Research article

The genetic relationships of Indian jujube (Ziziphus mauritiana Lam.) cultivars using SSR markers

Chu-Ying Chiou a,1, Huei-Chuan Shih b,1, Chi-Chu Tsai a,c,1, Xiao-Lei Jin d, Ya-Zhu Ko d, Junaldo A. Mantiquilla d,e, I-Szu Weng b, Yu-Chung Chiang f,

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ABSTRACT

The genetic relationships among 24 Indian jujube cultivars (Ziziphus mauritiana Lam.) were evaluated by genotyping the microsatellite loci using simple sequence repeat (SSR) markers. The SSR loci were scored by fluorescent labelling and automated detection systems for the high-throughput capillary electrophoresis and high-resolution gel electrophoresis. Out of the 29 newly characterized SSR loci, 26 were considered as polymorphic with a total of 181 alleles obtained. The number of alleles ranged from 2–12, while the polymorphism information content ranged from 0.08–0.83, and the expected and observed heterozygosity were 0.04–0.83 and 0.04–0.82, respectively. The allele pattern of Indian jujube for all SSR loci confirmed its karyotype as tetraploid. Similarity coefficients and UPGMA dendrogram revealed that the Taiwanese cultivars consisted of a large ‘A’ clade, which is further divided into ‘A1’ and ‘A2’ groups, and the ‘B’ clade where both are rooted by the wild accession, ‘Chad native’. These four genetic clusters were supported by the results of PCoA and the assignment test. The excess of heterozygotes based on F-statistics was attributed to its mating system as outcrossing and self-incompatible, and the introgression of the presumed mutation-derived cultivars with genetic admixture. Based on this study, SSR markers offer valuable information on the genetic relationship of this tropical fruit tree which is basically in agreement with the genealogy of its breeding history.

1. Introduction

Lesser known fruits are genetically very diverse groups grown in temperate, subtropical and tropical regions and have been recognized for their human health benefits. Most of these fruits have high content of non-nutritive, nutritive, and bioactive compounds such as flavonoids, phenolics, anthocyanins, phenolic acids, as well as nutritive compounds such as sugars, essential oils, carotenoids, vitamins, and minerals [1, 2, 3].

Indian jujube (Ziziphus mauritiana Lam.) is among those less recognized fruits globally. It belongs to the genus Ziziphus of the family Rhamnaceae that includes approximately 86 species found in the tropical and subtropical regions of the northern hemisphere. Chinese jujube (Ziziphus jujube Mill.) and Indian jujube are the most important commercialized species. Basic chromosome numbers of $x = 10$, 12 and 13 have been shown for Ziziphus species [4] of which $2n = 4x = 48$ for Indian jujube [4, 5]. Most of the Indian jujube varieties are tetraploid, but the cultivar ‘Illachi’ is an octaploid ($2n = 8x = 96$) [6]. Indian jujube had been used around 1,000 BC, and it is believed to originate from Central Asia and spread to North Africa, India, South China, Myanmar and Australia [7].

Recently, DNA markers have been widely used to assess genetic relationships and cultivar identification. These molecular markers were used for several Chinese jujube studies [8, 9, 10, 11, 12, 13, 14, 15], but few for Indian jujubes. Among those reported to evaluate genetic relationships, genetic diversity and cultivar identification of several Indian

a Kaohsiung District Agricultural Research and Extension Station, Pingtung 900, Taiwan
b Department of Nursing, Meho University, Pingtung 912, Taiwan
c National Pingtung University of Science and Technology, Pingtung 912, Taiwan
d Department of Biological Sciences, National Sun Yat-sen University, Kaohsiung 804, Taiwan
e Department of Biological Sciences and Environmental Studies, College of Science and Mathematics, University of the Philippines Mindanao, Mintal, Davao City 8022, Philippines
f Department of Biomedical Science and Environment Biology, Kaohsiung Medical University, Kaohsiung 807, Taiwan

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Indian jujube is an economically important crop that is also extensively cultivated in Taiwan [29]. There are several cultivars developed by breeders and farmers which show that the propagation of Indian jujube is incredibly active [29, 30, 31]. These cultivars are also propagated asexually, thus it is difficult to protect the right of the breeder. This study aims to address the needs of understanding the genetic background of Indian jujube cultivars by using molecular markers to effectively establish breeding strategies and legally protect the new cultivars. Specifically, SSR markers were used to investigate the genetic relationship among 24 Indian jujube cultivars in Taiwan.

2. Materials and methods

2.1. Plant materials

Twenty-four Indian jujube cultivars were collected and cultivated at the Kaohsiung District Agricultural Research and Extension Station (KDARES), Taiwan (Table 1). The J1 to J23 cultivars were obtained from the local tribes based on the differences of morphological characteristics from the inventory of tropical fruit trees, and hence, asexually propagated at the KDARES for breeding purposes. The J24 was a direct germplasm exchange from India by scientist cooperation through the Agricultural Technical Cooperation.

| No. | Cultivars  | Voucher  | Origin     | Genetic background |
|-----|------------|----------|------------|--------------------|
| J1  | Kaolang 1  | C. C. Tsai 3101 | Taiwan     | unknown            |
| J2  | Kaolang 2  | C. C. Tsai 3102 | Taiwan     | mutant from Kaolang 1 |
| J3  | Kaolang 3  | C. C. Tsai 3103 | Taiwan     | mutant from Kaolang 1 |
| J4  | Tsueimi     | C. C. Tsai 3104 | Taiwan     | mutant from Kaolang 1 |
| J5  | Mejao       | C. C. Tsai 3105 | Taiwan     | unknown            |
| J6  | Gento       | C. C. Tsai 3106 | Taiwan     | mutant from Mejao  |
| J7  | Gingtao     | C. C. Tsai 3107 | Taiwan     | mutant from Mejao  |
| J8  | Dayeh       | C. C. Tsai 3108 | Taiwan     | mutant from Mejao  |
| J9  | Chungyeh    | C. C. Tsai 3109 | Taiwan     | unknown            |
| J10 | Tsueishiang | C. C. Tsai 3110 | Taiwan     | mutant from Mejao  |
| J11 | Tioumi      | C. C. Tsai 3111 | Taiwan     | unknown            |
| J12 | Kaoshiung 2 | C. C. Tsai 3112 | Taiwan     | unknown            |
| J13 | Kaoshiung 3 | C. C. Tsai 3113 | Taiwan     | unknown            |
| J14 | Kaoshiung 5 | C. C. Tsai 3114 | Taiwan     | unknown            |
| J15 | Yuguan      | C. C. Tsai 3115 | Taiwan     | unknown            |
| J16 | Biyuan      | C. C. Tsai 3116 | Taiwan     | unknown            |
| J17 | Hongyung    | C. C. Tsai 3117 | Taiwan     | unknown            |
| J18 | Hinsuchi    | C. C. Tsai 3118 | Taiwan     | unknown            |
| J19 | Huangguan   | C. C. Tsai 3119 | Taiwan     | unknown            |
| J20 | Telong      | C. C. Tsai 3120 | Taiwan     | unknown            |
| J21 | Roulong     | C. C. Tsai 3121 | Taiwan     | unknown            |
| J22 | Kaoshiung 6 | C. C. Tsai 3122 | Taiwan     | unknown            |
| J23 | Shuem i     | C. C. Tsai 3123 | Taiwan     | unknown            |
| J24 | Chad native | C. C. Tsai 3124 | India      | native species     |

Voucher specimens were deposited at the herbarium of the National Museum of Natural Science, Taiwan (TNM).
The PCR conditions were as follows: total volume 25 ul with 20 ng of template DNA, 1x PCR buffer, 0.2 mM of each dNTP, 0.2 mM of each SSR xu/4 specific primer and 0.25 U Taq DNA polymerase (Promega, Madison, Wisconsin, USA). A two-step PCR amplification was conducted. The first thermocycling profiles included: initial denaturation at 94 °C for 3 min, followed by 20 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C, 40 s extension at 72 °C and a final extension for 7 min at 72 °C. Subsequently, 0.075 mM M13 primer 5'-labelled with IRDye for LI-COR 4300 DNA analyzer was added in this PCR reaction mixture. The second thermocycling profiles were as follows: initial denaturation at 94 °C for 3 min, followed by 10 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C, 40 s extension at 72 °C and a final extension for 7 min at 72 °C. Samples were denatured in the loading dye (10 mg/ml blue dextran in formamide) and separated using 6.5% polyacrylamide gel (19:1, 7 M urea) electrophoresis in a LI-COR 4300 DNA analyzer. Fragment lengths were determined with the aid of an external standard (50–500 bp, GE Healthcare, USA) and an in-house amplified standard allele (Allele Locator 1.03 software; Amersham Biosciences, India). Also, the forward primer of these SSR loci added 18 bp tail sequence (5’-TGTAAACGACGGCCAGT-3’) for the detection by ABI PRISM 3700 DNA Sequencer. These additional sequences are complementary to universal primer M13, which could be labelled with three fluorescent primers: 6-FAM (blue), HEX (green) and NED (yellow). The PCR conditions and thermocycling profiles were the same as enumerated above. The amplification products were analyzed further with the ABI PRISM 3700 DNA Sequencer and coded by using GeneMapper v.3.7 (Applied Biosystems) to confirm the accuracy of the data.

### 2.3. Data analysis

In this study, the degree of polymorphism for the 29 newly characterized SSR loci, including the number of alleles (Nₐ), expected heterozygosity (gene diversity) corrected for sample size (Hₑ), observed heterozygosity (H), the three fixation indices (Fₛ, Fᵣ, and Fₛₑ) of F-statistics [34], the coefficient of gene differentiation (Gₑ) [35] and fixation indices (Rₑ) of R-statistics [36] were calculated using SPAGeDi [37]. To evaluate the selective neutrality of microsatellite markers, the Evens-Watterson neutrality test was performed by Popgene 1.31 (Yeh et al. 1997). The polymorphism information content (PIC) [38] was calculated using PowerMarker version 3.25 [39]. There were three types of test conducted for bottleneck analysis: sign test, standardized difference test and Wilcoxon signed rank test under different mutation models such as Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and Two Phase Model (TPM) using Bottleneck 1.2.02 (Cornuet and Luikart, 1996).

The dissimilar genetic distance between Indian jujube accessions was estimated according to Bowcock et al. (1994) and Ciampolini et al. (1995) [40,41]. This distance was calculated based on the pairwise comparison between individuals causing a multilocus genetic similarity value complementary to the multilocus genetic distance (Dm). It is then converted as the dissimilar genetic distance with 1–Dm. The cluster dendrogram reconstruction was derived from the pairwise dissimilar distance matrix using the unweighted pair-group method (UPGMA) (MEGA version 5.05) [42].

The Principal Coordinates Analysis (PCoA) was carried out to evaluate the relationship among tetraploid Indian jujube cultivars and the genotypic group structures using the Lynch genetic distance matrix [43] with the POLYSAT software [44]. The Bayesian-clustering assignment test in STRUCTURE ver. 2.3.4 [45, 46, 47] was used to estimate the genetic compositions and genotypic group structures that evaluate best for 24 Indian jujube cultivars/lines of their genotypic grouping and degree of genetic admixture. The posterior probability of K from 1 to 16 was estimated by the Markov chain Monte Carlo (MCMC) method using the admixture model [48] in 10,000,000 steps with a 1,000,000-step burn-in for each run. The posterior probability of each grouping number was replicated 20 independent runs to evaluate the consistency of the results. The Delta K (ΔK) method [49] (STRUCTURE HARVESTER v. 0.6.8) [50] was performed to generate the dynamic plot of ΔK and the mean of LnP (K) to evaluate the best fit on the number of grouping. The number of K with the largest ΔK was considered the best fit.

### 3. Results

#### 3.1. Characterization of SSR loci, SSR-PCR products

Twenty-nine newly characterized SSR loci were tested for 24 Indian jujube cultivars after optimization, of which 26 SSR loci were observed as polymorphic and the other 3 were monomorphic (Table 2). Some SSR loci showed either one or two PCR products in each sample. This indicates homogeneity or heterogeneity of the sample, respectively (Figure 1). Other SSR loci had more than two alleles (Figure 2). PCR amplification was used for 29 SSR loci to carry out the genetic diversity analysis of 24 Indian jujube cultivars.

#### 3.2. Genetic diversity of Indian jujube cultivars in Taiwan

For the 26 polymorphic SSR loci, a total of 181 alleles were obtained ranging from 2 to 12 per locus (Table 3) with a mean of 5.24 (Table 3). The Hₑ and Hₑₑ ranged from 0.04 (Zma173) to 0.82 (Zma282) and 0.04 (Zma173) to 0.83 (Zma282) with a mean of 0.48 and 0.41, respectively. Moreover, the PIC value of the locus ranged from 0.08 (Zma30, Zma57, Zma173, and Zma187) to 0.83 (Zma282) with a mean of 0.46 (Table 3). Among the loci, the Zma282 had the most variations at 12 alleles, 0.83 for Hₑ, 0.82 for Hₑₑ and 0.83 for PIC indicating a locus with the highest polymorphism. The three fixation indices, Fₛₗ, Fᵣₗ and Fₛₑₑₗ had ranges from -0.78 to 0.47, -1 to -0.2 and 0 to 0.59, respectively. The corresponding means of the three indices are as follows: -0.17, -0.57 and 0.26, all of which were detected statistically significant. Nevertheless, the Evens-Watterson test indicates that the polymorphic SSR loci were selectively neutral except for Zma73 and Zma282 (Table 4). The results of the bottleneck tests were shown to be significant suggesting that recent bottleneck was shaping these Indian jujube cultivars (Table 5). This was confirmed based on the results of Sign test and Wilcoxon test involving the 24 cultivars as shown by the excess of heterozygosity based on the IAM, TPM, and SMM mutation models (Table 5).

#### 3.3. Cultivar identification and similarity test among the cultivars

For all the 29 SSR markers tested, estimates of all possible pair-wise genetic similarity ranged from 1 to 0.30 with an average of 0.74 (Table 6). Accessions ‘Kaoshiung 6’ and ‘Chad native’ were found to be less similar genetically with a value of 0.30 (Table 6). In contrast, two groups exhibited the highest genetic similarity of 1. First, ‘Kaolang1’, ‘Kaolang2’, ‘Kaolang3’ and ‘Tsueim’ belonged to one group, and the other group consisted of ‘Mejao’, ‘Cento’, ‘Gingtao’, ‘Daych’, ‘Chungyeh’ and ‘Tsueishiang’. The construction of dendrogram was based on UPGMA analysis to evaluate relationship patterns (Figure 3). All cultivars were divided into two major clades and rooted by ‘Chad native’ due to long genetic distance. The ‘A’ clade included 14 cultivars while the ‘B’ clade had 9 cultivars. The ‘A’ clade was further subdivided into ‘A1’ and ‘A2’ groups. However, some cultivars cannot be separated from these groups as a presumed mutation-derived cultivar. For instance, ‘Kaolang 2’, ‘Kaolang 3’ and ‘Tsueim’ were likely derived from ‘Kaolang 1’, while ‘Cento’, ‘Gingtao’, ‘Daych’, ‘Chungyeh’ and ‘Tsueishiang’ from ‘Mejao’.

The PCoA and assignment test were used to reassign the clustering of Indian jujube cultivars without any assumptions from prior classification. PCoA results revealed the separation between the wild ‘Chad native’ and the other 23 cultivars (Figure 4A). Furthermore, the admixture patterns of all domesticated and commercial cultivars in three axes can explain 79.90% of the variation (31.61%, 21.78%, and 21.76% of the first, second and third axis, respectively) (Figure 4A). Excluding the wild accession, three other major groups were separated.
Table 2. Characteristics of the 29 polymorphic microsatellite loci isolated from Indian jujube (T2a, optimized annealing temperature).

| SSR Locus | Primers (5′→3′) | Fluorophore for Li-Cor system | Fluorophore for ABI system | Allelic size (bp) | Repeat Motif | T_a (°C) | GenBank Accession No. |
|-----------|-----------------|------------------------------|------------------------------|------------------|-------------|---------|-----------------------|
| Zma30 F   | ATATTTTGCGCTCTCAACGG | IRDye 6-FAM | (GA)_15 | 325-374 | 58 MG385144 |
| R          | ATGGAAGATGACCGAGGG |                         |                              |                  |             |         |                       |
| Zma32 F   | GTTTTTCCTCTATTGACACG | IRDye HEX | (AG)_26N (CT)_14 (AT)_14 | 222-350 | 58 MG385145 |
| R          | ATGACGAAGATCCCTGAGG |                         |                              |                  |             |         |                       |
| Zma41 F   | GTGAGAAGTTTTGTAACGCCG | IRDye NED | (GA)_23 | 180  | 58 MG385146 |
| R          | GGAGTAATTGAACTGCGAG |                         |                              |                  |             |         |                       |
| Zma51 F   | TGTTTTCTTCTTACGGGCCG | IRDye 6-FAM | (AG)_19 | 290-314 | 58 MG385147 |
| R          | ATGGATGACGGAGCTGACCT |                         |                              |                  |             |         |                       |
| Zma52 F   | TGAATTGAGCGACGGGCTT | IRDye HEX | (AG)_18 | 188-204 | 58 MG385148 |
| R          | TATTTGCTGCCCTGAGTG |                         |                              |                  |             |         |                       |
| Zma57 F   | CCTGACTTCATCTTGTCCTCC | IRDye HEX | (AG)_17 | 196-226 | 58 MG385149 |
| R          | CGCAAGATCCCCAACGTCGT |                         |                              |                  |             |         |                       |
| Zma58 F   | TACCATGTTGCAAGCTCTT | IRDye 6-FAM | (TC)_29 (AC)_4 | 288-310 | 58 MG385150 |
| R          | GAGATGGAAAGCGATGCTGA |                         |                              |                  |             |         |                       |
| Zma59 F   | CAACAAATTGCGCTACTCCT | IRDye NED | (AG)_18 | 165-181 | 58 MG385151 |
| R          | GAGAGAAGAGCGATGCTGT |                         |                              |                  |             |         |                       |
| Zma73 F   | AATTTGATGCTGACGGTGA | IRDye NED | (AG)_27 | 176-200 | 58 MG385152 |
| R          | TTGAACTCAAGGAAAAGAAGG |                         |                              |                  |             |         |                       |
| Zma93 F   | GCCACTTCCTCTACACAGCG | IRDye HEX | (AG)_18 | 233-275 | 58 MG385153 |
| R          | TCGAAATCAGCAATCTGGACA |                         |                              |                  |             |         |                       |
| Zma122 F  | ACCATAAGCCACCCCTGT | IRDye 6-FAM | (AG)_28 | 254-296 | 58 MG385154 |
| R          | CCTGATCCACTGACAGGTT |                         |                              |                  |             |         |                       |
| Zma131 F  | TCTGCTGTAGGGCTAGCTG | IRDye NED | (AG)_22 | 143-174 | 58 MG385155 |
| R          | CCTGCAAACACCGGCTTCC |                         |                              |                  |             |         |                       |
| Zma147 F  | TCTCCTGTAGCGGACAGTG | IRDye HEX | (AG)_25 | 294-265 | 58 MG385156 |
| R          | AAGCGACCTCTGGTGAGTGA |                         |                              |                  |             |         |                       |
| Zma148 F  | GGATGAGAAAGACGCTAAATTC | IRDye HEX | (AG)_29 | 214-250 | 58 MG385157 |
| R          | CGTTGGCAGTAACTAGGTCGA |                         |                              |                  |             |         |                       |
| Zma158 F  | CCGGGAGATTCTCACGGCT | IRDye NED | (AG)_18 | 125-135 | 58 MG385158 |
| R          | TTGCGCCATCAACGCAGGAGG |                         |                              |                  |             |         |                       |
| Zma166 F  | AGAGGCGATCTTCTGGTGGA | IRDye HEX | (AG)_18 | 231-255 | 58 MG385159 |
| R          | TCCAAAGCTCAACGGCTGTTG |                         |                              |                  |             |         |                       |
| Zma167 F  | CGAGCCTTTAGCCAGCTTACTG | IRDye 6-FAM | (AG)_19 | 290-322 | 58 MG385160 |
| R          | TTCCACTTCCCCCAACGAGG |                         |                              |                  |             |         |                       |
| Zma173 F  | AGAGGAAAAACGGGGGTTG | IRDye NED | (AG)_29 | 182-190 | 58 MG385161 |
| R          | CATTATGGCAACGGCGAGAG |                         |                              |                  |             |         |                       |
| Zma180 F  | CGACGCTGTAGCTGTGTTG | IRDye NED | (AG)_24 | 155  | 58 MG385162 |
| R          | GGCGACGTCTGAAGCCACACA |                         |                              |                  |             |         |                       |
| Zma185 F  | TCCCTCTCTGGAATTGCA | IRDye HEX | (AG)_20 | 188-200 | 58 MG385163 |
| R          | TCAAACTCCTCAAAACCCA |                         |                              |                  |             |         |                       |
| Zma187 F  | AAGAGAATCTCCAGCGTCCT | IRDye HEX | (AG)_21 | 194-241 | 58 MG385164 |
| R          | TGTCAATAGGCTGGCTACTCA |                         |                              |                  |             |         |                       |
| Zma202 F  | CATGTGCTCTGTGCTGTTG | IRDye NED | (AG)_19 | 172-182 | 58 MG385165 |
| R          | TAAAAAGGCGCGCCAGGTT |                         |                              |                  |             |         |                       |
| Zma206 F  | AGATCGAAAACAGAGCGGGGA | IRDye NED | (AG)_20 | 156-180 | 58 MG385166 |
| R          | CGAGAACTAGCGAACCTGGCC |                         |                              |                  |             |         |                       |
| Zma258 F  | GTTATAATCAACGCAAAACCCAC | IRDye 6-FAM | (AG)_19 | 270-310 | 58 MG385167 |
| R          | AGTGCTCTGCTTGGTACCACCA |                         |                              |                  |             |         |                       |
| Zma262 F  | TCTAAGGAGCGCTCACA | IRDye NED | (AG)_31 | 166  | 58 MG385168 |
| R          | TCGACAAAAGTGCCAGGAG |                         |                              |                  |             |         |                       |
| Zma264 F  | AAGAGGATAGCTGCTGTTG | IRDye HEX | (AG)_32 | 220-255 | 58 MG385169 |
| R          | CACCGCTCTGCGTACTGGGC |                         |                              |                  |             |         |                       |
| Zma265 F  | AAGAGGTTGGAGGAGAGTG | IRDye HEX | (AG)_17 | 190-212 | 58 MG385170 |
| R          | GCTCTTCTGACCCCAACA |                         |                              |                  |             |         |                       |
| Zma282 F  | AGGGTGTGCTCCTGCTGACCC | IRDye HEX | (AG)_21 | 204-236 | 58 MG385171 |
| R          | GATGCAACCTGGCAGTGGAA |                         |                              |                  |             |         |                       |
| Zma283 F  | CCAATTGTCAAGGGTGGAGACT | IRDye NED | (CT)_10N [32]10 (TG)_7 | 166-190 | 58 MG385172 |
| R          | TCACCTGGCCACATTACAGAC |                         |                              |                  |             |         |                       |
in the PCoA plot (Figure 4B) which is consistent with the dendogram (Figure 3). The ‘A1’ group in UPGMA dendrogram was distributed between second and third quadrants, and ‘A2’ group in the first quadrant, while the ‘B’ group in the fourth quadrant (Figure 4B). Specifically, ‘A1’ group was further divided into two subgroups that distinctly distributed ‘Kaolang1’, ‘Kaolang 2’, ‘Kaolang 3’ and ‘Tsueimi’ in the second quadrant, and ‘Tianmi’, ‘Kaohsiung2’, and ‘Kaohsiung3’ in the third quadrant. Apparently, the best fit for the number of grouping was implied as four with \( \Delta K = 162.292 \) (STRUCTURE HARVESTER v. 0.6.8) [50] based on the 29 microsatellite loci (Figure 5A). When \( K = 4 \), four genetic

### Table 3. The summary of genetic variation and fixation index based on 29 SSR loci for all strains of *Ziziphus mauritiana* Lam. calculated by SPAGeDi which include mean of \( N_a \) (number of alleles), \( H_e \) (expected heterozygosity, corrected for sample size), \( H \) (observed heterozygosity), \( F_{ST}, F_{IS}, F_{ST}, G_{ST}, R_{ST}, \) and PIC (polymorphism information content) by PowerMarker.

| Locus   | \( N_a \) | \( H_e \) | \( H \) | \( F_{ST} \) | \( F_{IS} \) | \( F_{ST} \) | \( G_{ST} \) | \( R_{ST} \) | PIC   |
|---------|---------|---------|---------|----------|----------|----------|--------|--------|-----|
| Zma30   | 2       | 0.05    | 0.06    | 0.43*    | -0.20*   | 0.52*    | 0.25   | 0.69   | 0.08|
| Zma32   | 10      | 0.79    | 0.78    | -0.27*   | -0.33*   | 0.05*    | -0.21  | 0.22   | 0.78|
| Zma41   | 1       | 0.00    | 0.00    | -        | -        | -        | -      | -      | 0.00|
| Zma51   | 5       | 0.37    | 0.38    | 0.41*    | -0.20*   | 0.51*    | -0.25  | 0.50   | 0.63|
| Zma53   | 3       | 0.52    | 0.52    | 0.47*    | -0.26*   | 0.58*    | 0.18   | 0.58   | 0.43|
| Zma57   | 2       | 0.54    | 0.53    | -0.78*   | -1.00*   | 0.11*    | -0.73  | 0.56   | 0.08|
| Zma58   | 9       | 0.74    | 0.74    | -0.15*   | -0.62*   | 0.29*    | -0.10  | 0.23   | 0.80|
| Zma59   | 4       | 0.68    | 0.67    | -0.44*   | -0.50*   | 0.04*    | -0.34  | 0.02   | 0.22|
| Zma73   | 3       | 0.62    | 0.62    | -0.20*   | -0.20*   | 0.00*    | -0.19  | 0.01   | 0.15|
| Zma93   | 6       | 0.42    | 0.42    | 0.10*    | -0.32*   | 0.32*    | -0.02  | 0.15   | 0.73|
| Zma122  | 7       | 0.68    | 0.67    | -0.26*   | -1.00*   | 0.37*    | -0.22  | 0.45   | 0.64|
| Zma131  | 6       | 0.64    | 0.63    | -0.30*   | -1.00*   | 0.35*    | -0.29  | 0.44   | 0.70|
| Zma147  | 4       | 0.71    | 0.71    | -0.24*   | -0.25*   | 0.01*    | -0.17  | 0.00   | 0.28|
| Zma148  | 8       | 0.73    | 0.73    | -0.14*   | -0.62*   | 0.29*    | -0.25  | 0.51   | 0.76|
| Zma158  | 8       | 0.57    | 0.56    | -0.17*   | -1.00*   | 0.41*    | -0.40  | 0.58   | 0.79|
| Zma166  | 9       | 0.75    | 0.74    | -0.12*   | -0.33*   | 0.16*    | -0.17  | 0.20   | 0.74|
| Zma167  | 10      | 0.80    | 0.80    | 0.00*    | -1.00*   | 0.50*    | -0.09  | 0.39   | 0.82|
| Zma173  | 2       | 0.04    | 0.04    | 0.43*    | -0.20*   | 0.52*    | 0.00   | 0.52   | 0.08|
| Zma180  | 1       | 0.00    | 0.00    | -        | -        | -        | -      | -      | 0.00|
| Zma185  | 7       | 0.73    | 0.73    | 0.08*    | -1.00*   | 0.54*    | -0.08  | 0.32   | 0.70|
| Zma187  | 2       | 0.69    | 0.68    | -0.44*   | -0.50*   | 0.04*    | -0.39  | 0.07   | 0.08|
| Zma202  | 4       | 0.62    | 0.62    | -0.34*   | -0.33*   | 0.00*    | -0.33  | -0.02  | 0.34|
| Zma206  | 3       | 0.57    | 0.56    | -0.71*   | -1.00*   | 0.14*    | -0.60  | 0.58   | 0.15|
| Zma258  | 6       | 0.73    | 0.73    | -0.21*   | -0.38*   | 0.12*    | -0.27  | 0.12   | 0.67|
| Zma262  | 1       | 0.00    | 0.00    | -        | -        | -        | -      | -      | 0.00|
| Zma264  | 5       | 0.61    | 0.61    | 0.17*    | -1.00*   | 0.59*    | -0.09  | 0.41   | 0.49|
| Zma265  | 8       | 0.64    | 0.64    | 0.21*    | -0.40*   | 0.43*    | -0.20  | 0.45   | 0.79|
| Zma282  | 12      | 0.83    | 0.82    | -0.21*   | -0.33*   | 0.09*    | -0.19  | 0.02   | 0.83|
| Zma283  | 4       | 0.61    | 0.61    | 0.02*    | -1.00*   | 0.51*    | -0.24  | 0.37   | 0.48|
| All loci| 5.24    | 0.41    | 0.48    | -0.17*   | -0.57*   | 0.26*    | -0.23  | 0.24   | 0.46|

Note: Asterisks indicate statistical significance (\( p < 0.05 \)).

\( N_a \) number of alleles, \( N_e \) number of effective alleles, \( H_e \) expected heterozygosity, PIC polymorphism information content.
compositions were calculated using the Bayesian-clustering method. Hence, the 24 Indian jujube cultivars were separated into four groups (Figure 5B) based on four different compositions (Table 7). This is consistent with the results of UPGMA dendrogram and PCoA plot except for 'Tianmi', 'Kaohsiung2', 'Kaohsiung3' and 'Roulong'. Along with 'Shuemi', these five cultivars were detected to have a genetic admixture of two compositions (Figure 5B) suggesting a possible hybrid origin [24, 48].

Table 4. The Ewens-Watterson test for the selective neutrality of 29 SSR loci by using Popgene.

|       | Obs. F | SE  | L95  | U95  |
|-------|--------|-----|------|------|
| Zma30 | 0.93   | 0.04| 0.38 | 0.98 |
| Zma32 | 0.22   | 0.03| 0.22 | 0.81 |
| Zma41 | 1.00   |     |      |      |
| Zma51 | 0.34   | 0.03| 0.24 | 0.87 |
| Zma53 | 0.62   | 0.03| 0.29 | 0.92 |
| Zma57 | 0.66   | 0.03| 0.25 | 0.86 |
| Zma58 | 0.37   | 0.03| 0.23 | 0.84 |
| Zma59 | 0.43   | 0.03| 0.28 | 0.93 |
| Zma73 | 0.17*  | 0.03| 0.23 | 0.82 |
| Zma93 | 0.29   | 0.02| 0.20 | 0.75 |
| Zma122| 0.40   | 0.02| 0.22 | 0.81 |
| Zma131| 0.53   | 0.03| 0.26 | 0.85 |
| Zma147| 0.22   | 0.04| 0.28 | 0.93 |
| Zma148| 0.33   | 0.02| 0.24 | 0.81 |
| Zma158| 0.34   | 0.03| 0.26 | 0.87 |
| Zma166| 0.40   | 0.03| 0.29 | 0.92 |
| Zma167| 0.26   | 0.03| 0.25 | 0.88 |
| Zma173| 0.99   | 0.03| 0.50 | 0.99 |
| Zma180| 1.00   |     |      |      |
| Zma185| 0.34   | 0.03| 0.30 | 0.93 |
| Zma187| 0.46   | 0.02| 0.24 | 0.81 |
| Zma202| 0.32   | 0.04| 0.32 | 0.95 |
| Zma206| 0.66   | 0.03| 0.24 | 0.86 |
| Zma258| 0.45   | 0.02| 0.20 | 0.77 |
| Zma262| 1.00   |     |      |      |
| Zma264| 0.63   | 0.03| 0.25 | 0.87 |
| Zma265| 0.27   | 0.02| 0.22 | 0.81 |
| Zma282| 0.20*  | 0.03| 0.25 | 0.88 |
| Zma283| 0.68   | 0.04| 0.38 | 0.99 |

These statistics were calculated using 1000 simulated samples. Obs. F - Observed sum of the square of allelic frequency. SE- Standard error of the mean. L95- Lower 95% confidence limit. U95- Upper 95% confidence limit. * p < 0.05.

Table 5. Tests for bottleneck under three microsatellite mutation models.

|       | IAM           | TPM           | SMM           |
|-------|---------------|---------------|---------------|
| Sign Test |               |               |               |
| He/He  | 14.5/26*      | 15.06/26*     | 15.29/26*     |
| P      | 0.22          | 0.41          | 0.00          |
| Standardized |             |               |               |
| T2     | 2.48          | -0.67         | -7.43         |
| P      | 0.01          | 0.25          | 0.00          |
| Wilcoxon test |           |               |               |
| F (one tail for H deficiency) | 0.99 | 0.38 | 0.00 |
| P (one tail for H excess)     | 0.01* | 0.63* | 0.99* |

IAM = infinite allele model; TPM = two-phase model; SMM = stepwise mutation model; Sign Test = number of loci with heterozygosity excess; Wilcoxon test = Wilcoxon rank test with probability of heterozygosity excess; Ho/He = observed and expected number of loci with heterozygosity excess under the infinite allele model (IAM), the two-phase model (TPM), and the stepwise mutation model (SMM); P = probability. H = heterozygosity. * p < 0.05.
4. Discussion

Chiou et al. (2012) selected 14 higher polymorphic SSR loci [Zma-25, Zma-29, Zma-107, Zma-161, Zma-168, Zma-181 (Figure 1), Zma-182, Zma-189, Zma-192, Zma-210, Zma-230, Zma-236, Zma-257, and Zma-279] as the standard polymorphic molecular markers to detect genetic variation and reconstruct the genetic relationship of the 24 Indian jujube cultivars [28]. In this study, however, 26 out of the 29 newly characterized SSR loci were found to be polymorphic and consequently selected to evaluate the genetic relationship of these cultivars. Although the stutter bands of PCR products were resolved [52, 53], most SSR loci (89.66%) (26/29) were observed to have more than two alleles. This result confirmed the karyotype of Indian jujube as tetraploid (2n = 4x = 48) [4,5]. Besides, SSR loci duplication also shows more than two bands in PCR products which can be a source of inaccuracy. Other possible causes of stutter bands in SSR-PCR products are commonly found among dinucleotide repeat units particularly those with larger repeat numbers [52, 54]. The stutter products are results from the slippage of DNA polymerase which is a natural process for SSR mutations [51]. It is common for SSR markers interfered with a high ratio of stutter products, especially in polyploid plants [55]. However, most stutter bands were shown to be minor and shorter bands compared to the major bands [56]. Therefore, bands considered as minor and stutter upon careful inspection were not included in the analysis of this study.

The obtained HE values of Indian jujube were closer to other tropical fruit trees such as mango cultivars [27], wax apple [26] and camu-camu [57], but much lower compared to Japanese plum cultivars [58], apple cultivars [59], Tunisian orange [60], Asian pear [61] and Chinese jujube. 

| J1 | J2 | J3 | J4 | J5 | J6 | J7 | J8 | J9 | J10 | J11 | J12 | J13 | J14 | J15 | J16 | J17 | J18 | J19 | J20 | J21 | J22 | J23 | J24 |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| J1 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| J2 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| J3 | 1.00 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| J4 | 1.00 | 1.00 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| J5 | 0.76 | 0.76 | 0.76 | 0.76 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| J6 | 0.76 | 0.76 | 0.76 | 0.76 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |    |    |
| J7 | 0.76 | 0.76 | 0.76 | 0.76 | 1.00 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |    |    |
| J8 | 0.76 | 0.76 | 0.76 | 0.76 | 1.00 | 1.00 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |    |
| J9 | 0.76 | 0.76 | 0.76 | 0.76 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |    |
| J10 | 0.76 | 0.76 | 0.76 | 0.76 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |    |    |    |    |    |    |    |    |
| J11 | 0.83 | 0.83 | 0.83 | 0.83 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 | 0.79 |    |    |    |    |    |    |
| J12 | 0.86 | 0.86 | 0.86 | 0.86 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.78 | 0.86 |    |    |
| J13 | 0.80 | 0.80 | 0.80 | 0.80 | 0.73 | 0.73 | 0.73 | 0.73 | 0.73 | 0.73 | 0.73 | 0.82 | 0.8 |    |
| J14 | 0.73 | 0.73 | 0.73 | 0.73 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.83 | 0.77 | 0.78 |    |
| J15 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.72 | 0.83 | 0.77 | 0.78 | 0.78 |
| J16 | 0.72 | 0.72 | 0.72 | 0.72 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.75 | 0.78 | 0.78 | 0.78 |
| J17 | 0.72 | 0.72 | 0.72 | 0.69 | 0.69 | 0.69 | 0.69 | 0.69 | 0.69 | 0.69 | 0.69 | 0.73 | 0.73 | 0.73 | 0.73 |
| J18 | 0.73 | 0.73 | 0.73 | 0.73 | 0.76 | 0.76 | 0.76 | 0.76 | 0.76 | 0.76 | 0.76 | 0.83 | 0.81 | 0.87 | 0.87 |
| J19 | 0.78 | 0.78 | 0.78 | 0.78 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.85 | 0.85 | 0.84 | 0.88 |
| J20 | 0.72 | 0.72 | 0.72 | 0.72 | 0.76 | 0.76 | 0.76 | 0.76 | 0.76 | 0.76 | 0.76 | 0.84 | 0.82 | 0.77 | 0.74 |
| J21 | 0.66 | 0.66 | 0.66 | 0.66 | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 | 0.71 | 0.72 | 0.72 | 0.69 | 0.69 |
| J22 | 0.76 | 0.76 | 0.76 | 0.76 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 | 0.77 | 0.94 | 0.85 | 0.74 | 0.72 |
| J23 | 0.70 | 0.70 | 0.70 | 0.70 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.74 | 0.68 | 0.69 | 0.78 | 0.77 |
| J24 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.33 | 0.35 | 0.40 | 0.32 | 0.34 |

Figure 3. Dendrogram showing the genetic relationships among 24 Indian jujube cultivars using SSR markers. Scale bar represents the genetic distance.
These fruit cultivars have strong self-incompatibility [62, 63, 64] including Indian jujube [7]. Self-incompatibility is a mechanism for outcrossing that tends to maintain a high degree of heterogeneity in crops [65]. However, these Indian jujube cultivars were shown otherwise relative to other self-incompatible fruit trees likely due to narrow genetic base when first introduced to Taiwan [29]. Several other variants eventually might have risen as cultivated mutants (Table 1) made by artificial selection. Moreover, the results in this study revealed that microsatellite primers were consistent with that of genomic microsatellite studies in mango (0.0–0.756 with a mean of 0.525) [66] and Chinese jujube (0.25–0.88 with a mean of 0.56) [62]. This indicates that SSR as a molecular marker is useful in investigating genetic relationships of Indian jujube cultivars in Taiwan.

The negative values of $F_{IS}$ and $F_{IT}$ would mean an excess of heterozygotes as a result of mating between more distant relatives than the average within subpopulations [67, 68]. These values among domesticated cultivars also suggest outcrossing due to self-incompatibility based on the genetic evidence. The results are likewise in agreement with that of spontaneous mutations where alleles always have identical genetic composition using SSR markers [69, 70, 71]. Nevertheless, several studies demonstrated that retrotransposon markers effectively detect spontaneous mutations [72]. These reports imply that SSR markers are less suitable for these mutations, but are favorable for cultivar identification [24, 25, 27]. Because of this limitation, it is suggested that the commercial cultivars like ‘Kaolang2’, ‘Kaolang3’, ‘Tsueimi’, ‘Cento’, ‘Gingtao’, ‘Daych’, ‘Chungyeh’ and ‘Tsueishiang’ are considered descendants of either ‘Kaolang1’ or ‘Mejao’ by spontaneous mutations.

Indian jujube cultivars were domesticated from wild jujube (Z. rotundifolia) [73, 74] between 1500 BC to 300 AD based on Indian archaeological and literary records [51]. The ability of different taxa such as Z. nummularia, Z. oenoplia, Z. rugosa, Z. sativa, Z. vulgaris and Z. xylopyrus to cross freely had formed Z. rotundifolia that enriched the gene pool of Z. mauritiana by increasing the genetic variations of their adaptability to different environments [51]. Breeders cultivated different varieties and selected descendants based on disease resistance, fruit size and sugar contents [51]. It was introduced from India to Indochina several times and cultivated in Southern Taiwan around 1944 [75]. This shows that the genetic composition of SSR loci might be contributed by various pollen flow resulting to admixture in some cultivated varieties such as ‘Tianmi’, ‘Kaohsiung2’, ‘Kaohsiung3’, ‘Roulung’ and ‘Shuemi’ in Taiwan. Other cultivars that might have been generated by spontaneous mutations include ‘Kaolang1’, ‘Mejao’ and their mutation-derived cultivars that had exactly identical compositions based on SSR markers [69, 70, 71].

The Indian jujube is one of the commercially important fruit crops in Taiwan. Lately, several new cultivated varieties were developed by breeders using both spontaneous mutation technology and the traditional artificial hybridization. The standard identification system of SSR marker is an important method to aid breeders in the improvement of commercial cultivars. In this study, we developed 29 primer sets consisting of 26 polymorphic and 3 monomorphic microsatellite loci from Indian jujube. These SSR markers detected that spontaneous mutations might have risen recently exhibiting identical genetic compositions with their potential progenitor. The cultivated varieties with genetic admixture

Figure 4. The PCoA analysis performed by POLYSAT based on 29 microsatellite loci from 24 strains of Ziziphus mauritiana Lam. (A) The PCoA plot with the Chad-native strain. (B) The PCoA plot without the Chad-native strain.
Figure 5. Plots of (A) the log likelihood and ΔK and (B) the best clustering at K = 4 estimated by the program Structure analysis of the full data set of 24 strains of Ziziphus mauritiana Lam. The vertical lines represent the 24 strains.

Table 7. Genetic compositions of Indian jujube cultivars based on the Bayesian-clustering method.

| Cultivars       | Composition 1 | Composition 2 | Composition 3 | Composition 4 |
|-----------------|---------------|---------------|---------------|---------------|
| Kaolang 1       | 0.997         | 0.001         | 0.001         | 0.001         |
| Kaolang 2       | 0.997         | 0.001         | 0.001         | 0.001         |
| Kaolang 3       | 0.997         | 0.001         | 0.001         | 0.001         |
| Tsueimi         | 0.997         | 0.001         | 0.001         | 0.001         |
| Mejao           | 0.001         | 0.996         | 0.001         | 0.001         |
| Cento           | 0.001         | 0.996         | 0.001         | 0.001         |
| Gingtao         | 0.001         | 0.996         | 0.001         | 0.001         |
| Dayeh           | 0.001         | 0.996         | 0.001         | 0.001         |
| Chungyeh        | 0.001         | 0.996         | 0.001         | 0.001         |
| Tsueishiang     | 0.001         | 0.996         | 0.001         | 0.001         |
| Tianmi          | 0.287         | 0.010         | 0.688         | 0.016         |
| Kaohsiung 2     | 0.252         | 0.004         | 0.670         | 0.074         |
| Kaohsiung 3     | 0.379         | 0.003         | 0.610         | 0.008         |
| Kaohsiung 5     | 0.005         | 0.013         | 0.976         | 0.005         |
| Kaohsiung 6     | 0.064         | 0.004         | 0.849         | 0.082         |
| Yuguans         | 0.010         | 0.066         | 0.918         | 0.006         |
| Biyuan          | 0.002         | 0.002         | 0.992         | 0.004         |
| Hongyung        | 0.002         | 0.002         | 0.992         | 0.003         |
| Hsinuchi        | 0.002         | 0.005         | 0.949         | 0.043         |
| Huangguans      | 0.012         | 0.017         | 0.963         | 0.009         |
| Telongs         | 0.003         | 0.002         | 0.990         | 0.005         |
| Roulongs        | 0.006         | 0.034         | 0.461         | 0.499         |
| Shuemis         | 0.003         | 0.633         | 0.023         | 0.341         |
| Chad native     | 0.001         | 0.001         | 0.001         | 0.998         |
were results of the past hybridization or introgression caused by artificial or natural occurrence. Therefore, the 29 new primer sets for Indian jujube SSR loci reported here are useful for evaluating genetic diversity, developing a standard system for cultivar identification and analyzing lineage of this tropical fruit tree.

Declarations

Author contribution statement

Chi-Yung Chio: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Huei-Chuan Shih: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Chi-Chu Tsai, Yu-Chung Chiang: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data.

Yi-Zu Ko: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jinluo A. Mantiquilla: Analyzed and interpreted the data; Wrote the paper.

I-Szu Weng: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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