability of TA to quantify fruit characteristics of variable pepper collection gathered from different Balkan regions that are difficult or impossible to measure using conventional phenotyping methods. We also explained the phenotypic basis of Balkan pepper fruit shape and size diversity as well as the variation in fruit shape, size, and color traits between and within varietal types. Detailed fruit trait characterization and phenotyping would be useful for further utilization in identification of genes related to these traits and could be helpful for pepper breeding.

Fig. 6. (continued)
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Author Contributions
- Amol Nankar conceptualize and designed the experiment, analyzed data, prepared figures and/or tables, prepared original draft, and reviewed drafts, approved final draft
- Ivanka Tringovska conceptualize and designed the experiment, performed experiments, analyzed data, authored or reviewed drafts of the paper, approved final draft
- Stanislava Grozeva analyzed data, authored or reviewed manuscript, approved final draft
- Velichka Todorova analyzed data, contributed material, authored or reviewed drafts of paper, approved final draft
- Dimitrina Kostova authored or reviewed draft of manuscripts, approved final draft

Data Statement
R code is available upon a request

Declaration of Competing Interest
None.

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Appendix A. Supplementary data
Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.scienta.2019.108862.

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