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Genetic diversity of cultivated and wild tomatoes revealed by morphological traits and SSR markers

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ABSTRACT. In the current study, morphological traits and molecular markers were used to assess the genetic diversity of 29 cultivated tomatoes, 14 wild tomatoes and seven introgression lines. The three components of the principal component analysis (PCA) explained 78.54% of the total morphological variation in the 50 tomato genotypes assessed. Based on these morphological traits, a three-dimensional PCA plot separated the 50 genotypes into distinct groups, and a dendrogram divided them into six clusters. Fifteen polymorphic genomic simple-sequence repeat (genomic-SSR) and 13 polymorphic expressed sequence tag-derived SSR (EST-SSR) markers amplified 1115 and 780 clear fragments, respectively. Genomic-SSRs detected a total of 64 alleles, with a mean of 4 alleles per primer, while EST-SSRs detected 52 alleles, with a mean of 4 alleles per primer. The polymorphism information content was slightly higher in genomic-SSRs (0.49) than in EST-SSRs (0.45). The mean similarity coefficient among the wild tomatoes was lower than the mean similarity coefficient among the cultivated tomatoes. The dendrogram based on genetic distance

divided the 50 tomato genotypes into eight clusters. The Mantel test between genomic-SSR and EST-SSR matrices revealed a good correlation, whereas the morphological matrices and the molecular matrices were weakly correlated. We confirm the applicability of EST-SSRs in analyzing genetic diversity among cultivated and wild tomatoes. High variability of the 50 tomato genotypes was observed at the morphological and molecular level, indicating valuable tomato germplasm, especially in the wild tomatoes, which could be used for further genetic studies.

Key words: Genetic diversity analysis; Morphological traits; Molecular marker; Wild tomato

INTRODUCTION

Tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Mill.) is one of the most economically important vegetables widely grown for its fruit, and consumed either fresh or processed. The annual global production of tomato exceeds 161 million tons (FAO, 2012).

Cultivated tomatoes typically have low genetic diversity due to population bottlenecks (Rick, 1976), and intensive selection of a few desired traits during domestication has led to further loss of genetic diversity among the commercial tomato varieties (Williams and Clair, 1993). The genetic base of tomato varieties is narrow, which makes it difficult to identify different varieties using molecular markers. Fortunately, the model tomato vegetable from the family Solanceae has abundant genomic information, including a complete reference genome sequence and a rapidly developing comparative genomic database (http://solgenomics.net/). Therefore, many molecular markers generated from the sequence database have been successfully applied for identification and genetic diversity analysis of tomato cultivars (Park et al., 2004; García-Martínez et al., 2006; Benor et al., 2008; Korir et al., 2014).

There are 16 wild species of tomato, including *S. habrochaites*, *S. pennellii*, *S. pimpinellifolium*, *S. cheesmaniae*, *S. galapagense*, *S. peruvianum*, *S. corneliomulleri*, *S. chilense*, *S. chmielewskii*, *S. arcanum*, *S. neorickii*, *S. huaylasense*, *S. lycopersicoides*, *S. ochranthum*, *S. jugandifolium*, and *S. sitiens* (Knapp et al., 2009; Bedinger et al., 2011). These species in the tomato clade are all diploid with the same chromosome number (2n = 24) and are considered to have evolved primarily by genic changes rather than large-scale chromosomal rearrangements (Anderson et al., 2010). The wild species of tomato harbor many valuable genes, which may have been lost among cultivated tomatoes. As wild species can enlarge the gene pool of cultivated species, wild species are very useful in breeding programs as sources of genetic variability (Hanson et al., 2007). It is of great importance to have a clear understanding of the genetic diversity and relationship between cultivated tomatoes and their wild species for effective conservation, classification, and further utilization of tomato germplasm resources.

Morphological description and classification is a traditional approach used to quantify genetic differences, and is often used for genetic diversity analysis (Khadivi-Khub et al., 2008; Terzopoulos and Bebeli, 2008; Nikoumanesh et al., 2011). However, morphological traits are easily altered by environmental conditions; thus, quantification of genotypic variation is not always possible (Cooke et al., 2003). By comparison, molecular markers are generally recognized as a reliable method for the identification of genotypes, such as amplified

fragment length polymorphisms (AFLPs) (Park et al., 2004), randomly amplified polymorphic DNA (RAPD) (Nikoumanesh et al., 2011; Cao et al., 2015), and simple-sequence repeats or microsatellites (SSRs) (Benor et al., 2008; Wohrmann et al., 2011). Among these molecular markers, SSR markers are very popular due to their characteristics of high reproducibility, co-dominance, and polymorphism (Powell et al., 1996). EST-SSRs, the SSRs derived from expressed sequence tags (ESTs), are rapidly developing markers and have been used for genetic diversity analysis of a wide range of plant species (Jia et al., 2007; Caruso et al., 2008; Huang et al., 2010; Korir et al., 2014). Moreover, using a combination of morphological and molecular markers to identify plant genetic diversity has become more common (Khadivi-Khub et al., 2008; Terzopoulos and Bebeli, 2008; Mazzucato et al., 2010; Nikoumanesh et al., 2011).

However, evaluation of the genetic relationship of cultivated and wild tomatoes using genomic-SSR and EST-SSR has not been undertaken to date. Taking the phenotypic diversity among the cultivated and wild tomatoes into account, a dual strategy was employed in this study to investigate the genetic diversity of the tomato germplasm based on both morphological and genetic markers (genomic-SSRs and EST-SSRs). The aim was to test the applicability of EST-SSRs in tomato genetic diversity assessments and clarify the variability of cultivated and wild tomatoes at the morphological and molecular level.

MATERIAL AND METHODS

Plant material

A total of 29 cultivated tomatoes (*S. lycopersicum*), 14 wild tomatoes, and seven introgression lines (ILs) developed from a cross between *S. pennellii* and *S. lycopersicum* were donated by the Tomato Genetics Resource Center (TGRC, Davis, CA, USA). The details of the tomato material are shown in Table 1.

Morphological data

Details of the nine morphological traits recorded for the tomatoes are shown in **Table S1**. Briefly, the traits recorded were growth habit, plant height (cm), stem diameter (mm), hypocotyl color, leaf shape, leaf area (cm^2), leaf length (cm), leaf width (cm), and pubescence of stem and leaf. These descriptors mainly conform to the guidelines of the International Plant Genetic Resources Institute (Mazzucato et al., 2010). The flower and fruit traits were also investigated, but these data were not used for further analysis as some wild tomatoes in our collection were self-incompatible.

Genotypic data

Total genomic DNA was isolated from young leaves of 50-day-old seedlings using a DNA extraction kit (Takara, Shanghai, China) following the manufacturer protocol, and the quality and quantity of DNA was assessed against known concentrations of unrestricted lambda DNA on a 1% agarose gel.

Genomic-SSR and EST-SSR markers were commercially provided by the Biotech Company (Invitrogen, Shanghai, China). Details of the 15 genomic-SSR and 13 EST-SSR primers used in the study were shown in **Table S2**. Amplification reactions were performed

in a total volume of 20 µL, containing 11.6 µL ddH_2O, 1 µL gDNA (20 ng), 1 µL forward and reverse primers (10 µM), 2 µL 10X buffer, 1.2 µL $MgCl_2$ (25 mM), 2 µL dNTPs (2.5 mM), and 0.2 µL Taq DNA polymerase (5 U/µL) (Takara). The reactions were carried out on 96-well plates in an Authorized Thermal Cycler (Eppendorf, Hamburg, Germany) with the following program: 94°C for 3 min, then 35 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min, with a final step at 72°C for 5 min. Annealing temperature was changed based on the primers used (**Table S2**). The PCR products were verified by 8% polyacrylamide gel electrophoresis and visualized by silver staining. The DL 500-bp DNA marker (Takara) was included on each gel to estimate band size. Electrophoresis was run at 100 V and its expendable time depended on the size of each marker. Amplification and verification was repeated twice for every polymorphic marker to ensure reliability of the data.

Table 1. Number, Tomato Genetics Resource Center (TGRC) code, taxon, and type (cultivated or wild tomato) of the 50 tomato genotypes assessed.

Number	Code	Taxon	Type	Number	Code	Taxon	Type
1	LA4090	*Solanum lycopersicum*	Cultivated	26	LA3668	*S. lycopersicum*	Cultivated
2	LA1019	*S. lycopersicum*	Cultivated	27	LA3847	*S. lycopersicum*	Cultivated
3	LA1023	*S. lycopersicum*	Cultivated	28	LA4355	*S. lycopersicum*	Cultivated
4	LA1222	*S. lycopersicum*	Cultivated	29	LA4440	*S. lycopersicum*	Cultivated
5	LA1500	*S. lycopersicum*	Cultivated	30	LA1223	*S. habrochaites*	Wild
6	LA1563	*S. lycopersicum*	Cultivated	31	LA1777	*S. habrochaites*	Wild
7	LA1698	*S. lycopersicum*	Cultivated	32	LA3915	Introgression line	-
8	LA1994	*S. lycopersicum*	Cultivated	33	LA3916	Introgression line	-
9	LA1996	*S. lycopersicum*	Cultivated	34	LA3917	Introgression line	-
10	LA2006	*S. lycopersicum*	Cultivated	35	LA3918	Introgression line	-
11	LA2013	*S. lycopersicum*	Cultivated	36	LA3919	Introgression line	-
12	LA2019	*S. lycopersicum*	Cultivated	37	LA3920	Introgression line	-
13	LA2413	*S. lycopersicum*	Cultivated	38	LA3932	Introgression line	-
14	LA2661	*S. lycopersicum*	Cultivated	39	LA0716	*S. pennellii*	Wild
15	LA2662	*S. lycopersicum*	Cultivated	40	LA1926	*S. pennellii*	Wild
16	LA2683	*S. lycopersicum*	Cultivated	41	LA1598	*S. pimpinellifolium*	Wild
17	LA2706	*S. lycopersicum*	Cultivated	42	LA2093	*S. pimpinellifolium*	Wild
18	LA2838	*S. lycopersicum*	Cultivated	43	LA0923	*S. cheesmaniae*	Wild
19	LA3006	*S. lycopersicum*	Cultivated	44	LA0483	*S. galapagense*	Wild
20	LA3120	*S. lycopersicum*	Cultivated	45	LA1627	*S. galapagense*	Wild
21	LA3183	*S. lycopersicum*	Cultivated	46	LA 0454	*S. peruvianum*	Wild
22	LA3320	*S. lycopersicum*	Cultivated	47	LA 1274	*S. corneliomulleri*	Wild
23	LA3473	*S. lycopersicum*	Cultivated	48	LA 1969	*S. chilense*	Wild
24	LA3475	*S. lycopersicum*	Cultivated	49	LA 2748	*S. chilense*	Wild
25	LA3538	*S. lycopersicum*	Cultivated	50	LA 2408	*S. lycopersicoides*	Wild

Data analysis

The morphological data were used in principal component analysis (PCA) and cluster analysis in SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). For genotypic data, the presence or absence of the band was scored as 1 or 0, respectively. Based on analysis of bands, the polymorphism information content (PIC) for each SSR was calculated with the PIC-CALC software, according to the formula:

$$PIC = 1 - \sum p_{ij}^2 \qquad \text{(Equation 1)}$$

where, p_{ij} is the frequency of the i^{th} allele of the j marker (Weir, 1990). Pairwise genetic simi-

larities (S_{ij}) among accessions *i* and *j* were estimated using the similarity coefficient described by Nei and Li (1979) as follows:

$$S_{ij} = 2N_{ij}/(N_i + N_j) \quad \text{(Equation 2)}$$

where, N_{ij} is the number of bands present in the *i* and *j* cultivars, with N_i and N_j representing the number of bands present in cultivar *i* and *j*, respectively. A dendrogram was generated using the unweighted pair group mean analysis method based on genetic distance with the NTSYS software. In addition, dissimilarity matrices were calculated by Mantel tests using the NTSYS software.

RESULTS

Morphological characterization and clustering

PCA of the morphological traits showed that the three components represented 78.54% of the total phenotypic variation (Table 2). The first component accounted for 47.46% of the phenotypic variation where the width, length, and area of leaf had the highest loadings. Leaf traits were predominant in the first component and contributed to most of the phenotypic variation. The second component, which featured plant height as the principal trait, explained an additional 20.78% of the phenotypic variation. Finally, the third component explained a further 10.30% of the variation in which hypocotyl color was predominant. The three-dimensional PCA plot shows the distribution of tomato genotypes based on the morphological traits (Figure 1). Cultivated tomatoes mainly clustered into two groups (S1, S2), along with the ILs. The wild tomatoes, except number 31 (*S. habrochaites*), clustered separately from the cultivated tomatoes.

Morphological cluster analysis showed six distinct clusters at a Euclidean distance of five (Figure 2). Cluster I included 14 cultivated tomatoes and four ILs; cluster II comprised ten wild tomatoes and three cultivated tomatoes; cluster III was composed of three wild tomatoes; cluster IV was composed of two cultivated tomatoes; cluster V comprised ten cultivars and three ILs; and cluster VI consisted of a single wild tomato (number 48).

Table 2. Eigenvectors of the three principal component (PC) axes from the principal component analysis of the 50 tomato genotypes based on morphological traits.

Morphological traits	PC1	PC2	PC3
Growth habit	0.327	-0.911	-0.026
Plant height	-0.452	0.833	-0.062
Stem diameter	0.801	0.173	0.116
Hypocotyl color	0.559	-0.114	0.570
Pubescence of stem and leaf	0.730	-0.011	0.267
Leaf shape	0.549	0.475	0.325
Leaf length	0.885	0.187	-0.224
Leaf width	0.909	0.204	-0.202
Leaf area	0.750	-0.007	-0.563
Cumulative eigenvalues	47.46	68.24	78.54

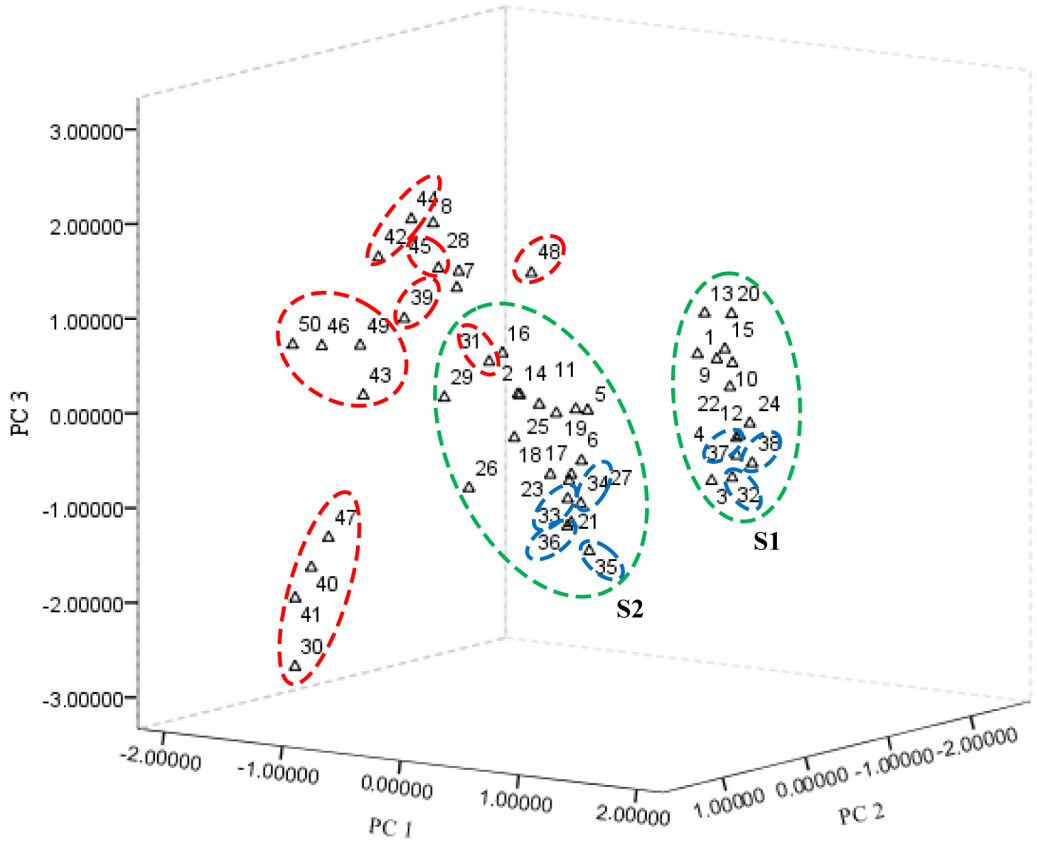

Figure 1. Three-dimensional principal component (PC) analysis plot of the 50 tomato genotypes based on morphological traits. Numbers correspond to the tomato genotypes in Table 1. The two sections (S1, S2) within the green dashed lines show the main distribution of the cultivated tomatoes in this study. Tomatoes within the blue dashed lines are introgression lines, while those within the red dashed lines are wild tomatoes.

Allelic variation based on SSR markers

The allelic variation of the genomic-SSR and EST-SSR markers is shown in Table 3. The number of clear fragments detected by 15 polymorphic genomic-SSRs in the 50 tomato genotypes was 1115, with a mean of 74 fragments per primer. Genomic-SSRs detected 64 alleles in total, with a mean of 4 alleles per primer and ranging from 2 to 8. The 13 EST-SSR primers amplified clear polymorphic products in the 50 tomato genotypes, with 780 clear fragments and a mean of 60 fragments per primer. The number of alleles ranged from 2 to 7, with a total of 52 alleles for all primers and a mean of 4 alleles per primer. DNA fingerprinting by the EST-SSR35 primer is shown in **Figure S1**. The mean PIC for the genomic-SSR loci was 0.49, ranging from 0.08 to 0.80, and the mean PIC for EST-SSR loci was 0.45, ranging from 0.14 to 0.71. Genomic-SSR and EST-SSR markers jointly amplified 1895 clear fragments with a mean of 68 fragments per primer. There were a total of 116 alleles, with a mean of 4 alleles per primer for the SSR markers.

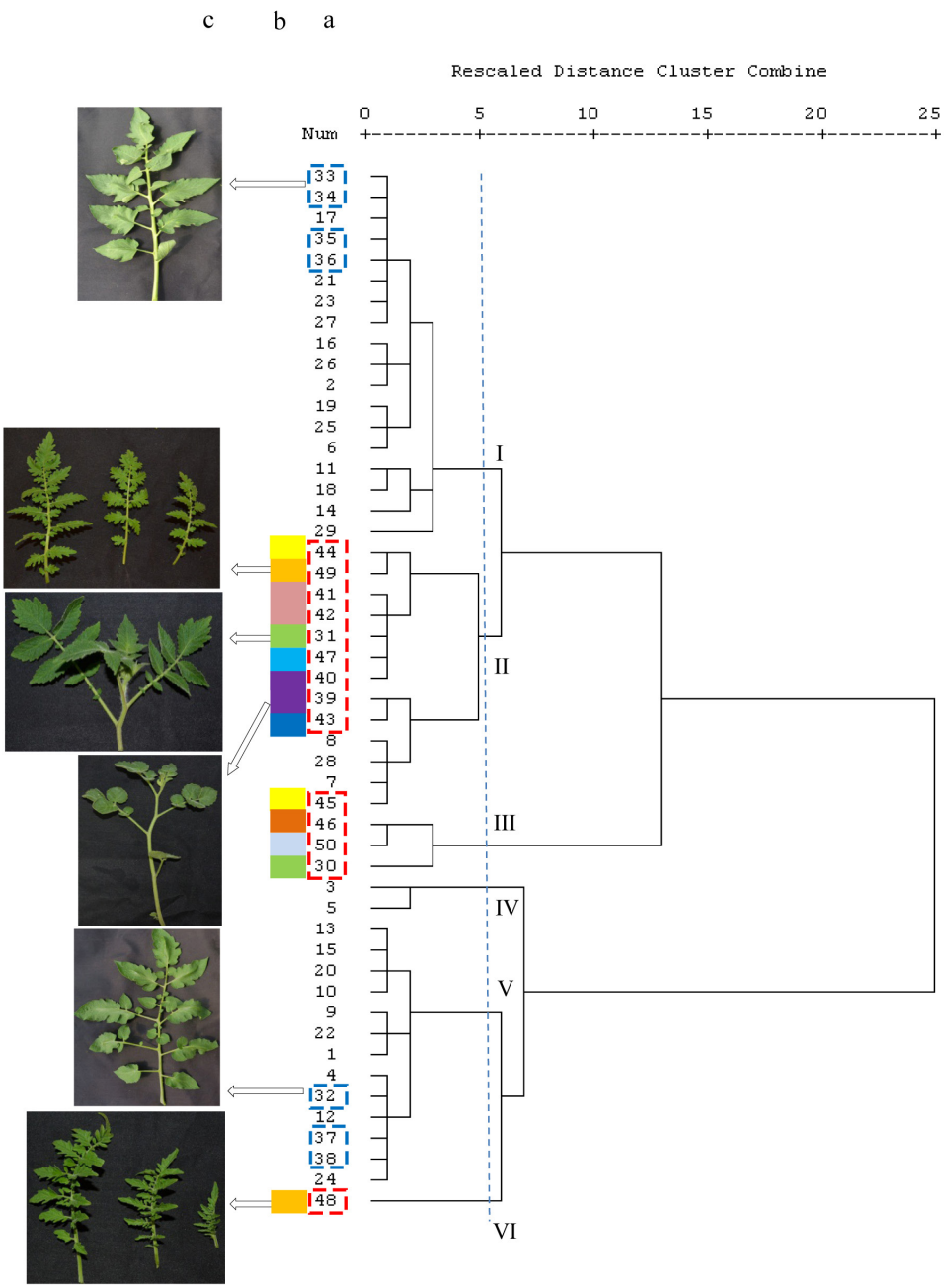

Figure 2. Cluster analysis of the 50 tomato genotypes based on morphological traits. The Roman numerals denote the six clusters. **(a)** Numbers correspond to the tomato genotypes in Table 1. Tomatoes within the blue dashed lines are introgression lines, while those within the red dashed lines are the wild tomatoes. **(b)** Different colored boxes to the left of the numbers represent different wild tomatoes. **(c)** Images of leaves from some tomato genotypes used in this study to show the leaf type variations.

Table 3. Allelic variation revealed by the polymorphic genomic simple sequence repeat (genomic-SSR) and expressed sequence tag-derived SSR (EST-SSR) among the 50 tomato genotypes.

Item	Genomic-SSR	EST-SSR	In total
Number of polymorphic primers	15	13	28
Total number of fragments	1115	780	1895
Mean number of fragments per primer pair	74	60	68
Total number of alleles	64	52	116
Mean number of alleles per primer pair	4	4	4
Polymorphism information content (range/mean)	0.08-0.80/0.49	0.14-0.71/0.45	0.08-0.80/0.47

Similarity coefficient and clustering of tomato genotypes based on SSR markers

The similarity coefficients of the 50 tomato genotypes based on SSR markers are shown in **Table S3**. These values ranged from 0.422 between *S. lycopersicum* and *S. pennellii* (numbers 5 and 40) to 0.994 between ILs (numbers 33 and 34), with a mean of 0.737 (**Table S3**). For the cultivated tomatoes, the minimum similarity coefficient was 0.724 (between numbers 6 and 16), with a mean of 0.845 (Table 4). For the ILs, the minimum and maximum similarity coefficients were 0.845 (between numbers 35 and 38) and 0.994 (between numbers 33 and 34), respectively (Table 4). The similarity coefficients of the wild tomatoes ranged from 0.457 (between numbers 40 and 41) to 0.836 (between numbers 43 and 44) (Table 4).

Genetic cluster analysis based on the genetic distance matrix is shown in Figure 3. There were eight clusters among all tomato genotypes at a distance coefficient of 0.66. Cluster I comprised 36 genotypes, including the 29 cultivated tomatoes and seven ILs. The wild tomatoes clustered together in the lower section of the dendrogram in clusters II to VI. There was only the one wild tomato within cluster VIII.

Table 4. Minimum (min), maximum (max), and mean values of the similarity coefficient among all tomato genotypes, cultivated tomatoes, introgression lines, and wild tomatoes.

Similarity coefficient	Sample No.	Min	Max	Mean
All tomato genotypes	50	0.422	0.994	0.737
Cultivated tomatoes	29	0.724	0.994	0.845
Introgression lines	7	0.845	0.994	0.932
Wild tomatoes	14	0.457	0.836	0.627

Correlation between morphological, genomic-SSR, and EST-SSR analysis

The Mantel test correlation coefficient (r) was 0.710 between genomic-SSR and EST-SSR matrices (Table 5). Combined data from both sets of molecular markers shows a closer relationship to the data from only the genomic-SSR markers (r = 0.952) than that observed from only the EST-SSR markers (r = 0.887; Table 5). However, the correlation coefficients between the morphological and molecular data were less than 0.2 (Table 5).

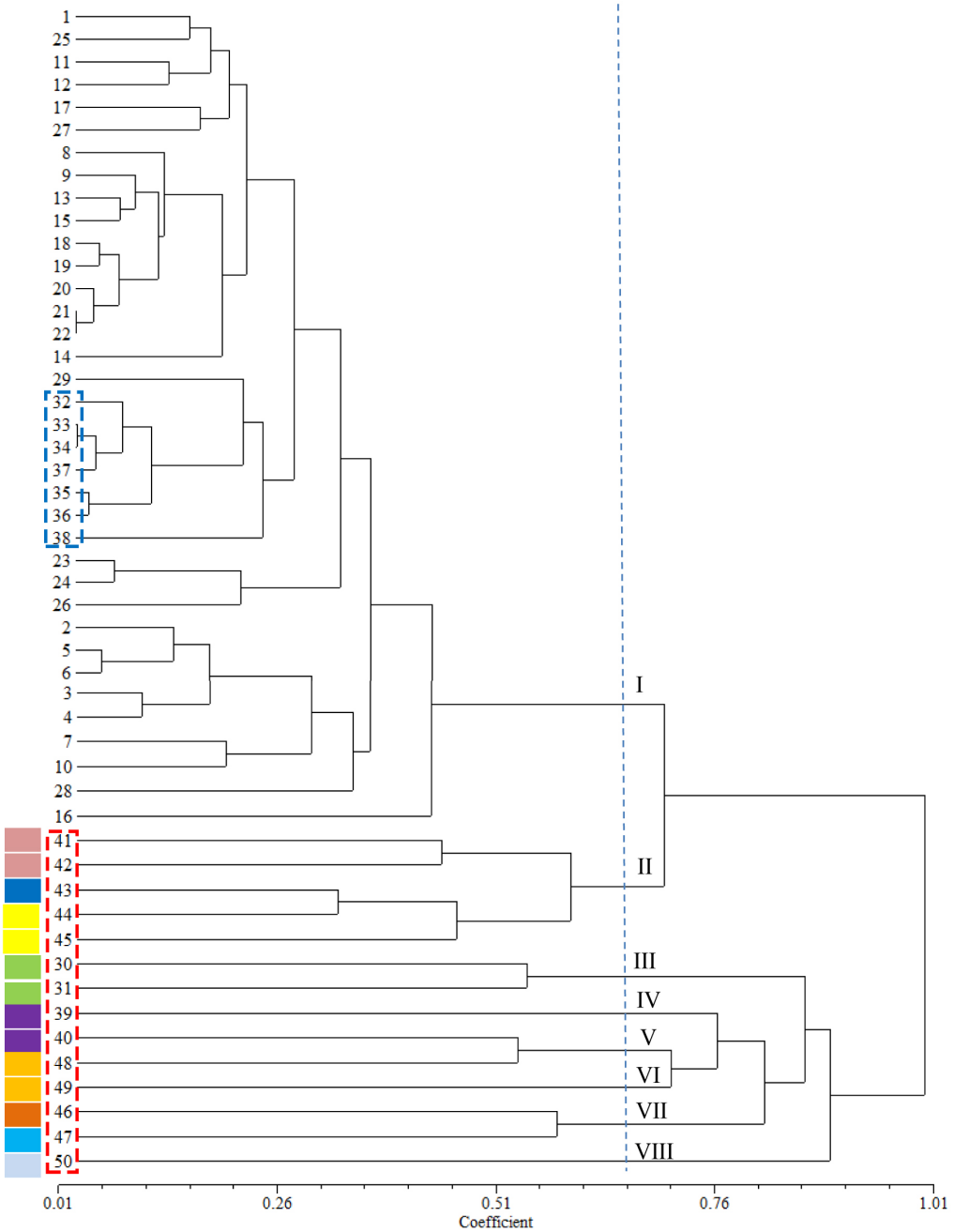

Figure 3. Dendrogram constructed using the unweighted pair group mean analysis method of the 50 tomato genotypes based on the genomic and expressed sequence tag simple-sequence repeat markers. The Roman numerals denote the eight clusters. Arabic numbers correspond to the tomato genotypes in Table 1. Tomatoes within the blue dashed lines are introgression lines, while those within the red dashed lines are the wild tomatoes. Different colored boxes to the left of the numbers represent different wild tomatoes.

Table 5. Mantel test correlation coefficient between the morphological matrices and the molecular matrices of the 50 tomato genotypes.

Correlation coefficient	Morphology	Genomic-SSR	EST-SSR	Genomic-SSR and EST-SSR
Morphology	1.000	-	-	-
Genomic-SSR	0.157	1.000	-	-
EST-SSR	0.128	0.710	1.000	-
Genomic-SSR and EST-SSR	0.157	0.952	0.887	1.000

SSR, simple sequence repeat; EST, expressed sequence tag.

DISCUSSION

Using a combination of morphological traits and molecular markers has been shown to lead to more reliable conclusions in assessments of genetic diversity (Khadivi-Khub et al., 2008; Nikoumanesh et al., 2011; Acosta-Quezada et al., 2012). According to the morphological results presented in the current study, leaf variation is crucial for distinguishing wild tomatoes from cultivated tomatoes as a result of i) most of the wild tomatoes possess specific leaf characteristics; ii) the results of PCA showed leaf traits to be the major contributors in the first component. The morphological cluster analysis was effective for classifying the cultivated and wild tomatoes. Based on morphological traits, the 50 tomato genotypes were divided into six clusters with the wild tomatoes gathered in clusters II, III, and VI. The morphological cluster of some wild tomatoes was in good agreement with their botanical classification. For example, two tomato genotypes from *S. pimpinellifolium* (numbers 41 and 42) showed a close relationship in the morphological cluster. However, there was a large distance between the two tomato genotypes from *S. chilense* (numbers 48 and 49). This indicates that some wild tomatoes cannot be discriminated clearly based on morphological characteristics alone, as morphological characteristics can easily be affected by the environment.

The cluster analysis based on the genomic-SSR and EST-SSR markers was more effective in comparison to the morphological cluster analysis. The cultivated tomatoes and ILs comprised cluster I, while the wild tomatoes comprised the other seven clusters. The ILs were expected to cluster together with the cultivated tomatoes as the ILs have a genetic background of a *S. lycopersicum* chromosome with a single restriction fragment length polymorphism-defined *S. pennellii*-chromosome segment. This also explains why the ILs showed a high similarity coefficient (0.932).

The EST-SSR markers were shown to be applicable for genetic diversity studies of tomato, which is in accordance with Muñoz-Falcón et al. (2011), Wohrmann et al. (2011), and Korir et al. (2014). In this study, the mean PIC of genomic-SSR markers was higher than the mean PIC for EST-SSR markers (0.49 and 0.45, respectively), which was also observed in eggplant (Muñoz-Falcón et al., 2011) and cucumber (Hu et al., 2011). High, medium, and low locus polymorphism is defined as PIC > 0.5, 0.5 > PIC > 0.25, and PIC < 0.25, respectively (Xie et al., 2010). Therefore, in our case, genomic-SSR and EST-SSR markers detected medium locus polymorphism among the 50 tomato genotypes, indicating that both markers are of great utility for genetic diversity studies of tomatoes.

The Mantel test revealed a low correlation between morphological matrices and molecular matrices of the cultivated and wild tomatoes (r = 0.157), which was also reported in Korean tomato varieties (Kwon et al., 2009), Greek tomato landraces (Terzopoulos and Bebeli, 2010), and almond genotypes and related *Prunus* species (Nikoumanesh et al., 2011). However, a good correlation between genomic-SSR and EST-SSR matrices (r = 0.710) was

observed, which further confirms the applicability of EST-SSR markers for analyzing genetic diversity in tomatoes. Furthermore, morphological indictors provide fundamental phenotypic information of tomato plants that could be complementary to molecular markers.

Domestication of tomatoes with the aim of selecting a few desired traits has resulted in a narrow genetic base and low genetic diversity of cultivated tomatoes. This is indicated by the high similarity coefficient of the 29 cultivated tomatoes (0.845) in this study. By contrast, wild tomatoes, with a lot of valuable genes, are important resources for improving the tolerance to both biotic and abiotic stress, such as *S. habrochaites* (Hanson et al., 2007; Sifres et al., 2011), which could expand the genetic base of cultivated tomatoes. The 14 wild tomatoes that were included in this study enriched the genetic diversity as indicated by i) their scattered distribution in the three-dimensional PCA plot (Figure 1); ii) the large genetic distance between them and the cultivated tomatoes (Figure 3); and iii) the lower similarity coefficient of the wild tomatoes (0.627) as compared to the similarity coefficient of the cultivated tomatoes.

In the current study, we used morphological descriptors and molecular markers, including genomic-SSR and EST-SSR markers, to assess the diversity of cultivated and wild tomatoes. Our results indicate that EST-SSR markers are effective in identifying tomato genotypes and analyzing genetic diversity of cultivated and wild tomatoes. Considerable genetic diversity among the cultivated and wild tomatoes was observed at both the morphological and molecular levels, which is of importance for germplasm classification, management, and further utilization.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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