Genetic diversity and population structure of Chinese jujube (*Ziziphus jujuba* Mill.) and sour jujube (*Ziziphus acidojujuba* Mill.) using inter-simple sequence repeat (ISSR) Markers

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The Chinese jujube (*Ziziphus jujuba* Mill.) originates from sour jujube (*Ziziphus acidojujuba* Mill.) and is an economically important genus in the Rhamnaceae family. However, little is known about the genetic relationship between jujube cultivars and wild species. In this study, we estimated the genetic variation and relationships between 85 jujube cultivars and 55 sour jujube individuals by ISSR markers. Of 216 ISSR primers, 110 were able produce amplified product(s) and 28 showed polymorphisms, accounting for 50.9% and 25.5% of total primers respectively. A total of 89 loci were amplified with 28 primers, of which 42 loci (47.2%) were polymorphic, and most of primers exhibited highly PIC values. Cluster analysis and population structure analysis roughly divided the 140 accessions into two major groups. One group included all jujube cultivars and some sour jujube individuals, and the other group included remaining sour jujube individuals. Most jujube cultivars have a certain correlation with their origin, and there are obvious gene exchanges between sour jujube and jujube cultivars. The results provide a useful basis for jujube germplasm conservation, genetic improvement and evolution research.

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Abstract

The Chinese jujube (*Ziziphus jujuba* Mill.) originates from sour jujube (*Ziziphus acidojujuba* Mill.) and is an economically important genus in the Rhamnaceae family. However, little is known about the genetic relationship between jujube cultivars and wild species. In this study, we estimated the genetic variation and relationships between 85 jujube cultivars and 55 sour jujube individuals by ISSR markers. Of 216 ISSR primers, 110 were able produce amplified product(s) and 28 showed polymorphisms, accounting for 50.9% and 25.5% of total primers respectively. A total of 89 loci were amplified with 28 primers, of which 42 loci (47.2%) were polymorphic, and most of primers exhibited highly PIC values. Cluster analysis and population structure analysis roughly divided the 140 accessions into two major groups. One group included all jujube cultivars and some sour jujube individuals, and the other group included remaining sour jujube individuals. Most jujube cultivars have a certain correlation with their origin, and there are obvious gene exchanges between sour jujube and jujube cultivars. The results provide a useful basis for jujube germplasm conservation, genetic improvement and evolution research.

Keywords *Ziziphus jujuba* Mill, *Ziziphus acidojujuba* Mill, ISSR, Genetic diversity, Population structure

Introduction

Chinese jujube (*Ziziphus jujuba* Mill.) and sour jujube (*Ziziphus acidojujuba* Mill.) belong to the family Rhamnaceae. Chinese jujube (hereafter referred to as jujube) is an economically and ecologically important species that is a popular fruit tree in Asia (Qu & Wang, 1993). According to archaeological evidence, jujube, which has been cultivated for more than 3,000 years, originated in China (Qu & Wang, 1993; Liu, 2003; Liu & Wang, 2009; Li et al., 2013). As one of the oldest cultivated fruit trees, the germplasm resources of jujube are abundant, with more than 900 cultivars reported thusTable far (Liu & Wang, 2009). Jujube fruits have high nutritional value and a long history of usage as an edible fruit and in herbal medicine, and constitute a rich source of vitamin C, cAMP, flavonoids, triterpenic acids, and polysaccharides (Gao et al., 2013). Recent phytochemical and pharmacological studies have revealed that the main biologically active components of jujube fruits are beneficial to the human health (Choi et al., 2012; Chen et al., 2017a). Sour jujube, also known as wild jujube, is another important species that is regarded as the wild ancestor of jujube. It is widely planted as the rootstock for jujube and its seeds have high medicinal value (Qu & Wang, 1993; Liu & Wang, 2009; Islam et al., 2006; Zhang et al., 2015a). Research on the genetic diversity and phylogenetic relationships of jujube is beneficial for jujube breeding and will help to elucidate the evolutionary history of jujube.

With the development of molecular biology theory and technology, the genetic diversity and

genetic structure of jujube have been studied using molecular markers, including amplified fragment length polymorphism (AFLP), chloroplast microsatellite (cpSSR), random amplified polymorphic DNA (RAPD), sequence-related amplified polymorphisms (SRAPs), simple sequence repeat (SSR), single nucleotide polymorphism (SNP), and so on (Peng et al., 2000; Bai, 2008; Ma et al., 2011; Soliman et al., 2013; Li et al., 2014; Wang et al., 2014; Xie, 2014; Huang et al., 2015; Xiao et al., 2015; Zhang et al., 2015c; Fu et al., 2016; Xu et al., 2016; Chen et al., 2017b). For example, 30 main cultivars were divided into six groups based on AFLP analysis (Xie, 2014). The genetic diversity of 76 jujube cultivars was analyzed using 31 SSR markers, and the cultivars were divided into three main groups based on cluster analysis (Wang et al., 2014). One hundred and fifty accessions were clustered into two groups by STRUCTURE Software 2.3.4 (http://web.stanford.edu/group/pritchardlab/structure.html) and principal coordinate analyses (PCoA, https://www.xlstat.com/en/ solutions/features/ principal-coordinate-analysis) based on SNPs (Chen et al., 2017b). However, only a few studies involving the genetic diversity and genetic structure of sour jujube and the genetic relationship between jujube and sour jujube have been reported (Huang et al., 2015; Zhang et al., 2015a).

The inter simple sequence repeat (ISSR) technique is a polymerase chain reaction (PCR)-based method that involves the amplification of regions between adjacent, inversely oriented microsatellites using single sequence repeats, usually 16-25 bp long, as primers (Zietkiewicz et al., 1994). It is a rapid, simple, and inexpensive way to study genetic diversity, phylogeny, and evolutionary biology (Reddy et al., 2002). The jujube genome contains high-density simple

sequence repeats (Liu et al., 2014); therefore, it is suitable for genetic diversity analysis using ISSR markers. In the present study, the genetic diversity and population structure of 85 jujube cultivars and 55 sour jujube individuals were analyzed by ISSR markers. The results revealed the level of genetic diversity in the collections and the genetic relationships between jujube and sour jujube.

Materials & Methods

Plant materials

In total, 140 samples included 85 cultivars from Chinese jujube and 55 individuals from sour jujube (Table S1). These materials were planted in jujube germplasm resources of Luoyang Normal University (Luoyang, Henan), which were acquired with permissions from the National Chinese Jujube Germplasm Repository (Taigu, Shanxi), the National Foundation for Improved Cultivar of Chinese Jujube (Cangzhou, Hebei) and the Xinzheng Jujube Academy of Science (Xinzheng, Henan). Fresh young leaves for each accession were collected in May 2017, brought back to the laboratory in an ice box, and stored at -70°C freezer for further analysis.

Genomic DNA Extraction and PCR Analysis

Genomic DNA was extracted using a modified CTAB method (Lian et al., 2006). The DNA quality was assessed using a NanoDrop2000 and the DNA was diluted to 50 ng/μL. Sequences of 216 ISSR primers were obtained from the Biotechnology Laboratory at the University of British Columbia (Vancouver, Canada) (Table S2). Polymerase chain reaction (PCR) was performed in

a 10 μL reaction mixture containing 2.0 μL of template DNA, 0.4 μL of primers (10 μM), 0.8 μL of dNTP (2.5 mM), 1.0 μL of 10×Buffer, 0.2 μL of Taq DNA Polymerase (Solarbio, Beijing, China), and 5.6 μL of deionized water. PCR amplifications were performed in 96-well plates on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C°C for 3 min; 35 cycles at 94°C for 30 s, 40-60°C (melting temperature depends on the primer sets as listed in Table S2) for 30 s, and 72°C for 1.5 min; and a final extension at 72°C for 10 min. The amplified products were separated by electrophoresis on 2.0% (w/v) agarose gels under UV light.

Genetic diversity analysis

Based on the relative position of the ISSR amplification product on the agarose gel, the presence and absence of bands at the same position were scored as "1" and "0", respectively. The following parameters were calculated using GenALEx 6.5 (Peakall & Smouse, 2012): the number of different alleles (Na), the effective number of alleles (Ne), the Shannon index (I), and the polymorphic information content (PIC).

The cluster analysis was performed using the sequential, agglomerative, hierarchical, and nested clustering (SAHN) module and the unweighted pair-group method arithmetic average (UPGMA) method of NTSYS-pc 2.10e software, and a cluster plot was generated by the Tree plot module (Rohlf, 1998).

Population structure analysis

A Bayesian clustering analysis was implemented in Structure 2.3.4 (Falush et al., 2003; Hubisz et al., 2009) to evaluate population genetic structure. An admixture model and correlated allele frequencies were applied to estimate the ancestry fractions of each cluster attributed to each accession. For each value of K (range 1-25), 10 independent runs were performed with a burn-in period of 100,000 followed by 1,000,000 MCMC repetitions. Parameters were set to default values, and all accessions were considered to have unknown origins. The delta K method (Evanno et al., 2005) was implemented in Structure Harvester program (Earl & Vonholdt, 2012) to determine the most probable K-value. The accessions with membership probabilities ≥ 0.50 were considered to belong to the same group (Chen et al., 2017). A principal coordinate analysis (PCoA), based on the standardized covariance of genetic distances was performed using GenAlEx v 6.5.

Results

Detection of Polymorphisms

All 216 of the ISSR primers were evaluated for successful PCR amplification by testing three accessions. Among them, 110 primers (50.9%) successfully amplified at least one clear and stable fragment from the jujube and sour jujube genome. To test the polymorphism of the 110 ISSR primers, 12 jujube cultivars and 12 sour jujube individuals were further analyzed. Of the 110 ISSR primers, 28 primers (25.5%) were polymorphic (Fig. 1) and produced a total of 89 DNA fragments (Table 1). The number of amplified fragments varied from 2 to 6 with an average of 3.19 amplicons per primer, and their sizes ranged between 200 and 1,500 bp (Table 1).

The polymorphism per primer ranged from 16.7 (ISSR60) to 100% (ISSR-11 and ISSR-13) and the average number of polymorphic bands per primer was 1.5 (Table 1). Based on genetic variation standards (BOTSTEIN et al., 1980), the polymorphism information content (PIC) values ranged from 0.168 to 0.777, and most of the primers exhibited high PIC values (Table 1). Thus, our results indicated that ISSR markers could be used to assess the genetic diversity and population structure in these germplasms.

Genetic Diversity and Cluster Analysis

To examine the genetic diversity of 140 accessions in detail, we calculated their genetic relationships using Unweighted Pair Group Method and Arithmetic Mean (UPGMA) cluster analysis. Based on the unweighted neighbor-joining clustering, 140 accessions were divided into two major groups (Fig. 2).

Group I (G1) contained all of the jujube cultivars and seven sour individuals, and could be further divided into four subgroups. The subgroups I (G1-I), III (G1-III), and IV (G1-IV) included three jujube cultivars and two sour jujube individuals; two jujube cultivars and one sour jujube individual; and one jujube cultivar and two sour jujube individuals, respectively. Subgroup II (G1-II) included the vast majority of the jujube cultivars and one sour jujube individual, and could be further divided into three clusters. The 41 cultivars in cluster I (C1) mainly originated from northwest China; the 18 cultivars in cluster II (C2) mainly originated from eastern China; and the 14 cultivars in cluster III (C3) mainly originated from central China. Group II (G2) contained the other sour jujube individuals, and could be further divided into four

subgroups. These four subgroups (G2-I-IV) included 27, 16, four, and one individuals, respectively. The results showed that the genetic relationships among the different jujube varieties correlated somewhat with the origin of the variety, but there was no significant correlation with the variety use (Fig.2 and Table S1).

Population Structure

Based on the ISSR analysis data obtained above, we used STRUCTURE 2.3.4 software (Jakobsson et al., 2007) to analyze the population structure of the jujube and sour jujube accessions. The mean LnP(K) values for the different Ks ranged from one to 25, and exhibited a rapid incremental trend before reaching a peak value at K = 2. After K = 2, the mean LnP(K) values gradually increased to K = 25, but variation was observed among the replicate runs. Furthermore, our results showed that the highest value of ΔK was observed for K = 2, where all of the accessions could be roughly divided into two major clusters (Fig. 3). Using a membership probability threshold of 0.6 (Chen et al., 2012), 94 accessions were assigned to group I, which contained 85 jujube cultivars and nine sour jujube individuals. The remaining 46 sour jujube individuals were assigned to group II (Fig. 4 and Table S3).

Statistical analysis indicated that the majority of accessions showed strong membership values (Table S4). In group I, 71 accessions (75.5%), including 68 jujube cultivars and three sour jujube individuals, demonstrated shared ancestry. Similarly, 37 individuals (80.4%) had a high proportion of membership in group II. The other accessions showed mixed ancestry from both groups.

PCoA also roughly divided the 140 accessions into two clusters (Fig. 5), which was consistent with the assignments generated by UPGMA clustering (Fig. 2) and population structure analysis (Fig. 4). The majority of sour jujube accessions belonging to cluster I were distributed in the left half of the resulting plot. The rest of the sour jujube and all of the jujube accessions belonging to cluster II were distributed in the right of the plot. The distribution of cluster I was more widely scattered than cluster II, indicating that sour jujube had higher diversity than the jujube cultivars.

Discussion

Numerous studies over the past few decades have focused on elucidating the complex genetic relationships among different jujube varieties. In the present study, the genetic diversity of a wide variety of jujube germplasm resources was evaluated, which provides an important scientific basis for the efficient use of these germplasms.

Twenty-eight ISSR markers were used in this study to analyze the genetic diversity of 85 jujube and 55 sour jujube accessions. The results showed that the Shannon's Information Index (I: 0.492) and marker diversity (90.48%) of sour jujube were both higher than in jujube (Table S5). One probable explanation is that the genetic diversity of the jujube varieties has been reduced as a result of long-term evolution and artificial domestication.

Morphological, biological, and cytological evidence indicates that sour jujube is a wild species of jujube and that jujube is derived from sour jujube. Zhang et al. (2015b) used seven SSR makers to classify 17 sour jujubes and 16 jujube varieties into wild, semi-wild, and cultivar

196 species, with frequent genetic exchanges observed among the three groups. Huang et al. (2015)
197 used chloroplast microsatellite (cpSSR) markers to analyze jujube, sour jujube, and Indian jujube.
198 The results also showed that a genetic exchange existed between sour jujube and jujube. In this
199 study, the cluster analysis showed that there were obvious genetic clusters between sour jujube
200 and jujube, but some of the sour jujube individuals had a closer genetic relationship with the
201 jujube cultivars. Therefore, we divided the 140 samples into wild, semi-wild, and cultivar species
202 (Fig. 2). Population structure analysis showed that there was gene flow between the sour jujube
203 and jujube varieties (Fig. 4). Our results validated previous research results and provided
204 molecular biological evidence for the cultivation of jujube from sour jujube.

205 Previous studies have shown that the genetic relationships between different jujube varieties
206 correlate, to an extent, with the origin of the variety (Liu et al., 2016). The genetic variation of
207 jujube mainly emanates from intra-population variation, and the contribution rate from among-
208 population variation is low. Among the 85 jujube varieties used in this study, four accessions
209 with a Q-value of less than 0.6 accounted for only 4.7%, and most of the species had a single
210 kinship ($Q \geq 0.8$), which indicated that the majority of the varieties are dominated by intra-
211 population or intra-geographic variation (Table S6). The above results indicate that the existing
212 germplasm resources of jujube may originate from different regions. Frequent gene exchange
213 and recombination have occurred among the intraspecific cultivars during the evolution of the
214 species, resulting in a more varied population structure composition.

215 Genome sequencing showed that the jujube genome contains a very high density of SSRs.

The SSR repeats exhibited a strong bias toward A/T, AT/TA, and AAT/ATT motifs, whereas C/G and CG/CG motifs were present at very low levels (Xiao et al., 2015; Fu et al., 2016). Interestingly, the analysis of SSR and ISSR markers showed that AG/GA, CT/TC, and AC/CA repeat motifs had high amplification efficiency, while A/T, AT/TA, and AAT/ATT repeat motifs had low amplification efficiency. The SSRs in our study included 10 AG/GA-, eight CT/TC-, four GT/TG-, and three AC-type primers, which respectively corresponded to 35.7%, 28.6%, 14.3%, and 10.7% of the total SSRs (Table 1). The above results indicate that the simple sequence repeats in the jujube genome are dominated by A/T, AT/TA, and AAT/ATT repeat motifs, but the polymorphic sites are mainly AG/GA, CT/TC, and AC/CA repeat motifs. Therefore, using AG/GA, CT/TC, and AC/CA repeats in primer design could greatly improve primer screening efficiency. This should inform future genetic diversity analyses and the molecular breeding of jujube.

Conclusions

In this study, 28 polymorphic ISSRs were obtained using 85 jujube cultivars and 55 sour jujube individuals. By analyzing the genetic diversity and population structure, we concluded that jujube and sour jujube have a closely genetic relationship and most jujube cultivars have a certain correlation with their origin. These results will provide reliable and efficient genetic information for the study of jujube genetic relationship and new variety selection.

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Figure 1

Amplification products from 12 jujube cultivars and 12 sour jujube individuals using the ISSR-25 primer. M: D2000 plus DNA Ladder (Solarbio, Beijing, China).

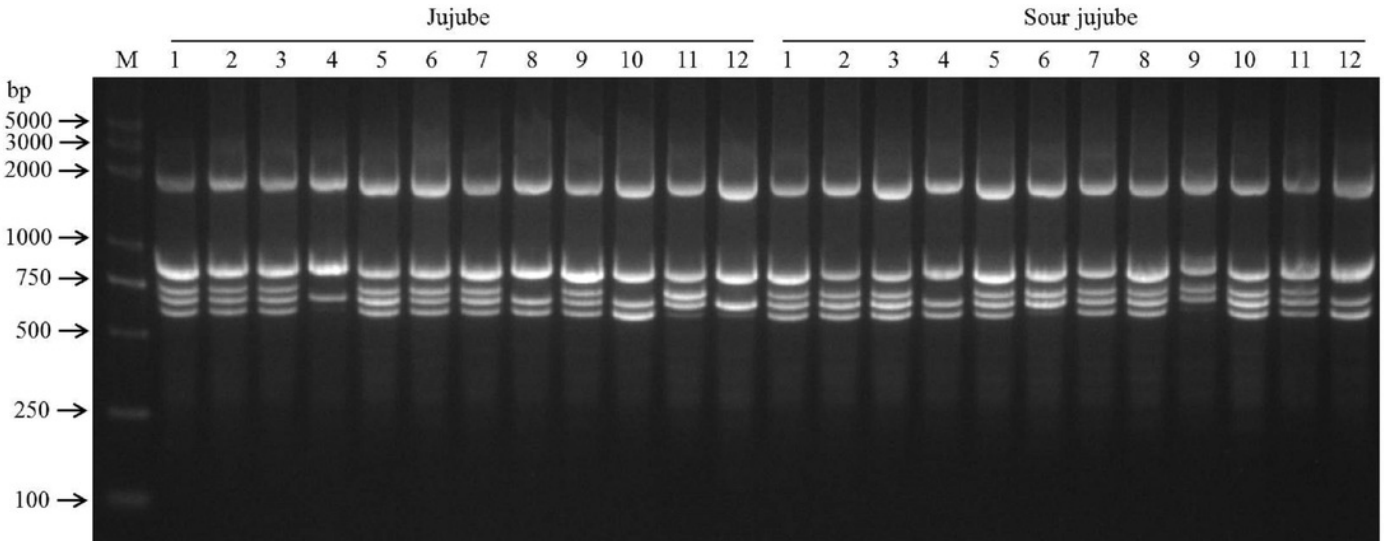

Figure 2

Dendrogram of 140 accessions based on 28 ISSR primers.

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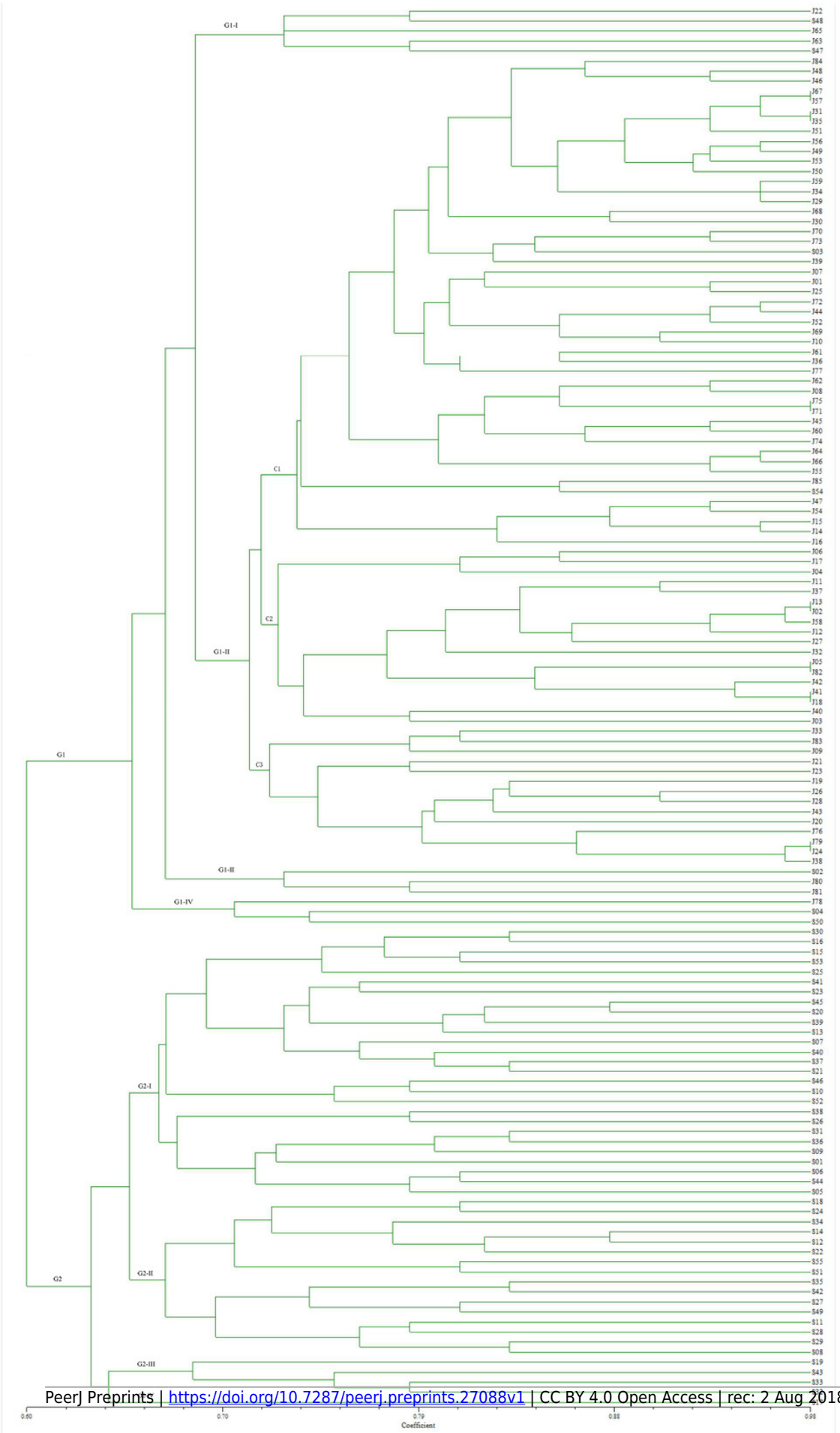

Figure 3

STRUCTURE estimation of the number of populations for K values ranging from 1 to 25, by delta K (ΔK) values.

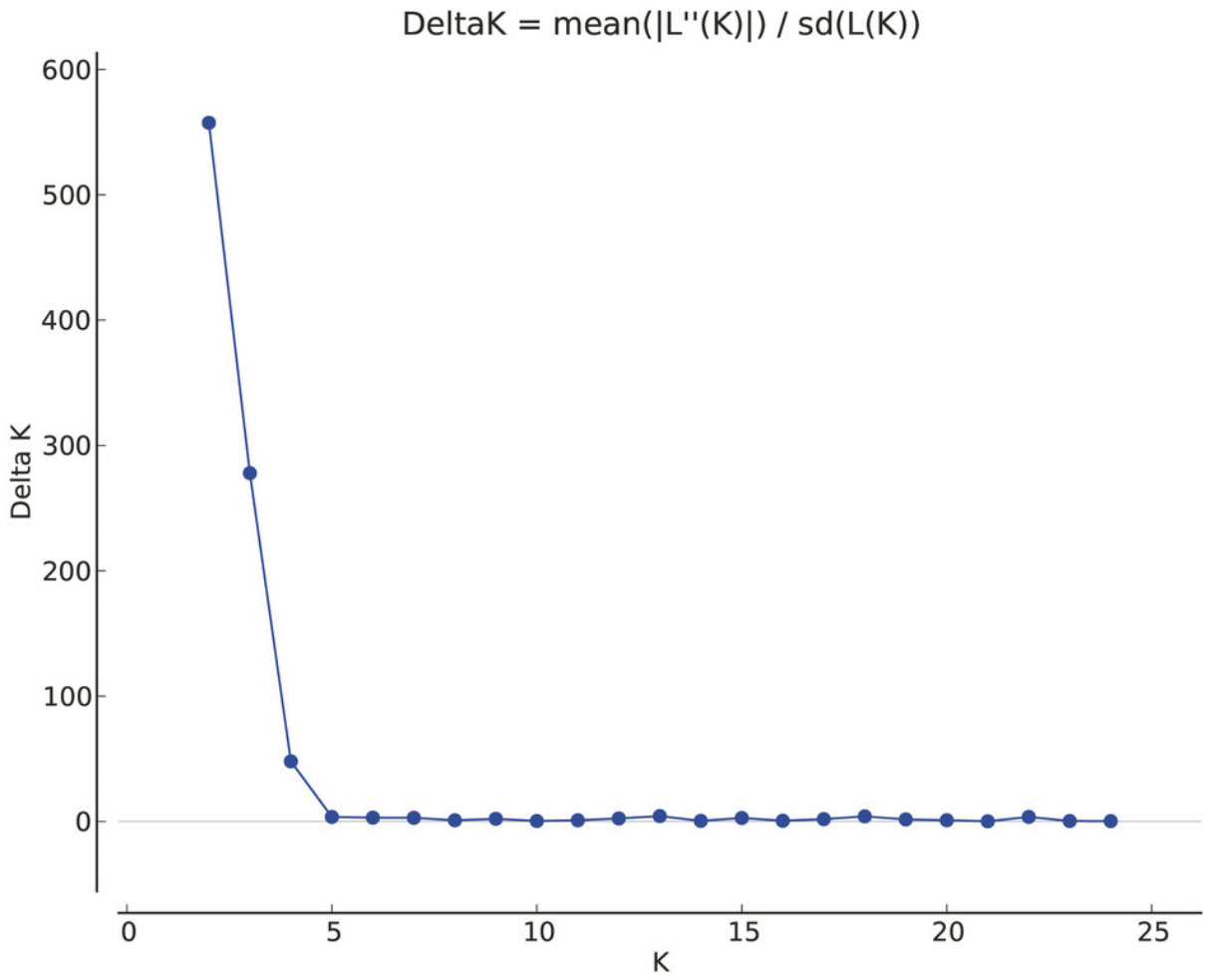

Figure 4

Population structure (K = 2) of 140 accessions.

Figure 5

The principal coordinate analysis (PCA) of 140 accessions using ISSR primers.

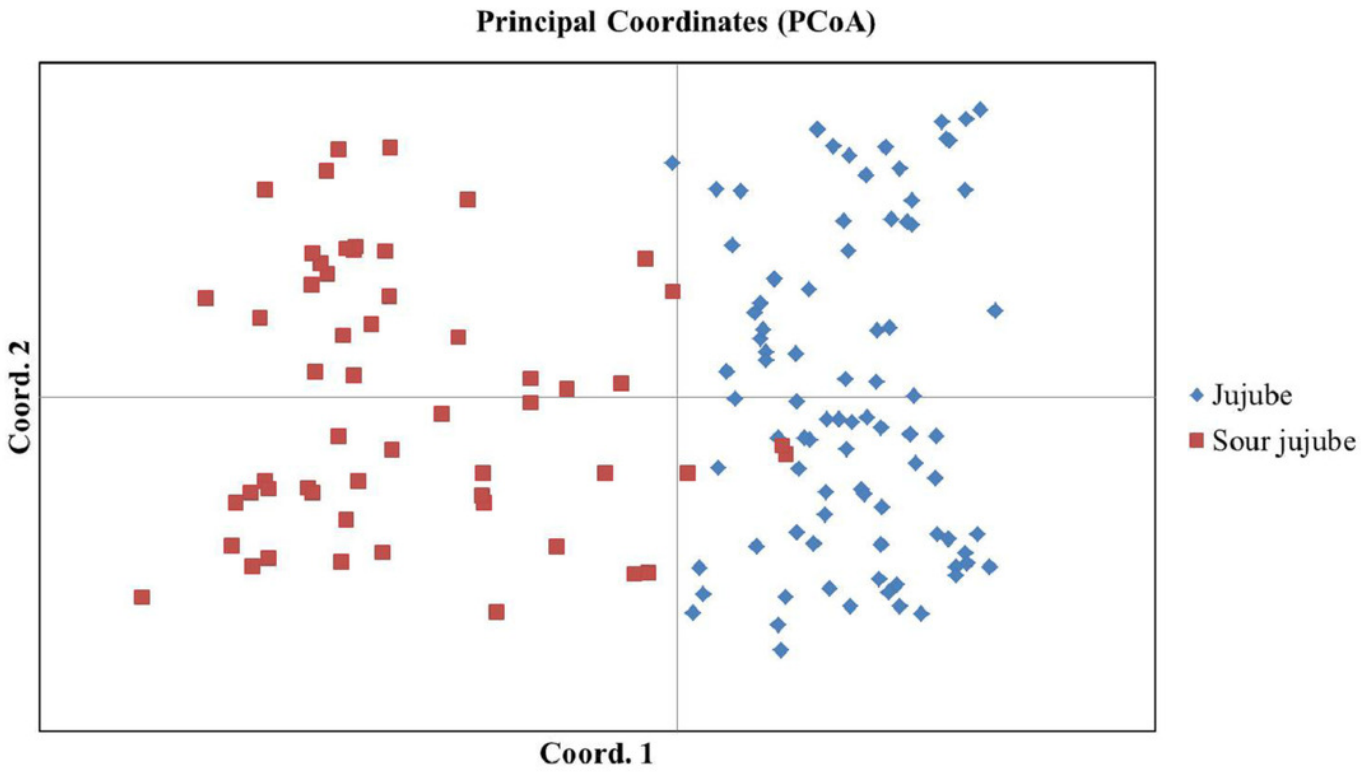

Table 1(on next page)

The 28 ISSR primers selected for this study.

Table 1. The 28 ISSR primers selected for this study

Primer name	Primer	Annealing temperature (°C)	Allele range (bp)	Total no. of bands	No. of polymorphic bands	PIC
ISSR11	GAGAGAGAGAGAGAGAC	50	550-600	2	2	0.684
ISSR13	CTCTCTCTCTCTCTCTT	50	700-800	2	2	0.507
ISSR22	TCTCTCTCTCTCTCTCA	50	700-900	2	1	0.396
ISSR23	TCTCTCTCTCTCTCTCC	50	600-1,000	3	1	0.436
ISSR25	ACACACACACACACACT	50	650-950	5	3	0.771
ISSR27	ACACACACACACACACG	50	450-950	3	2	0.722
ISSR40	AGAGAGAGAGAGAGAGTT	55	500-750	2	1	0.382
ISSR43	AGAGAGAGAGAGAGAGTC	55	350-600	5	3	0.777
ISSR46	AGAGAGAGAGAGAGAGTA	55	400-750	3	2	0.693
ISSR47	AGAGAGAGAGAGAGAGGA	55	550-1,500	4	2	0.678
ISSR48	AGAGAGAGAGAGAGAGCA	55	350-1,500	3	1	0.426
ISSR55	GAGAGAGAGAGAGAGATT	55	200-400	2	2	0.639
ISSR57	GAGAGAGAGAGAGAGACT	55	200-350	2	1	0.311
ISSR60	GAGAGAGAGAGAGAGACC	55	200-500	6	1	0.235
ISSR63	GAGAGAGAGAGAGAGACG	55	250-600	4	1	0.414
ISSR66	CTCTCTCTCTCTCTCTAC	55	550-700	2	2	0.64
ISSR68	CTCTCTCTCTCTCTCTAG	55	600-1,500	2	1	0.414
ISSR69	CTCTCTCTCTCTCTCTGG	55	250-500	5	1	0.235
ISSR81	GTGTGTGTGTGTGTGTCC	55	200-1,500	5	1	0.467
ISSR82	GTGTGTGTGTGTGTGTTG	55	300-1,000	3	1	0.275
ISSR88	TCTCTCTCTCTCTCTCGT	55	300-1,000	3	1	0.168
ISSR89	TCTCTCTCTCTCTCTCAG	55	300-700	6	2	0.629
ISSR95	ACACACACACACACACGA	55	600-1,500	2	1	0.402
ISSR103	TGTGTGTGTGTGTGTGGC	55	400-500	2	1	0.488
ISSR105	TGTGTGTGTGTGTGTGGA	55	400-700	2	1	0.496
ISSR121	GATAGATAGACAGACA	50	350-750	3	2	0.733

ISSR124	CTTCACTTCACTTCA	50	400-750	3	2	0.628
ISSR126	GGGTGGGGTGGGGTG	55	550-700	3	1	0.467
Total				89	42	
Average				3.19	1.5	0.504

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