

## Genetic background of citrus landrace ‘Huarongdao Zhoupigan’ revealed by molecular marker and genomic analysis

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### Background

Citrus is the largest fruit crop in the world, both in area and yield. Traditional crossbreeding strategies are very difficult due to long juvenile period, polyembryony, self-incompatibility and male sterility (Guo et al., 2013). Exploiting and utilizing natural hybrids or bud mutation resource is an ideal method for citrus breeding. As a cultivated landrace, ‘Huarongdao Zhoupigan’ is originated in Hunan province (a local variety of Huarong county) with a history of more than a hundred years. It is very popular among local people for its crumpled pericarp, soft juicy pulp and slightly bitter taste, which have a potential of medicine or food therapy. In 2011, it had been approved as geographically indicated products (Ministry of Agriculture and Rural Affairs, PRC., 2011). As the production and planting areas gradually increased, the origination and genetic background of ‘Huarongdao Zhoupigan’ not only arouses local farmers’ curiosity but also draws the attention of researchers.

To unravel the genetic background of ‘Huarongdao Zhoupigan’, 21 accessions of citrus species were evaluated based on molecular markers and genomic sequence alignment as well as morphology traits. This study provided evidences that ‘Huarongdao Zhoupigan’ might be originated from an interspecific hybridization with sour orange (a sexual hybrid between pummelo and mandarin) as seed parent and mandarin as pollen parent.

### Methods

#### Plant samples

‘Huarongdao Zhoupigan’ were collected from Dongshan town, Huarong county, Hunan province, China. 20 accessions of diversified citrus materials of different genus such as mandarins, sweet orange, sour orange, pummelo, citron, Ichang papeda and Poncirus, Fortunella.

#### Morphological traits and molecular marker analysis

A total of 20 morphological traits of ‘Huarongdao Zhoupigan’ and ‘Yuanjian Nanju’ were measured. A total of sixteen nuclear SSR and eight chloroplast SSR markers were obtained from previous works which proved highly efficient in parentage analysis of citrus and related genera (Cheng et al., 2005; Wang and Jiang, 2010). Following procedures of marker analysis previously reported (Chai et al., 2013).

## Genome resequencing and analysis

DNA of young fresh leaf tissues were sequenced at 30 × genome coverage. Raw data were processed using the Trimmomatic and filtered for high quality clean reads. Then, the clean reads were aligned to the sweet orange reference genome with BWA (Li and Durbin, 2009). Finally, the sequence alignment file was analyzed using the input of the method for genetic background.

## Results

### Comparison of the morphological traits of ‘Huarongdao Zhoupigan’ and ‘Yuanjian Nanju’

The protected area of this geographically indicated products was mainly in Dongshan town, Huarong county, Hunan province (Fig. 1). The morphological traits of ‘Huarongdao Zhoupigan’ and ‘Yuanjian Nanju’ were showed in Table 1.

Table 1 Comparative morphological characterization and fruit quality between ‘Huarongdao Zhoupigan’ and ‘Yuanjian Nanju’.

Characters	Huarongdao Zhoupigan	Yuanjian Nanju
Crown diameter (m)	4.8×4.6	5.4×5.2
Tree height (m)	3.64±0.19	4.87±0.12
Tree age (y)	18	37
Spring twigs (cm)	10.4±1.7a	13.7±0.9b
Thorn length (mm)	Nearly without thorn	5.8±1.2
Leaf length (mm)	91.1±6.2a	71.1±3.6b
Leaf width (mm)	33.7±2.1 a	33.0±1.4 a
Leaf thickness ( mm )	0.27±0.01 a	0.27±0.01 a
Length of petiole wing (mm)	7.9±1.1 a	8.6±1.6 a
Width of petiole wing (mm)	1.7±0.1 a	2.3±0.7 a
Fruit maturity	December–January	November–December
Fruit weights (g)	231.5±20.4	113.3±12.4
Fruit polar diameter (mm)	68.7±2.6	52.3±2.8
Fruit equatorial diameter (mm)	91.8±3.5	69.8±3.7
Fruit shape index	0.75±0.03	0.75±0.05
Pericarp thickness (mm)	6.9±0.9a	3.3±0.2b
Segments of fruit	10.2±0.4	9.8±0.8
Seed numbers	13.7±3.6	26.6±6.3
Peeling	Easy-peeling	Easy-peeling
Pericarp surface	Crumpled	Smooth
Fruit surface color	Yellow-orange	Red-orange
Juice color	Yellow	Orange
TSS (Total soluble solids) (%)	8.33±0.06 a	8.83±0.06 a
TA (Titratable acid) (%)	1.00±0.01 a	0.80±0.01b
Vitamin C (mg ·mL <sup>-1</sup> )	17.2±0.08	8.97±0.15

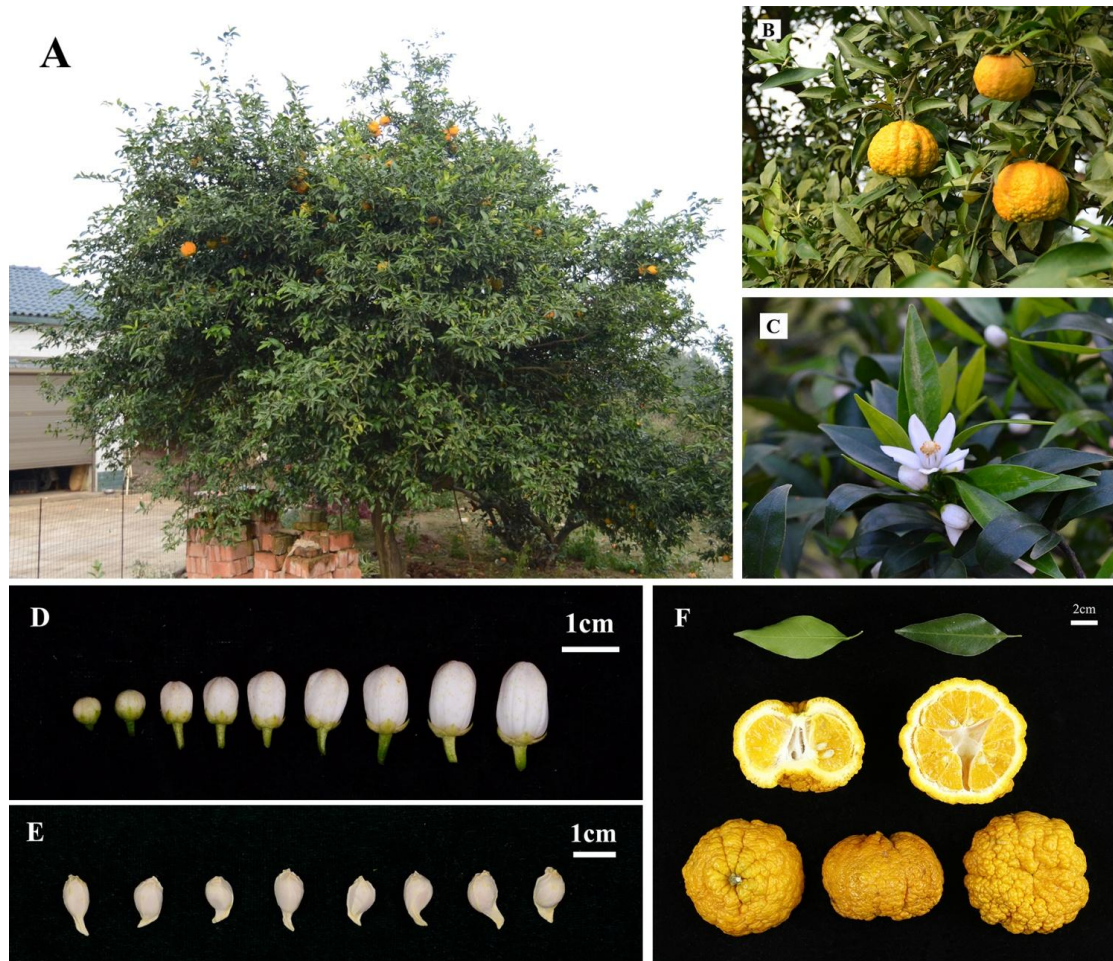


Fig. 1. Morphological features of 'Huarongdao Zhoupigan'.

A. Tree; B. Fruits on the tree; C. Flowers; D. Flowers of different periods; E. Seeds; F. Leaves and fruits.

### Phylogenetic analysis based on chloroplast and nuclear SSR markers

174 bands were amplified by 16 pairs of nSSR primers, with the average bands of 10.88 in each loci. From the dendrogram constructed by nSSR marker, the cluster analysis revealed that all 21 studied genotypes were clearly differentiated, and they fell into five major clusters when the coefficient was 0.75. 'Huarongdao Zhoupigan' was grouped with mandarins and closely stay together with 'Yuanjian Nanju' which indicating that its male parent might be mandarin (Fig. 2). 46 bands were amplified by 8 pairs of cpSSR primers, with the average bands of 5.75 in each loci. With regarding to dendrogram based on cpSSR marker, 21 accessions were grouped into four distinct clusters with a similarity value of 0.75. The group of 'Huarongdao Zhoupigan' contained pummelo, sour and sweet oranges, furthermore it was sub-grouped with Goutou sour orange, Dahong and Newhall sweet oranges. This analysis revealed that the female parent might be orange (Fig. 3).

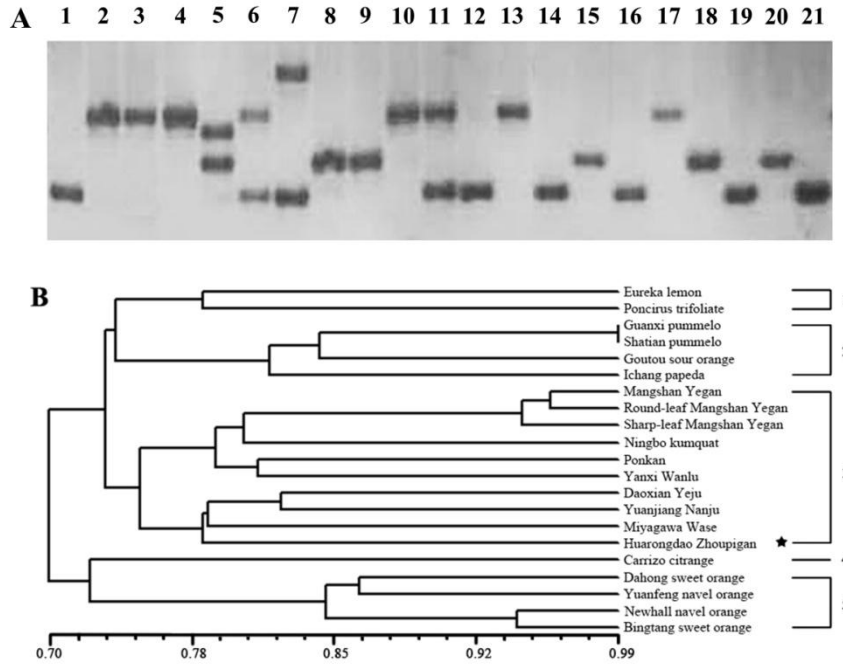


Fig. 2. Phylogenetic analysis based on nuclear SSR markers

A. Silver-stained polyacrylamide gel showing polymorphism among 21 tested Citrus and related genera using the nSSR primer CS\_065.

B. UPGMA dendrogram analysis of the 21 citrus and its related genera based on 16 nSSR markers. The asterisk refers to the sample of 'Huarongdao Zhoupigan'.

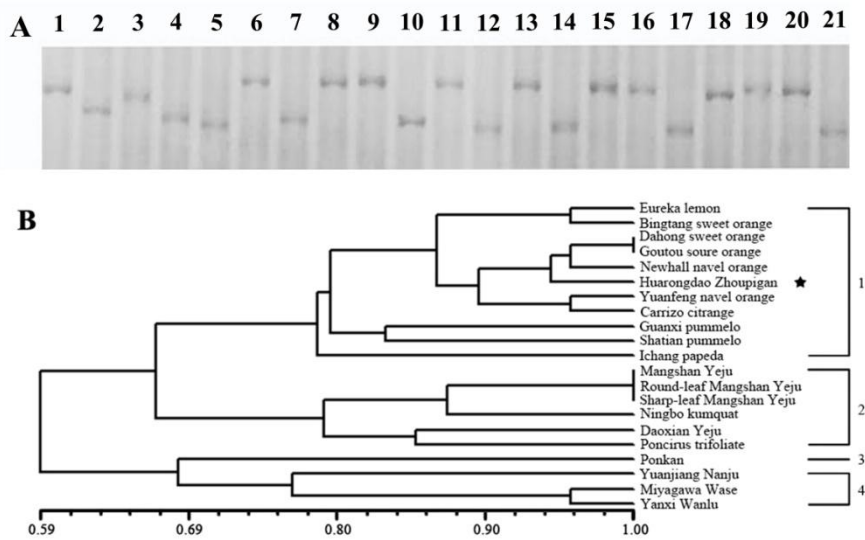


Fig. 3. Phylogenetic analysis based on chloroplast SSR markers

A. Silver-stained polyacrylamide gel showing polymorphism among 21 tested Citrus and related genera using the cpSSR primer NTCP9.

B. UPGMA dendrogram analysis of the 21 citrus and its related genera based on 8cpSSR markers. The asterisk refers to the sample of 'Huarongdao Zhoupigan'.

## Genomic sequence analysis

The genome admixture pattern showed that the result of ‘Huarongdao Zhoupigan’ with the hybrid of mandarin and pummelo contribute to 46.50%, pure mandarin 36.09%, pummelo 1.46%, and the remaining unknown part 19.11% (Fig. 4). Sequence alignment showed the major genomic component are hybrid region and mandarin. The roughly ratio of the hybrid region and mandarin are nearly 1:1, except the unknown and pummelo parts.

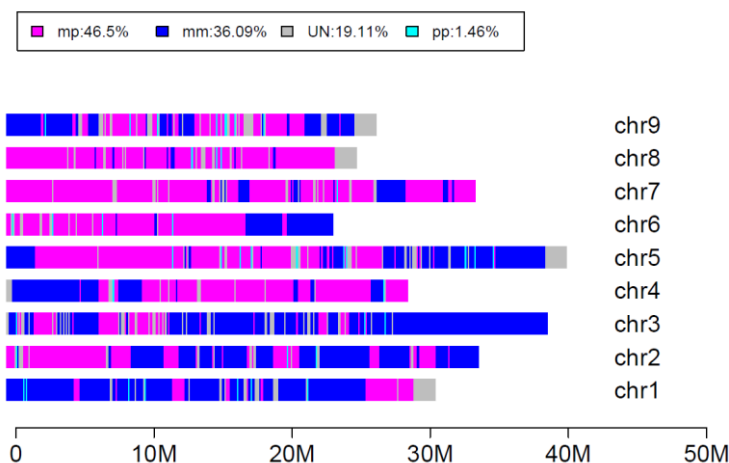


Fig. 4. ‘Huarongdao Zhoupigan’ genome admixture pattern.

Blue indicated mandarin background, cyan represents pummelo background, magenta represents hybrid of mandarin and pummelo background, grey represents unknown part.

## Conclusion

In this study, nSSR and cpSSR molecular markers were both used, and the primers showed rich polymorphism. In nSSR analysis, ‘Huarongdao Zhoupigan’ grouped with Miyagawa Wase, Daoxian Yeju and Yuanjiang Nanju. Miyagawa Wase had been introduced into China in 1965 (Zhang, 1978) and Daoxian Yeju were naturally distributed in the primitive forest of Nanling mountains. Therefore, the most capable male parent should be ‘Yuanjiang Nanju’ which distributed surrounding the Dongting lake. cpSSR analysis showed that ‘Huarongdao Zhoupigan’ stay together with Goutou sour orange, Dahong and Newhall sweet orange. It can be speculated that its female parent might be orange. It is difficult to distinguish the certain female parent just through molecular markers. Not only genetic relationship of citrus materials could be rapidly identified through genome resequencing analysis (Wu et al., 2018), but also the ingredient and proportion of basic species in candidate genome could be calculated by genomic analysis (Wei et al., 2020). The genomic admixture pattern of ‘Huarongdao Zhoupigan’ is 36.09% mandarin, 46.50% of mandarin and pummelo hybrid. Sour orange is an F1 hybrid of pure *C. maxima* and *C. reticulata* parents (Wu et al., 2014), and its cultivation surrounding the Dongting lake can be traced back to the Warring States Period (Li, 1990). Finally, it can be speculated that ‘Huarongdao Zhoupigan’ might be a natural hybrid between a sour orange (a sexual hybrid between pummelo and mandarin) as seed parent and mandarin as pollen parent.

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