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Comparison of gSSR and EST-SSR markers for analyzing genetic variability among tomato cultivars (*Solanum lycopersicum* L.)

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ABSTRACT. In order to study genetic variability and develop better strategies for the utilization of 48 tomato cultivars from America, China, the Netherlands, and Portugal, genomic simple sequence repeat (gSSR) and EST-derived SSR (EST-SSR) markers were applied. In all, 15 of 82 gSSR and 18 of 115 EST-SSR markers showed polymorphic loci. There were 995 and 2072 clear fragments amplified by polymorphic gSSR and EST-SSR markers, respectively. The total and average number of alleles detected by EST-SSRs (75, 4.2) was more than gSSRs (54, 3.6) as a result of some multi-locus EST-SSRs. A lower polymorphism information content value was found in gSSRs (0.529) compared to EST-SSRs (0.620). Similarity coefficient matrixes of the 48 tomato cultivars were established based on the gSSRs and EST-SSRs, and UPGMA dendrograms were constructed from the gSSRs and EST-SSRs similarity coefficient matrixes. A high similarity was observed between the gSSRs and EST-SSRs dendrograms. Genetic variability of four tomato populations from different countries showed that the observed number of alleles and Nei's genetic diversity were highest in the American population, and the effective number of alleles was

highest in the Dutch population. The estimated genetic structure showed some tomato cultivars from different countries shared a common genetic background, which might be related to gene flow. It was inferred that both gSSR and EST-SSR markers were effective to assess genetic variability of tomato cultivars, and the combination of both markers could be more effective for genetic diversity analysis in tomato.

Key words: Tomato cultivars; gSSR; EST-SSR; Dendrogram; Estimated genetic structure; Genetic variability

INTRODUCTION

The tomato (*Solanum lycopersicum* L.), originating in the Andes mountains of South America, is one of the most important vegetables in the world and is widely grown both in fields and under protected cultivation. Most of the commercial cultivars of tomato have been developed through traditional breeding and phenotypic selection. The initial narrow genetic bases of tomatoes have been further restricted by the development of cultivars and diversity within cultivated tomatoes has been lost (Williams and Clair, 1993). The range of similarity among improved tomato cultivars is narrow (Terzopoulos and Bebeli, 2008). It is vital to analyze the genetic diversity of tomatoes for their further utilization in breeding programs (Mazzucato et al., 2010). A thorough analysis of the genetic variability and structure of germplasm accessions is a fundamental requirement for the effective use of plant materials for breeding and crop improvement.

Today, molecular markers are recognized as a reliable approach for germplasm identification among plant genotypes. Genetic diversity and variability studies of plants have been carried out using molecular markers such as amplified fragment length polymorphisms (AFLPs) (Park et al., 2004; García-Lampason et al., 2012), randomly amplified polymorphic DNAs (RAPDs) (Hend et al., 2009; Nikoiumanesh et al., 2011), inter-simple sequence repeats (ISSRs) (Christopoulos et al., 2010), and simple sequence repeats or microsatellites (SSRs) (Hormaza, 2002; Benor et al., 2008; Pirseyedi et al., 2010). SSRs have shown higher efficiency among these markers due to the reproducibility, codominance, and polymorphism of SSR markers (Powell et al., 1996). Genomic simple sequence repeats (gSSRs) distributed in exons or untranslated-regions have been applied to genetic identification and polymorphism analysis (Nas et al., 2011; Giovannini et al., 2012). Alternatively, SSRs could be developed from available expressed sequence tags (ESTs), which are specially associated with functional genes, with less cost and time. With the sharp development of ESTs, EST-SSRs have been applied in the genetic diversity analysis of various crops (Jia et al., 2007; Huang et al., 2010; Korir et al., 2014). Both gSSR and EST-SSR markers were effectively used in mapping and identifying quantitative trait loci (QTL) (Sraphet et al., 2011). In cucumber, the combination of gSSRs and EST-SSRs resulted in a more effective genetic diversity analysis (Hu et al., 2011).

Tomato is a cultivated species well suited to the analysis of the effect of breeding in shaping diversity (Sim et al., 2009). In this study, we collected 48 tomato cultivars from America, China, the Netherlands, and Portugal, where they are popular in local marketplaces for their organoleptic qualities. In all, 82 gSSR primers and 115 EST-SSRs were screened and the polymorphic markers were used for further genetic variability analysis. The aim of this study was to compare the gSSR and EST-SSR markers when analyzing genetic variability, and to characterize the genetic variability of the tomato cultivars from different countries. This information will be valuable for optimizing the management of tomato collections and for designing crosses that maximize variability in breeding programs.

MATERIAL AND METHODS

Plant material

The tomato germplasm collection used included 48 tomato cultivars. There were 28 cultivars from America, seven cultivars from Mainland of China, four cultivars from Taiwan of China, seven cultivars from the Netherlands, and two cultivars from Portugal. The abbreviations and cultivar names of the 48 tomato cultivars are shown in Table 1.

Table 1. Abbreviations and names of the 48 tomato cultivars.

Abbreviation	Cultivar name	Abbreviation	Cultivar name
AM01	Ace 55 UF	AM25	Tomato Bush Silvery Fir Tree
AM02	Amama Orange	AM26	Tomato Cherry Gardener's Delight
AM03	Beef Steak	AM27	Yellow Pear
AM04	Black Krim	AM28	Stupice
AM05	Bonnie Best	MA29	Di huang huang yin tao
AM06	Burpee's BigBoy Hybrid	MA30	Di huang fen tian shi
AM07	C Gardener's D	MA31	Ai guan sha
AM08	Cherry Rainbow Blend	MA32	Chao yan
AM09	Cherry Sugar Sweetie	MA33	Fu shi No. 3
AM10	Cherry Sun Gold	MA34	Fu shi No. 6
AM11	German Johnson	MA35	Fu shi qiang
AM12	Lemon Boy	TA36	V-185
AM13	Pole Black Krim	TA37	Gold Small Tomato
AM14	Pole Brandywine	TA38	Double Color Big Tomato
AM15	Pole Brandywine Red & Yellow	TA39	Double Color Small Tomato
AM16	Pole Speckled Roman	NE40	Improved Cairo
AM17	Prudens Purple	NE41	Hao na
AM18	Red Cherry	NE42	Ji na
AM19	Roma	NE43	Jia li
AM20	San Marzano	NE44	Cairo
AM21	Super Sweet 100 Hybrid	NE45	Luo man na
AM22	Tomato Cherry Supersweet	NE46	Sabata
AM23	Tomato Grape Telly Bean Red & Yellow	PO47	Portuguese Tomato
AM24	Tomato Pole Beefsteak	PO48	Ref Vermelho

The first two letters of the abbreviations referred to the origin of the cultivar (AM, America; MA, Mainland of China; TA, Taiwan of China; NE, Netherlands; PO, Portugal), followed by two serial numbers.

DNA isolation

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

DNA fingerprinting

The gSSR and EST-SSR markers were commercially synthesized by Invitrogen (Shanghai, China). A total volume of 20 µL was used for amplification reactions, containing 11.6 µL ddH_2O, 1 µL gDNA (20 ng), 1µL forward and reverse primer (10 µM), 2 µL 10X Buffer Mg(-), 1.2 µL $MgCl_2$ (25 mM), 2 µL dNTPs (2.5 mM), and 0.2 µL Taq DNA polymerase (5 U/µL) (Takara, Shanghai, China). The amplification reactions were carried out in 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; finally 72°C for 5 min. The PCR products were verified by 8% polyacrylamide gel electrophoresis and visualized by silver staining. In order to estimate band sizes, a DL 500 bp DNA marker (Takara, Shanghai, China) was included in each gel. Electrophoresis was run at 100 V for approximately two hours.

Data analysis

gSSR and EST-SSR markers showing no bands or consistent bands among the tomato cultivars were eliminated. For the polymorphic markers, the presence or absence of a band was scored as 1 or 0, respectively. The polymorphism information content (PIC) for the gSSR and EST-SSR markers was calculated using PIC-CALC software according to the formula:

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

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

As described by Nei and Li (1979), pairwise genetic similarities (S_{ij}) among accessions i and j were estimated 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^{th} and j^{th} cultivars, with N_i and N_j representing the number of bands present in cultivar i and cultivar j, respectively. Based on genetic similarity matrices, a dendrogram was constructed using NTSYSpc version 2.10 software by the unweighted pair group mean analysis method.

The number of polymorphic loci (NPL), observed number of alleles (Na), effective number of alleles (Ne), and Nei's genetic diversity (Nei) were calculated using POPGENE 1.32 (Nei, 1972). Gene flow (Nm) was calculated according to the following formula:

$$Nm = 0,25 \text{ x } (1\text{-}Fst) / Fst \quad \text{(Equation 3)}$$

The estimated genetic structure was constructed using the STRUCTURE program of Evanno et al. (2005).

RESULTS

PCR amplification and allelic variation

Of the 82 gSSR and 115 EST-SSR markers, 15 gSSR and 18 EST-SSR markers showed clear polymorphic loci (Table 2). The 67 gSSR and 97 EST-SSR markers showed a consistent strip or no clear strip among the 48 tomato cultivars. There were 18.3% gSSR and 15.7% EST-SSR markers with polymorphisms (Table 2). There were 995 clear fragments amplified by 15 polymorphic gSSR markers with an average of 66 fragments per primer in all tomato cultivars (Table 2). The number of alleles ranged from 2 to 5 with a total of 54 alleles and an average of

3.6 alleles for gSSR markers (Table 2). On the other hand, 2072 fragments were amplified by 18 polymorphic EST-SSR markers with an average of 115 fragments per primer in all tomato cultivars (Table 2). The number of alleles ranged from 3 to 6 with a total of 75 alleles and an average of 4.2 alleles for EST-SSR markers (Table 2). The average PIC value of the 15 gSSR markers was 0.529, ranging from 0.291 to 0.698 (Table 2). The average PIC value of 18 EST-SSR markers was 0.620 with a range of 0.391 to 0.800 (Table 2).

The sequence, annealing temperature, allele number, and expected size of the polymorphic gSSR and EST-SSR markers were shown in Table 3. The DNA fingerprints of the 48 tomato cultivars as given by EST-SSR7 are shown in Figure 1.

Table 2. Summary of microsatellite allele data revealed by polymorphic microsatellite loci in the 48 tomato cultivars.

Item	gSSR	EST-SSR
Percentage of polymorphic primers (%)	18.3	15.7
Number of polymorphic primers	15	18
Number of all fragments	995	2072
Average number of fragments per primer pair	66	115
Number of all alleles	54	75
Average number of alleles per primer pair	3.6	4.2
Polymorphism information content (range/mean)	0.291-0.698/0.529	0.391-0.800/0.620

Table 3. Polymorphic gSSR and EST-SSR primer pairs used for further analysis in this study.

Marker name	Forward primer (5' to 3')	Reverse primer (5' to 3')	Annealing temperature (°C)	Allele	Expected size (bp)
gSSR					
AI895937	CATAATCACAAGCTTCTTTCGCCA	CATATCCGCTCGTTTCGTTATGTAAT	54	3	150-200
X13437	GAGCACCCATTAATTTCGTTACG	GTGGCGGATCTAGAAATTTAAACTG	55	3	200-300
SSR111	GTCCAGTGTTTCCAAAGGGA	TTTGCTGCTATACTGCTGACA	50	4	150-300
Tom 31-32	AATGTCCTTCGTATCCTTTCGT	CTCGGTTTTAATTTTTGTGTCT	52	5	100-250
AI780156	TCCAATTTCAGTAAGGACCCCTC	CCGAAAACCTTTGCTACAGAGTAGA	56	3	100-250
AW037347	GCCACGTAGTCATGATATACATAG	GCCTCGGACAATGAATTG	54	2	100-250
LE21085	CATTTTATCATTTATTTGTGTCTTG	ACAAAAAAAGGTGACGATACA	54	4	100-250
SSR47	TCCTCAAGAAATGAAGCTCTGA	CCTTGGAGATAACAACCACAA	52	4	150-300
TOM236-237	GTTTTTTCAACATCAAAGAGCT	GGATAGGTTTCGTTAGTGAACT	54	4	100-200
AI491173	GCACGAGCACATATAGAAGAGAATCA	CCATTTCATCATATCTCTCAGCTTGC	52	3	150-200
AI897173	CCTCTCTTCACCTCTTTACAATTTCC	CACTGGTCATTAAGTCTACAGCC	54	2	50-150
TMS6	CTCTCTCAATGTTTGTCTTTC	GCAAGGTAGGTAGCTAGGGA	54	3	150-300
U2108	CATTTTATCATTTATTTGTGTCTTG	ACAAAAAAAGGTGACGATACA	53	4	100-200
LEMDDNa	ATTCAAGGAACTTTTAGCTCC	TGCATTAAGGTTCATAAATGA	54	4	200-250
LE20592	CTGTTTACTTCAAGAAGGCTG	ACTTTAACTTTATTATTGCCACG	53	5	150-300
EST-SSR					
EST-SSR1	ACCTACCTGTCTCCGCCTCT	TGACAAGGTAAAGCCAACCC	55	5	150-300
EST-SSR2	CTTATGTGAAAACACCTCGCTC	TTCAAAATTCCCCAAAGACG	54	4	50-150
EST-SSR7	GAAGAAGATGGTGGGGATGA	CTTGCAACAATCGTGAATGC	54	4	50-150
EST-SSR19	ACCTGCACACACCACACACT	GATCAAAGAAGCGGGATGAT	53	4	100-150
EST-SSR23	TAGACTGGGCCTGTGGTCTT	TGGTGAATCAATTTTGGGGT	52	3	100-150
EST-SSR25	ATTGGGGAATGGGTTTTCTC	AAACGAAGGCAACAACGAAG	54	4	100-200
EST-SSR26	TCAAATGGCTTCTCTTGTTCTTT	TTGTTGGAAACTCCTTTGGC	54	3	100-150
EST-SSR35	CATAAGAAGAAAGGTGTGAATGAGA	GTTGCTTTGTCTTTGTCGCC	52	6	100-200
EST-SSR42	CCAAAAGAAGTGGGTCCAAA	AAACTAGCGACAAATAAAAGCAGA	54	6	100-200
EST-SSR62	AATACCCGAAAATGACCGAA	TGGCTGGTGATACCACCTCT	54	3	100-200
EST-SSR71	GGACCAAGCGAAGTTGGATA	CGAGTGTTTCGCTTCTCCTC	54	3	100-200
EST-SSR74	GGATTTCGTCGATTTCCATAA	GATTCCTTCCAATACACAATTCAA	50	5	150-200
EST-SSR77	GAGGACGACAACAACAACGA	GACATGCCACTTAGATCCACAA	53	5	100-200
EST-SSR80	GGTTTAATATCATTCACATATGCTCG	GATCAGGGCCAAGAATTGAA	52	5	150-300
EST-SSR83	TTAGGCAGCTTACGACTGGA	CCACAAATTCTTTTCCCCAA	51	3	150-200
EST-SSR85	GCCCATATTAAGCCCAAAAA	AGACAGCATGAGGTCCGAAT	51	3	150-200
EST-SSR97	CTCGAACCCTGTACCACACC	TGCTGTCGCTTTCATTATCG	54	6	150-300
EST-SSR106	TGTCAATCCACCTGGCATAA	AGGAGGTGCGTAAGGAGGAT	54	3	50-150

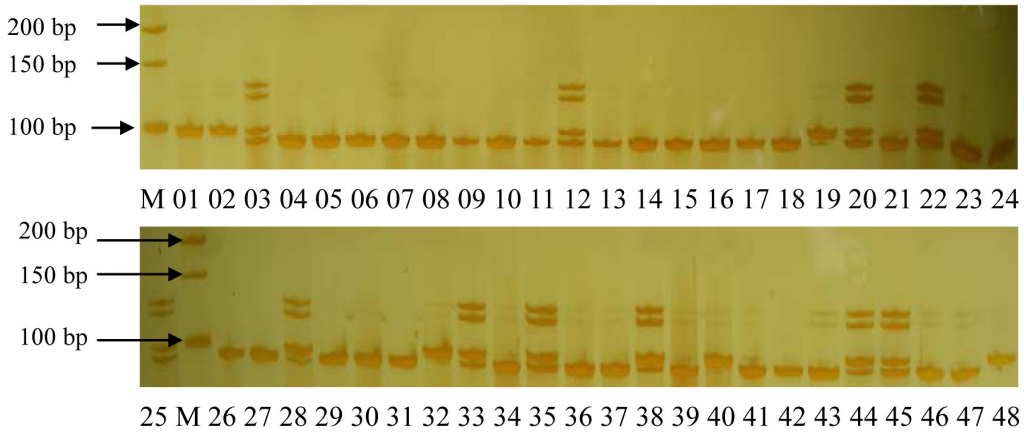

Figure 1. Profile amplified by EST-SSR7 in the 48 tomato cultivars. *Lane M*: DL 500 bp DNA marker; *Lanes 01-48*: cultivars AM1-PO48.

Dendrogram of tomato cultivars from gSSR and EST-SSR markers

The similarity coefficients detected by gSSRs ranged from 0.296 (AM21 and NE44) to 0.944 (AM09 and AM14), with an average of 0.637 (**Table S1**). And for EST-SSRs, the similarity coefficients ranged from 0.360 (AM13 and AM25) to 0.960 (AM23 and AM24), with an average of 0.671 (**Table S2**). Two dendrograms were constructed based on the similarity coefficients (Figure 2, 3).

The 48 tomato cultivars were classified into four major clusters with two cultivars (PO48 and AM10) considered as two individual clusters in the gSSRs dendrogram (Figure 2). Cluster I included nine American cultivars. Cluster II comprised 11 American cultivars, seven Chinese cultivars, five Dutch cultivars, and one Portuguese cultivar. Five American cultivars gathered together in cluster III. Eight cultivars from different regions formed Cluster IV.

The 48 tomato cultivars were divided into four clusters in the EST-SSRs dendrogram (Figure 3). There were 27 tomato cultivars from different regions in Cluster I. Cluster II included five American cultivars. Cluster III contained seven American cultivars, two Dutch cultivars, and three Chinese cultivars. Two American, one Chinese, and one Portuguese cultivar were grouped in Cluster IV.

Genetic diversity of four populations from different countries

The 48 tomato cultivars were classified into four populations by their geographical origins. Analysis of the genetic diversity among the American, Chinese, Dutch, and Portuguese populations are shown in Table 4. The mean number of polymorphic loci (NPL) was 13.75 for the four populations. The highest observed number of alleles (N_A) was found in the American population (3.467), while the highest effective number of alleles (N_E) was found in the Dutch population (2.579). The highest Nei's genetic diversity (Nei) was observed in the American population (0.5599). The estimated genetic structure was sensitive to the number of loci and the population sample size. The model with K = 5 gave the most favorable result in this study. The proportion of the five bases in each cultivar is shown in **Table S3**. The estimated genetic structure derived from the proportion of the five bases showing the genetic background of the 48 tomato cultivars is represented by bars of different colors (Figure 4).

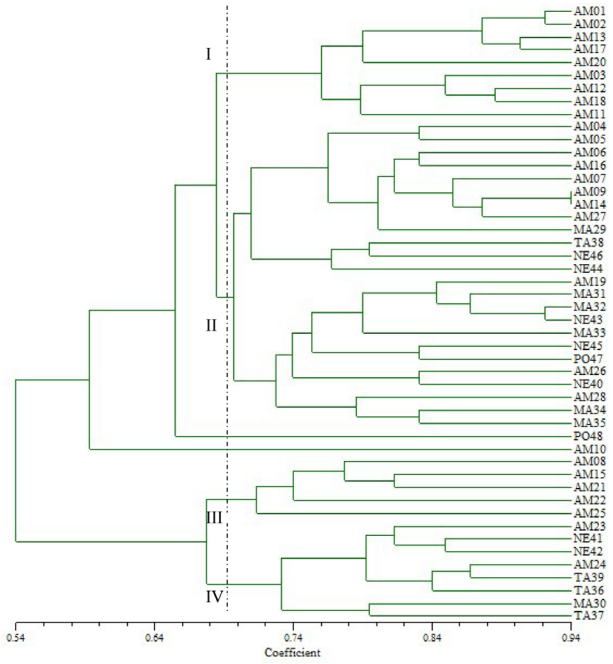

Figure 2. UPGMA dendrogram of 48 tomato cultivars using gSSR markers. The codes on the right refer to the abbreviations of the tomato cultivars in Table 1.

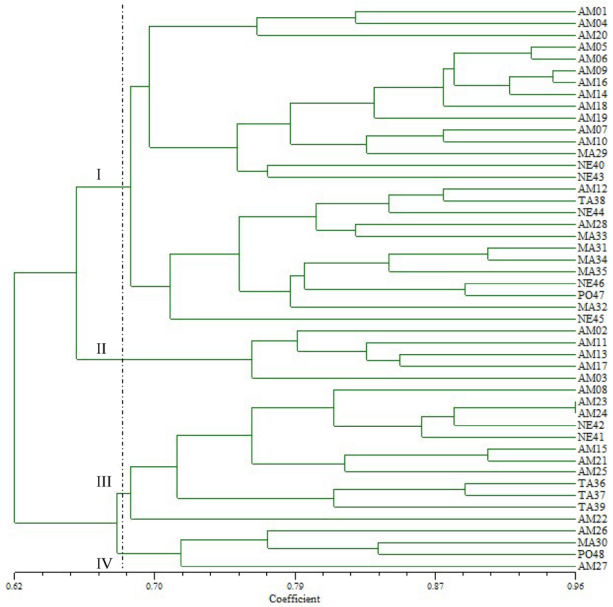

Figure 3. UPGMA dendrogram of 48 tomato cultivars using EST-SSR markers. The codes on the right refer to the abbreviations of the tomato cultivars in Table 1.

Table 4. Genetic diversity of four populations from different countries.

Population	NPL[a]	Na[b]	Ne[c]	Nei[d]	Nm[e]
America	15	3.467	2.559	0.560	-
China	15	3.267	2.392	0.519	-
Netherlands	15	3.200	2.579	0.544	-
Portugal	10	2.000	1.892	0.404	-
Mean	13.75	2.983	2.355	0.507	-
Overall	-	3.600	2.579	0.566	1.547

[a]number of polymorphic loci, [b]observed number of alleles, [c]effective number of alleles, [d]Nei's genetic diversity, [e]gene flow.

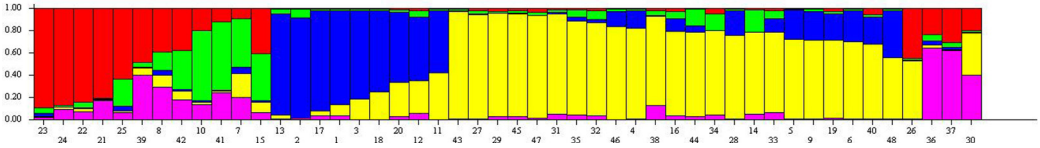

Figure 4. Estimated genetic structure for *K* = 5 for tomato genotypes based on gSSR variation. Each vertical bar represents one cultivar listed in Table 1 and the different colors represent different genetic backgrounds.

DISCUSSION

gSSR and EST-SSR marker selection

There has been much attention paid to the development and evaluation of SSR markers in tomato. Ohyama et al. (2009) developed nearly 700 SSR markers based on genome databases. Shirasawa et al. (2010) developed EST-derived SSR markers (TES markers), genome-derived SSR markers (TGS markers), and EST-derived intronic polymorphism markers (TEI markers) in tomato. Among the SSR markers, EST-SSR markers have been developed for a wide range of plant species and used for genetic studies with multiple purposes (Caruso et al., 2008; Tehrani et al., 2009; Sraphet et al., 2011; Korir et al., 2014). In this study, 15 out of 82 gSSR primers (18.3%) and 18 of 115 EST-SSR primers (15.7%) were polymorphic, indicating that the polymorphism rate of gSSR is higher than that of EST-SSR. A low polymorphism rate in gSSR and EST-SSR among tomato cultivars is probably due to the autogamous nature of tomato. Some markers detected more than two clear and stable bands in some cultivars in our study, and these were considered as multi-locus markers. The total and average number of alleles detected by EST-SSR (75, 4.2) is slightly higher than for gSSR (54, 3.6). In cucumber, gSSR markers detected more alleles than EST-SSR (Hu et al., 2011). Most of EST-SSRs are multi-locus markers in our study, which might explain why more alleles were detected by EST-SSR than by gSSR.

Polymorphic information content (PIC) was used to measure genetic diversity. Values of PIC > 0.5, 0.5 > PIC > 0.25, and PIC < 0.25 were designated as high, medium, and low locus polymorphism, respectively (Xie et al., 2010). The mean values of PIC obtained by gSSR and EST-SSR markers in this study were 0.529 and 0.620, respectively, demonstrating that both gSSRs and EST-SSRs could develop high locus polymorphism. The PIC of SSRs in cultivated and wild tomatoes was 0.687 (Meng et al., 2010) and the PIC of SSRs in tomato varieties was 0.628 (Kwon et al., 2009), which was similar to the results of this study. The PIC of SSRs in *Pomegranate* was 0.43 (Pirseyedi et al., 2010), relatively lower than the results of this study. The difference in the PIC of SSRs might be due to the different plant materials.

Cluster analysis and genetic relationships among tomato cultivars

Two dendrograms were constructed based on the similarity coefficients derived from the gSSR and EST-SSR markers. There was a clear consistency between gSSR and EST-SSR dendrograms in terms of the positioning of some tomato cultivars. Certain tomato cultivars had close relationships in both dendrograms, such as AM05 and AM06, AM13 and AM17, AM21 and AM25, NE41 and NE42, TA36 and TA39, and MA34 and MA35. The results demonstrated that certain tomato cultivars from the same geographical region had high similarity coefficients. The climate and market orientation in one geographical region might produce a specific breeding approach, which could be the reason for the high similarity coefficients between the tomato cultivars from the same country. However, tomato cultivars did not always cluster according to their geographical origins. For example, PO47 and NE45, and TA38 and NE44 were both close. This suggests that tomato varieties with different geographical origins probably have at least some common genetic bases as a consequence of gene flow.

The 48 tomato cultivars were divided into four populations according to their origins. The highest Ne was detected in the Dutch population, while the highest Na and Nei were detected in the American population. The Nm among the 48 tomato cultivars was 1.547, which was similar to the Nm in 75 pomegranate genotypes (1.451) (Parvaresh et al., 2012). The estimated genetic structure indicated that the tomato cultivars from different origins, such as MA29 and NE45, could have similar genetic backgrounds, possibly as a result of gene flow. The results of our study showed that gSSR and EST-SSR markers could be successfully used in the characterization of tomato cultivars and the analysis of genetic diversity, which was also discovered in studies of *Panicum virgatum* (Narasimhamoorthy et al., 2008) and *Prunus divaricata* (Wohrmann et al., 2011).

The intensive breeding of crops over the past half century with a focus on yield and special phenotypes has indirectly led to the loss of diversity. Modern tomatoes have been produced by the selective breeding process, leading to an inevitable reduction in their genetic variation (Yi et al., 2008). Estimation of genetic diversity in plant species with a narrow genetic base could be more efficient if different marker systems were used (Hu et al., 2011). gSSRs and EST-SSRs, two kinds of molecular markers, should yield similar cluster results and more reliable conclusions in the assessment of tomato genetic variability.

We compared the gSSR and EST-SSR markers in a genetic variability analysis of tomato cultivars from different countries. EST-SSRs detected more alleles and higher polymorphism information content than gSSRs among the 48 tomato cultivars as a result of multi-locus EST-SSRs. Both gSSR and EST-SSR markers could be efficiently applied in determining the genetic variability of cultivated tomato. A high similarity was observed in dendrograms based on the two markers. Our results are of importance not only for germplasm management, but also for parent selection and cross breeding of these modern tomato cultivars.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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