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A GENOME-SCALE MINING OF SINGLE-COPY NUCLEAR GENE MARKERS FOR *ZIZIPHUS JUJUBA* VAR. *SPINOSA* AND IMPLICATIONS FOR GENETIC STUDY

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Abstract

Nowadays, the breeding of *Ziziphus jujuba* encounters substantial difficulties in cultivation process. The wild relative of jujube, *Z. jujuba* var. *spinosa* (also called sour jujube), has long been treated as the important gene pool for the genetic breeding of cultivated jujube. However, the mining of important functional genes in this significant lineage has been seldom manipulated due to the lack of information about genetic diversity and evolutionary history. In the present study, a novel set of single-copy nuclear gene markers were developed and characterized. Thirteen single-copy nuclear gene markers were developed following a genome-wide scanning of jujube genome. The single-copy nuclear gene markers showed relatively high level of nucleotide variation (π= 0.00513, θ_w= 0.00531). None of the results of neutrally tests showed significant difference, indicating that all the markers conformed to the neutral evolution model. STRUCTURE and phylogenetic analysis showed admixture of these two lineages, supporting the domestication of cultivated jujube from sour jujube. The single-copy nuclear gene markers are powerful for genetic study of *Z. jujuba* var. *spinosa* and provide useful genetic information for future protection and conservation management of this important lineage. Furthermore, these markers could be useful for future population genetics, phylogeney and phylogeographic research in *Ziziphus*, even in Rhamnaceae.

Key words: Single-copy nuclear gene marker; *Ziziphus*; Nucleotide variation; Phylogeney.

Introduction

Jujube (*Ziziphus jujuba* Mill.) is not only an important member of family Rhamnaceae, but also a traditional herbal medicine and a dry fruit for more than one billion people around the world (Liu *et al.*, 2014). Recently, the draft genomic sequence of two jujube cultivars, `Dongzao' and `Junzao', were reported, which provided valuable genomic resource for jujube genetic improvement and offered great insight into jujube and fruit genome evolution (Huang *et al.*, 2016; Liu *et al.*, 2014). Nowadays, the breeding of jujube encounters substantial difficulties, such as the creation of hybrid progeny using conventional artificial hybridization and the selection of germplasms with high resistance to fruit cracking and various biotic/abiotic stress (Yan *et al.*, 2018; Yuan *et al.*, 2013). The wild relative of jujube, *Z. jujuba* var. *spinosa* (also called sour jujube), from which cultivated jujube was domesticated, harbors high level of economic and ecological values (Huang *et al.*, 2016). Furthermore, the resistance of *Z. jujuba* var. *spinosa* to various environmental stress is relatively high (Huang *et al.*, 2015). *Z. jujuba* var. *spinosa* has long been treated as the important gene pool for the genetic breeding of cultivated jujube (Liu *et al*., 2015). However, in jujube cultivation, sour jujube is only used as rootstock and directly transplanted from natural populations (Sun *et al.*, 2006). Until now, the mining of functional genes in this important germplasm resource has been seldom manipulated, partly due to that some basic issues concerning this lineage such as the genetic diversity and evolutionary history are still yet to be clarified.

The development and utility of various molecular markers have advanced researches concerning genetic diversity and evolutionary history (Zeng *et al*., 2010). Traditionally, some basic molecular markers such as nAFLPs and nSSRs were used to infer interspecific relationships and population history (Cervera *et al*., 2005; Heuertz *et al.*, 2004). However, the low resolution of these markers and the low level of intra-/interspecific divergence hinder the application of these methods in relative research. Some ribosomal DNA sequences (ITS, internal transcribed spacer) were also generally used (Hamzeh & Dayanandan, 2004; Wang *et al.*, 2015). Nevertheless, the low evolution rate and the lack of significant intra-/interspecific difference among closely related lineages or species may probably result in the failure of tracking population evolutionary history (Chen *et al.*, 2017). Recently, a novel set of single-copy nuclear gene markers were developed and applied in some population genetic studies of closely related or lately diverged species or lineages (Chen *et al.*, 2017; Du *et al.*, 2015; Du *et al.*, 2014). With the characteristics of containing a series of linked SNPs and no lineage sorting, the utility of these markers have further facilitated our understanding on the pattern of plant speciation and adaptive evolution (Curto *et al.*, 2012; Liu *et al.*, 2016). In this study, we developed a novel set of single-copy nuclear gene markers for *Z. jujuba* var. *spinosa* based on the genomic sequence of *Z. jujuba*. Furthermore, the application of these markers in population genetics and phylogeney was conducted. The development and further application of these single-copy nuclear gene markers will facilitate the phylogeography, phylogeny and population genetic study of *Ziziphus* and put a solid genetic foundation for application of protection procedures.

Materials and Methods

Materials: For marker development, ten *Z. jujuba* var. *spinosa* individuals distributed in two distant populations (5 individuals in each population) and 5 cultivated jujube individuals ('Hupingzao') were collected. Furthermore, one *Z. mauritiana* individual was collected and used as outgroup in following analysis (Table 1). The collected individuals in the same population were at least 50 m apart. Total genomic DNA of all sampled individuals were extracted from silica gel-dried leaves using the modified CTAB method (Doyle, 1987).

Table 1. The collected samples in this study.

Lineage	Population	Location	Latitude and longitude	Altitude (m asl)
Z. jujuba var. *spinosa*	TA	Taian, Shandong province	36.20°N 117.09°E	167
	JZ	Jinzhong, Shanxi province	37.70°N 112.71°E	912
Z. jujuba	TY	Taiyuan, Shanxi province	37.87°N 112.55°E	788
Z. mauritiana	GZ	Guangzhou, Guangdong province	23.13°N 113.26°E	18

Table 2. The primers for the single-copy gene markers developed in this study.

Locus	Primer sequences (5'-3')	Gene annotation	Ta (℃)
DSH2	F:TGGTACAGGATCTACAATTC R:CCTGACTTTCTAATTGCTTC	myb-like protein X	52
DSH11	F:ATGGCTTTTGCTTGCCTCTC R:GTGCATTCGGGTCATCAATG	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase MurE homolog	53
DSX2	F:GCTACTCGCTCTGGTTTCCAT R:GAAGAATCCTTGCCGGTTCAG	trichohyalin	54
DSX5	F:CAAATGGAAGCGGCCTAGTG R:GCATATGCCAAATGGGGTCC	DNA repair endonuclease UVH1	56
DSX7	F:GTCAGAAAGGCGCTTACAAAG R:GCTGTCAAGTTGGTGGTCAAT	pentatricopeptide repeat-containing protein	55
DSX8	F:TATTGAAGCCGCGCAAGTCAT R:ACTAAAGGAGGATGCAGTGGA	probable rRNA-processing protein EBP2 homolog	54
DSX11	F:TCACATCTTCTCGCCACCAAA R:ATCAAAGAAGTGCAACTGTAAATGC	50S ribosomal protein L4	53
DSX12	F:GCCCTTTCGCAAAGCTTTCTT R:CCCAACACTGAGATTACTGGAG	transcription termination factor MTEF18	54
DSX13	F:AAATGGAAGCGGCCTAGTGA R:TCCAGGAGTTTCCTCAGAGTC	DNA repair endonuclease UVH1	54
DSX14	F:CACTCCACTGCTCCTTCTCA R:GCTTTTCGGCTTCGGGTTGT	uncharacterized	52
DSX15	F:TCCCCTGACCAGAAAACCCT R:TCCCAAACCCGTATAACCCC	p21-activated protein kinase-interacting protein 1-like	55
DSX16	F:CTATGCCTTCAGCTTGCCAC R:GAGGGGCATCCTCACTTGTT	F-box/WD-40 repeat-containing protein At5g21040	59
DSX17	F:ACCTCGTGAAGTCAATCGGAG R:TCCCACCACGGTATACATCTTC	AP-4 complex subunit epsilon (LOC107431115), transcript variant X3	58

Single-copy gene mining and primer design: We firstly searched GENE (http://www.ncbi.nlm.nih.gov/gene) with the single-copy gene tags provided by Duarte *et al*., (2010), then turned to the KEGG page to obtain the nucleotide sequence of the single-copy gene. Afterwards, we ran the nucleotide BLAST against the *Z. jujuba* genomic sequence (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=OGP__326968__182558) with the nucleotide sequence of the single-copy gene as query sequence and with the option of "somewhat similar sequence". The length of the sequence ranging from 500- 1000 bp with the lowest E-value was taken as the final reference sequence and Primer Premier 5.0 (Premier Biosoft International, Silicon Valley, CA, USA) was employed to design primers for the reference sequence. All the primers developed in this study were listed in Table 2.

PCR amplification and sequencing: PCR was performed in a volume of 30 uL containing 10 to 15 ng genomic DNA, 2.4 μM of each primer, 0.8 μM of each dNTP, 2.0 mM $MgCl_2$, and 0.15 U ex Taq DNA polymerase (TaKaRa, Shiga, Japan). Amplifications were carried out in a temperature gradient 96 U thermocycler (Eppendorf, Germany) as follows: 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 52° to 59°C (based on the annealing temperature of specific primer pair), 90 s at 72°C, and a final extension at 72°C for 10 min. PCR product was examined on 0.8% agarose gel by electrophoresis. Afterwards, PCR product was purified using a DNA Purification kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The purified DNA was sequenced using an ABI 3730 DNA analyzer (Applied Biosystems). The same primers were utilized in amplification and sequencing.

Data analysis

Assembled contigs of each sample were aligned in Clustal X (Thompson *et al.*, 1997) and refined manually with BioEdit (Hall, 1999). The number of haplotypes (H) and segregating sites (S), haplotype diversity (H_d), nucleotide variation parameters, Watterson's θ_w (Watterson, 1975) and π (Nei, 1987), and the minimum number of recombination events (R_m) were analyzed for

each single-copy nuclear gene marker. Tajima's D (Tajima, 1989), Fu and Li's D^* and F^* (Fu & Li 1993) were also calculated for each markerto test whether the data conformed to the neutral evolution model. All the above parameters were analyzed using DNASP 5.10.0 (Librado & Rozas, 2009).

The phylogenetic tree containing all the individuals was estimated in Mrbayes 3.2.1 (Ronquist & Huelsenbeck, 2003). All markers were concatenated and used for analysis. Two independent Metropolis-coupled Markov chain Monte Carlo (MCMC) runs were simultaneously conducted, with each run comprising of one cold chain and three incrementally heated chains. Both runs started randomly in the parameter space. All the other parameters were set to default value. 2,000,000 generations were run and trees were sampled once every 1,000 generations. The program Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer) was utilized to check for stationary. The first 25% of sampled trees were discarded as burn-in and the posterior probabilities were calculated from the remaining trees. The phylogenetic tree was visualized in FigTree v 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

To test the application of the single-copy nuclear gene markers in clustering individuals from populations, data from all individuals were analyzed using STRUCTURE v2.3.4 (Pritchard *et al.,* 2000). An admixture model was utilized and correlated allele frequencies among populations were assumed. Twenty runs were performed at K= 2 (two clusters) with a burn-in of 15,000 followed by 15,000,000 iterations of data. Graphical display was handled with DISTRUCT 1.1 (Rosenberg, 2004).

Results

Nucleotide diversity and neutrally test: Aligned sequence of the single-copy nuclear gene markers ranged from 295 to 887 bp, with a concatenated length of 9806 bp. The locus DSX2 contained the most segregating sites and haplotypes (S=40, H=21) while DSX11 contained the least. Two important nucleotide diversity parameters, π and θ_w varied between 0.00073 and 0.01220, 0.00105 and 0.01209, with mean values of 0.00513 and 0.00531, respectively. Rm varied between 0 and 9, with a mean value of 3. None of the results of neutral tests showed significance, indicating that all the loci conformed to the neutral evolution model (Table 3).

Phylogenetic and genetic structure analysis: The phylogenetic tree constructed in this study using Mrbayes3.2.1 illustrated that all the individuals clustered in a clade, in which all the jujube and sour jujube individuals clustered in a single branch with relatively high posterior probability and the sour jujube individuals occupied basal and terminal positions of this branch (Fig. 1). Result of STRUCTURE analysis also supported the genetic admixture between these two closely related lineages (Fig. 2).

Table 3. The nucleotide diversity and neutral test of the single-copy nuclear gene markers.

Locus	L	S	H	H_d	π	θ_w	Rm	D	D^*	F^*	GenBank no.
DSH2	295	6	5	0.568	0.00494	0.00573	0	-0.43966	-0.1542	-0.2709	MK443561-MK443575
DSH11	887	19	13	0.851	0.00525	0.00542	2	-0.2775	-0.24248	-0.29826	MK443576-MK443590
DSX2	842	40	21	0.931	0.00979	0.01209	6	-1.03938	-0.03842	-0.43285	MK443601-MK443615
DSX5	671	20	19	0.959	0.00786	0.00752	4	-0.01649	-0.45410	-0.36971	MK443616-MK443630
DSX7	626	25	13	0.940	0.01220	0.01008	9	-0.74692	-1.68749	-1.63031	MK585408-MK585422
DSX8	780	4	5	0.685	0.00146	0.00131	0	0.30276	0.04507	0.13959	MK585423-MK585437
DSX11	739	3	4	0.469	0.00073	0.00105	0	-0.68160	-1.47512	-1.44395	MK585438-MK585452
DSX12	875	17	14	0.933	0.00524	0.00490	3	0.23343	0.86902	0.78456	MK443631-MK443645
DSX13	738	22	10	0.841	0.00682	0.00752	1	-0.33156	-0.93761	-0.87483	MK443646-MK443660
DSX14	830	23	13	0.830	0.00706	0.00700	6	0.03013	-0.55318	-0.43098	MK443661-MK443675
DSX15	839	9	8	0.657	0.00175	0.00271	2	-1.09504	-0.81375	-0.69793	MK585453-MK585467
DSX16	878	11	7	0.786	0.00273	0.00316	1	-0.44453	-0.56511	-0.61787	-MK651046-MK651060
DSX17	806	2	4	0.609	0.00090	0.00063	1	0.88047	0.80615	0.95514	-MK654061-MK651075
mean	754	16	11	0.7738	0.00513	0.00531	3	-	-	-	-

Fig. 1. The phylogenetic tree constructed using Bayesian inference with concatenated single-copy nuclear gene markers. Numbers next to nodes indicated posterior probability.

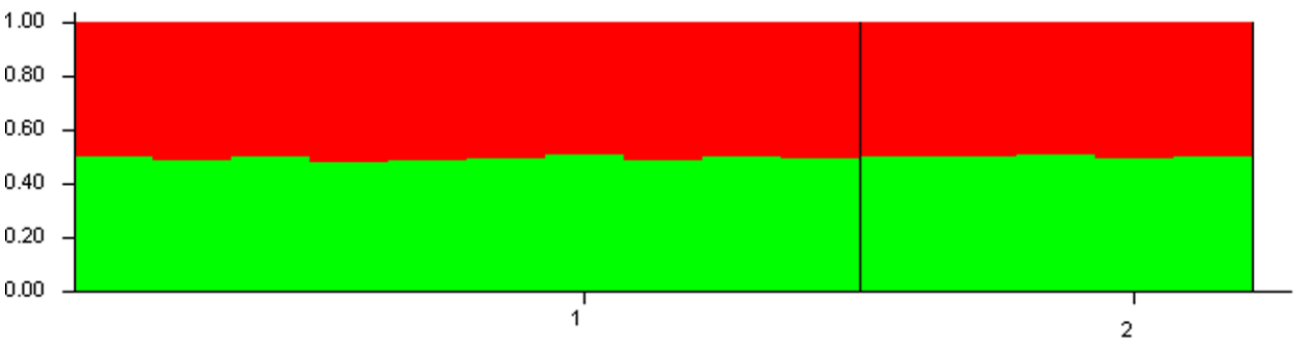

Fig. 2. STRUCTURE result when K=2. 1 indicated the sour jujube population and 2 indicated the cultivated jujube population.

Discussion

Reconstruction of taxon evolutionary history is one of the major issues of modern historical biogeographic research. However, the discontinuous geographical distribution and adaptation to various climatic conditions can result in genetically diverse strains within the same species or lineage (Leydet *et al.*, 2018). To our knowledge, there has been no population historical study of *Z. Jujuba* var. *spinosa* so far, partly due to the lack of appropriate molecular markers. Although some nSSRs markers have been developed and applied in genetic diversity research (Huang *et al*., 2015; Zhang *et al.*, 2015), relatively restricted sample areas and inappropriate method resulted in no population dynamic investigation.

The intrinsic characteristics of single-copy genes directly lead to difficulties in isolating and characterizing them. Gene duplication and/or high sequence variability may contribute to the difficulties in single-copy gene marker development. For example, among the 566 primer pairs of single-copy nuclear gene markers of Chinese white oaks, only 19 markers could produce orthologous products with moderate polymorphism (Chen *et al.*, 2017), which partly resulted from whole genome duplication or independent gene duplication events in oaks (Salse, 2012). Although no recent whole-genome duplication event occurred in jujube, it underwent frequent segmental duplication and inter-chromosome fusions (Liu *et al.*, 2014). During marker development, it was found that some query sequences blasted more than one sequence in the jujube genome and/or the result sequence of jujube was too short to be applicable for primer design (<200 bp) (data not shown). Furthermore, among the single-copy nuclear gene markers that can be amplified and sequenced, some of the results were too low to be analyzed because of frequent insertion and/or deletion (indels) within the sequences. So among all the single-copy genes we searched in the jujube genome, until now only 13 single-copy nuclear gene markers were developed and produced orthologous products. It has been estimated that in plant genome, about 10% of all the genes are single-copy, resulting from a detrimental dosage effect after duplication (Duarte *et al.*, 2010). So among the 32,808 genes annotated in jujube genome, there would be about 3,000 single-copy genes in general (Liu *et al.*, 2014). So it will be possible to develop more sing-copy nuclear gene markers and apply in relative study of this important lineage.

The genetic diversity and structure of *Z. jujuba* var. *spinosa* has been investigated by employing molecular markers, mainly based on nSSRs markers. For example, Zhang *et al*., (2015) revealed high level of genetic diversity (H_E=0.659 and H_S=0.674) and moderate differentiation (F_{ST}=0.091) among populations within this lineage. This may likely be attributed to its wide geographical distribution, various modes of reproduction that can accumulate more mutations in the genome (Zhang *et al*., 2015). In the present study, the nucleotide variation of sour jujube was moderately high, with π=0.00513 and θ_w=0.00531, respectively, which were similar to or higher than those of some deciduous tree species (Chen *et al.*, 2017; Du *et al.*, 2015). However, the number of individuals analyzed in this study is relatively restricted, the nucleotide variation of this lineage will be investigated after sampling more individuals covering the whole distribution range.

The taxonomic classification of *Z. jujuba* var. *spinosa* has been controversial based on various analysis. It has been inferred that sour jujube should be classified as a *varietas* (Wu, 1982). Another investigations suggested that sour jujube should be classified separately from cultivated jujube and as a single species, *Z. acidojujuba* (Huang *et al.*, 2017; Yan *et al.*, 2018; Zhang *et al*., 2015). Or the cultivated jujube was domesticated from sour jujube and it should be classified as a *varietas* (Peng, 1991; Song, 1999). In the present study, the sour jujube and cultivated jujube individuals did not show significant genetic differentiation in STRUCTURE analysis. Furthermore, phylogenetic analysis based on the concatenated single-copy nuclear gene markers illustrated that the branch comprising of all the cultivated jujube individuals occupied the terminal position of the phylogenetic tree with high posterior probability (Fig. 1). The preliminary investigation based on phylogenetic and genetic structure analysis both supported the domestication of cultivated jujube from sour jujube. Phylogenetic analysis based on genomic data also suggested that the two lineages should be classified as a single species and the cultivated jujube was derived from sour jujube, probably by long history of artificial selection and cultivation (Huang *et al*., 2016). The detailed domestication history of sour jujube and the precise functional genes under artificial selection will need further investigations with numerous studies and data. As in the current study, DNA extracted from a narrow range of species of *Ziziphus* were amplified and sequenced, the transferability of these single-copy nuclear gene markers to other *Ziziphus*, even Rhamnaceae species will need to be further testified with more samples in this family.

Z. jujuba var. *spinosa* possesses vital economic and ecological values, for example, kernel is widely used as a traditional medicine in China for about 2000 years (Li *et al.*, 2017). However, compared with other economic forest tree species such as chestnut and walnut, the direct economic benefit of sour jujube is relatively low. Within some distribution areas, some natural sour jujube populations were deforested and replaced by other economic forestry species (Li *et al.*, 2014). Accompanied with increasing human activities, the natural population of this important lineage is continuously decreasing. Therefore, the application of appropriate protection procedure of *Z. jujuba* var. *spinosa* is necessary to some extent. The development and application of the single-copy nuclear gene markers will provide a basic genetic foundation for future protection of this germplasm resource.

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