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Full Length Article

Analysis of the Origin of *Citrus changshan-huyou* by DNA Barcode

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Abstract

Citrus changshan-huyou Y. B. Chang is a high quality fruit with a therapeutic value. It is produced in the Changshan County of the Zhejiang Province in China, but to date its origin remains unclear. In this work, we studied six plant samples, including the "ancient plant" of *C. changshan-huyou*, the Cuihong cultivar of *C. changshan-huyou*, sweet orange (*Citrus sinensis* Osbeck), mandarin orange (*Citrus reticulata* Blanco), pomelo (*Citrus maxima*) and ponkan (*Citrus ponki* Hort). Specifically, we employed seven pairs of primers generally used in identifying species, including the characteristic gene fragments of chlorophyll (*rbcL*, *rpoC1*, *rpoB*, and trnH-psbA), ribosome (5.8S-28S rRNA/ITS1; 18S-5S rRNA/ITS2), and mitochondria (nad1 exonB-C). The phylogenetic tree of *C. changshan-huyou* was constructed after PCR, cloning, and sequencing of the six plant samples. It was found that (1) pomelo was a female parent of *C. changshan-huyou* based on the results of ITS2, *rbcL*, *rpoC1*, and *rpoB*; (2) sweet orange was a either a male or a female parent of *C. changshan-huyou* based on the results of ITS1, *rpoC1*, trnH-psbA, and nad1 exonB-C, and (3) ponkan was a male parent of *C. changshan-huyou* based on the results of ITS1 and nad1 exonB-C. From the results, it could be argued that the origin of *C. changshan-huyou* involved the hybridization of multiple sources, including pomelo, sweet orange and ponkan.

Keywords: *Citrus changshan-huyou*; DNA barcode; Phylogenetic tree; Origin

Introduction

Citrus changshan-huyou Y. B. Chang is a new species of the *Citrus* genus (Zhang, 1991). It is originated in the Changshan County of the Zhejiang Province in China. Its fruit is rich in amino acids, vitamins, and other nutrients (Guo *et al.*, 2018). The fruit also contains abundant therapeutic components and has various medicinal properties, such as relieving cough, clearing phlegm, moistening throat, lowering blood sugar etc. (Jiang, 1994; Zhao *et al.*, 2003; Fang *et al.*, 2015; Lin *et al.*, 2015). The fruit has a golden color with a unique aroma and taste. It is a key specialty food developed in the Zhejiang Province because of its excellent quality and high popularity among the consumers (Li *et al.*, 2011).

Citrus changshan-huyou is a natural hybrid species whose origin remains to be ascertained because of inconsistent arguments given in different studies. Since the botanical character of *C. changshan-huyou* lies between that of pomelo (*C. maxima*) and sweet orange (*C.* × *sinensis*), Shen (1963), Bei and Ye (1995) and Yu *et al.* (2006) examined the peroxidase isoenzyme (POD) and suggested that *C. changshan-huyou* might be a hybrid of pomelo and sweet orange. Chen *et al.* (2002) used random amplified polymorphic DNA (RAPD) to analyze ponkan (*C. poonensis*), mandarin orange (*C. aurantium* Linn), *Citrus changshan-huyou*, pomelo (*C. maxima*), and sweet orange (*C.* × *sinensis*) and suggested that pomelo probably firstly hybridized with ponkan or mandarin orange (*C. aurantium*) to give a resultant fruit that hybridized with sweet orange to afford *C. changshan-huyou*. Chen *et al.* (2006) found from inter-simple sequence repeat (ISSR) and RAPD analysis that *C. changshan-huyou* and *C. sinensis* formed a subcluster, and *C. sinensis* was an assured parent of *C. changshan-huyou*, but the large genetic distance between *C. grandis* and *C. changshan-huyou* suggested that *C. changshan-huyou* might have originated from the natural hybrid of multiple sources including *C. sinensis*, *C. grandis*, and other *Citrus* species. Xu *et al.* (2006) undertook RAPD and internal transcribed spacer (ITS) analysis to study existing *Citrus* species in the Changshan County, including three mandarin oranges, one sweet orange, one sour orange and one pomelo, and concluded that *C. changshan-huyou* was probably a natural hybrid of pomelo and sour orange.

In this work, we inspected the origin of *C. changshan-huyou* by DNA barcode for the characteristic spacers in related species, including the 5.8S-28S rRNA ribosomal gene spacer (ITS1), the 18S-5S rRNA ribosomal gene spacer (ITS2), the mitochondrial characteristic sequence nad1 exonB-C, and the characteristic *rbcL*, *rpoCl*, *rpoB*, and trnH-psbA chloroplast characteristic sequences. The results provided a theoretical basis for the further cultivation and development of *C. changshan-huyou*.

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Materials and Methods

Materials

The oldest citrus seedling plant of *C. changshan-huyou*, which currently grows in the Chentang Village of the Changshan County and ages 113 years as of 2019 (Li *et al.*, 2017), was examined (referred to as the "ancient plant"). In addition, the "Cuihong" cultivar of *C. changshan-huyou*, sweet orange (*Citrus sinensis* Osbeck), mandarin orange (*Citrus reticulata* Blanco), pomelo (*C. maxima*), and ponkan (*Citrus ponki* Hort) were also collected from the Changshan County.

Extraction and PCR Amplification of Plant Genomic DNA

The plant genomic DNA was extracted with the rapid DNA extraction kit (Sangon Biotech, China) according to the procedures given in the instruction manual. Seven PCR primers were designed by considering both the characteristic genomic sequence of the *Citrus* family (Fazekas *et al.*, 2008) and the comparative analysis by Clustal (https://www.ebi.ac.uk/Tools/psa) (Table 1) The PCR reaction was performed according to Du *et al.* (2015) using a PE9700 PCR amplifier (Applied Biosystems Inc., USA), with an initial denaturing step at 94°C for 5 min, then 30 amplification cycles (30 s at 94°C, 30 s at 55°C, and 2 min at 72°C), followed by the final elongation at 72°C for 10 min. The PCR products were subjected to electrophoresis for 60 min at 90 V in 1.5% agarose gel, then dyed with ethidium bromide and inspected with the Gel Imaging Analyzer (Bio-Rad, USA).

Purification, Cloning and Sequencing of PCR Products

The DNA fragments of the target genes were purified and collected using a PCR purification kit (Sangon Biotech, China). Purified DNA was linked to pMD18-T vector (TaKaRa, Japan) and transferred into DH5α competent cells by the freeze-thaw process. White single colonies were screened on LB plates containing X-Gal, IPTG and ampicillin (50 mg/L). Positive clone plasmids were extracted with a rapid plasmid extraction kit (Beijing Dinguo, China) and characterized by double digestion with *Eco*R Ⅰ plus *Bam*H Ⅰ. The digestion verification plasmid was sequenced by Shanghai Sunnybio Biotechnology Co., Ltd.

Sequencing Analysis and Construction of Phylogenetic Tree

The molecular phylogenetic tree was constructed with MEGA 7.0 (http://www.megasoftware.net) after online sequence comparison with Blast (http://www.ncbi.nlm.nih.gov/blast). Multi-sequences alignment was conducted by DNAMAN (https://www.lynnon.com). Default parameters were used in all cases.

Table 1: Sequences of primers

Gene/Location	Primer sequences（5′→3′）	Estimated length (bp)
ITS1/ribosome	AGAAGTCGTAACAAGGTTTCCGTA TCCTCCTCCGCTTATTGATATGC	750
ITS2/ribosome	GTGTTGCTGAGACATGCGCC ATATGGCGCAAGACGATTCC	1060
rbcL/chloroplast	TGTCACCAAAAACAGAGACT TTCCATACTTCACAAGCAGC	1380
rpoC1/chloroplast	GGCAAAGAGGGAAGATTTCG CCATAAGCATATCTTGAGTTGG	530
rpoB/chloroplast	ATGCAACGTCAAGCAGTTCC AAATAAGGCATATCTTGTCT	880
trnH-psbA/chloroplast	GTTATGCATGAACGTAATGCTC CGCGCATGGTGGATTCACAAATC	560
nad1 exonB-C/mitochondrion	GCATTACGATCTGCAGCTCA GGAGCTCGATTAGTTTCTGC	1660

Results

PCR Amplification and Cloning

For all six plant samples, the PCR amplification with each primer gave a single band, *i.e.*, at *ca.* 750 bp for ITS1, *ca.* 1000 bp for ITS2, *ca.* 1300 bp for *rbcL*, *ca.* 500 bp for rpoC1, *ca.* 900 bp for *roiB*, *ca.* 550 bp for trnH-psbA, and *ca.* 1600 bp for nad1 exonB-C (Fig. 1–7). All PCR products were connected to pMD18-T transferred *E. coli* to obtain monoclone plasmids, which were verified by the double digestion with *Eco*R Ⅰ plus *Bam*H Ⅰ (data not shown).

Sequencing and Analysis of ITS1

For the six plants, the PCR products gave ITS1 bands in 749–751 bp. Sequencing results showed that the ITS1 sequence in the ancient plant had 98.54% similarity with the Cuihong cultivar, 99.07% with sweet oranage, 98.27% with mandarin orange, 92.28% with pomelo, and 99.07% with ponkan. Similarity was the highest between the ancient plant and ponkan, and between the ancient plant and sweet orange.

The phylogenetic tree constructed based on ITS1 sequencing showed that the ancient plant was more related to sweet orange and ponkan, and less related to pomelo (Fig. 8). Therefore, the ancient plant was probably derived from the same ancestor of sweet orange and ponkan. On the other hand, the Cuihong cultivar was genetically closest to mandarin orange and less related to the ancient plant, indicating that hybridization with mandarin orange probably occurred during the breeding of the Cuihong cultivar.

Sequencing and Analysis of ITS2

The ITS2 bands of the six samples ranged in 1064–1069 bp. Sequencing results showed that the ancient plant had 99.81% similarity with the Cuihong cultivar, 99.25% with sweet orange, 99.35% with mandarin orange, 100% with pomelo, and 99.35% with ponkan. The ITS2 sequence was identical in the ancient plant and in pomelo.

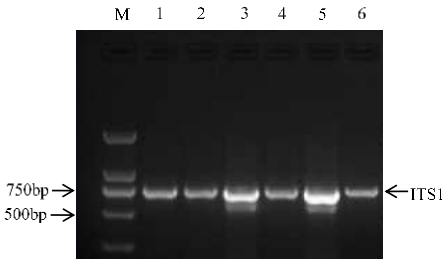

Fig. 1: Amplification of PCR for ITS1
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

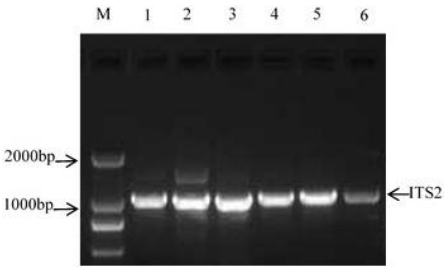

Fig. 2: Amplification of PCR for ITS2
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

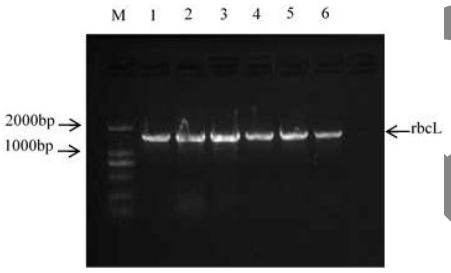

Fig. 3: Amplification of PCR for *rbcL*
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

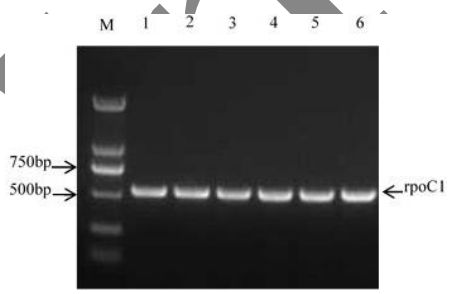

Fig. 4: Amplification of PCR for *rpoC1*
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

The phylogenetic tree constructed based on ITS2 sequencing showed that both the ancient plant and the Cuihong cultivar were closely related to pomelo, and less related to ponkan, sweet orange, or mandarin orange (Fig. 9).

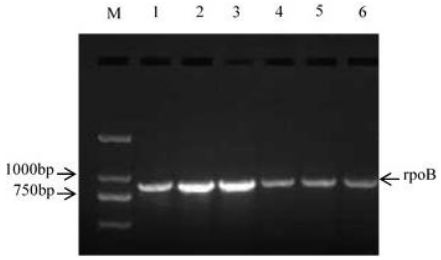

Fig. 5: Amplification of PCR for *rpoB*
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

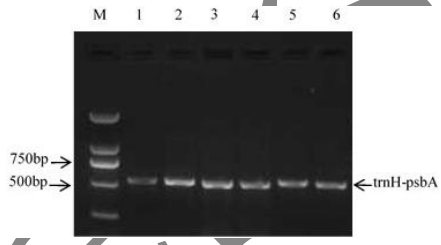

Fig. 6: Amplification of PCR for trnH-psbA
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

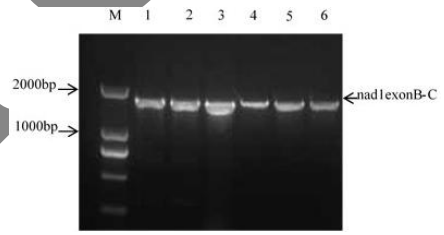

Fig. 7: Amplification of PCR for nad1exonB-C
M: DS2000 DNA marker; 1: "ancient tree" of *C. changshan-huyou*; 2: *C. changshan-huyou*; 3: *C. sinensis*; 4: *C. reticulata*; 5: *C. maxima*; 6: *Citrus ponki*

Sequencing and Analysis of *rbcL*

The *rbcL* bands of the six samples ranged in 1386–1387 bp. Sequencing results showed that the ancient plant had 99.78% similarity with the Cuihong cultivar, 99.64% with sweet orange, 99.35% with mandarin orange, 99.57% with pomelo, and 99.35% with ponkan. The highest similarity was observed between the ancient plant and the Cuihong cultivar. The phylogenetic tree constructed based on *rbcL* sequencing showed that the ancient plant was closely related to pomelo and less related to ponkan, sweet orange, or mandarin orange (Fig. 10). Besides, the Cuihong cultivar was closely related to the ancient plant, sweet orange, and pomelo, and less related to mandarin orange or ponkan.

Sequencing and Analysis of *rpoC1*

The *rpoC1* bands of the six samples ranged in 528–529 bp. Sequencing results showed that the ancient plant had 99.81% similarity with the Cuihong cultivar, 100% with sweet orange, 99.62% with mandarin orange, 100% with pomelo,

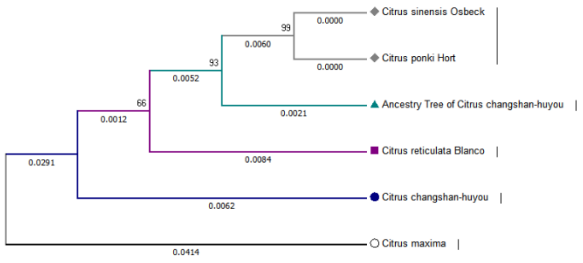

Fig. 8: Phylogenetic tree based on ITS1 sequences

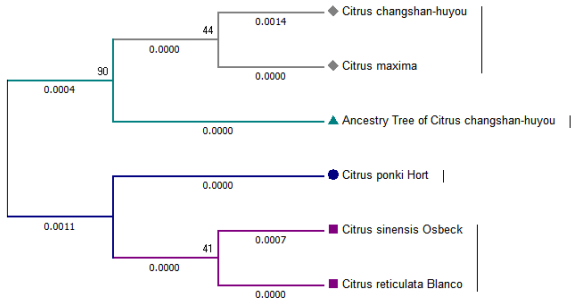

Fig. 9: Phylogenetic tree based on ITS2 sequences

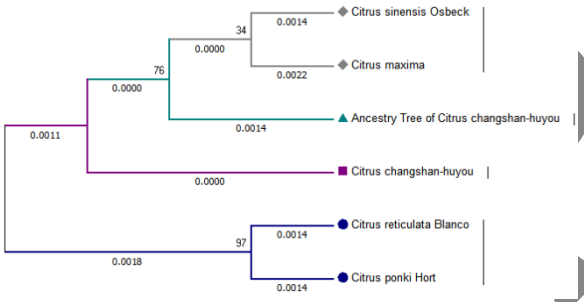

Fig. 10: Phylogenetic tree based on *rbcL* sequences

and 99.81% with ponkan. The rpoC1 sequence of the ancient plant was the same as that in pomelo and in sweet orange. The phylogenetic tree constructed based on *rpoC1* sequencing showed that the ancient plant was genetically closely related to sweet orange and pomelo, and less related to the Cuihong cultivar, ponkan, or mandarin orange (Fig. 11). In addition, the Cuihong cultivar was related most closely to the ancient plant.

Sequencing and Analysis of *rpoB*

The *rpoB* bands were 884 bp for all six samples. Sequencing results showed that the ancient plant had 99.66% similarity with the Cuihong cultivar, 99.77% with sweet orange, 99.32% with mandarin orange, 99.66% with pomelo, and 99.55% with ponkan. The highest similarity was observed between the ancient plant and sweet orange. The phylogenetic tree constructed based on *rpoB* sequencing showed that the ancient plant was genetically closely related to pomelo and ponkan, and less related to sweet orange or

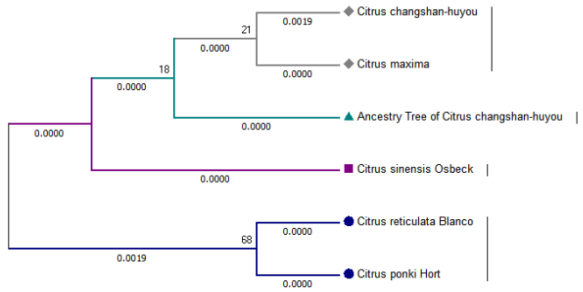

Fig. 11: Phylogenetic tree based on rpoC1 sequences

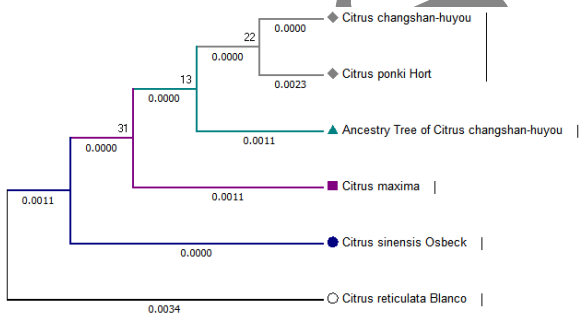

Fig. 12: Phylogenetic tree based on *rpoB* sequences

mandarin orange (Fig. 12). In addition, the Cuihong cultivar was closely related to the ancient plant, pomelo and ponkan, and less related to sweet orange or mandarin orange. Hence, the Cuihong cultivar was probably derived from the hybridization between the ancient plant and pomelo or ponkan.

Sequencing and Analysis of trnH-psbA

The trnH-psbA bands of the six samples ranged in 552–564 bp. Sequencing results showed that the ancient plant had 40.41% similarity with the Cuihong cultivar, 97.73% with sweet orange, 96.34% with mandarin orange, 97.73% with pomelo, and 95.99% with ponkan. The highest similarity was observed between the ancient plant and sweet orange, and between the ancient plant and pomelo. The phylogenetic tree constructed based on trnH-psbA sequencing showed that the ancient plant was genetically closely related to pomelo and sweet orange, and less related to mandarin orange or ponkan (Fig. 13). In addition, the Cuihong cultivar was related most closely to the ancient plant.

Sequencing and Analysis of nad1 exonB-C

The nad1 exonB-C bands of the six samples ranged in 1600–1663 bp. Sequencing results showed that the ancient plant had 99.88% similarity with the Cuihong cultivar, 99.76% with sweet orange, 99.64% with mandarin orange, 99.94% with pomelo, and 99.88% with ponkan. The highest similarity was observed between the ancient plant and pomelo. The phylogenetic tree constructed based on

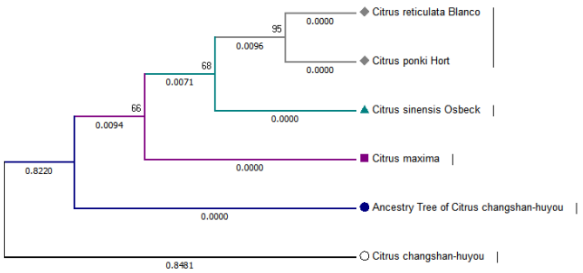

Fig. 13: Phylogenetic tree based on trnH-psbA sequences

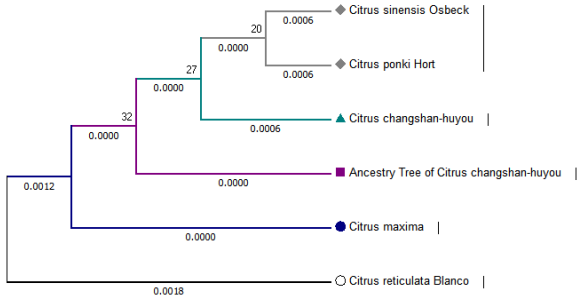

Fig. 14: Phylogenetic tree based on nad1 exonB-C sequences

nad1 exonB-C sequencing showed that the ancient plant was genetically closely related to the Cuihong cultivar, sweet orange, and ponkan, and less related to pomelo or mandarin orange (Fig. 14). In addition, the Cuihong cultivar was related closely to sweet orange and ponkan, probably because hybridization with sweet orange and ponkan took place during its breeding.

Discussion

Two species are considered to have close genetic relationship if their characteristic sequences are sufficiently similar and align well with each other, and the origin of species can thus be effectively inferred by studying the characteristic DNA sequences (Hall, 1999; Kumar *et al.*, 2004). Current research in plant classification and molecular identification commonly examined the characteristic gene fragments of chlorophyll (*rbcL*, *rpoC1*, *rpoB*, and trnH-psbA), ribosome (5.8S-28S rRNA/ITS1 and 18S-5S rRNA/ITS2), and mitochondria (nad1 exonB-C). Many studies of plant phylogeny and molecular identification used either a single fragment or the combination of multiple fragments (Tian and Li, 2002). It is commonly believed that the characteristic gene fragments of chlorophyll are the most suitable DNA barcode for studying the evolution of species, and the accuracy can be further enhanced by jointly considering other characteristic gene fragments (Gao *et al.*, 2008). For instance, the medicinal herb Ginseng was well identified by DNA barcode from the combined sequencing of trnH-psbA and ITS1 (Hollingsworth *et al.*, 2009). In addition, Bruni *et al.* (2010) examined 50 toxic plants by using three characteristic gene fragments of chlorophyll (trnH-psbA, *rpoB*, and *matK*) and two characteristic gene fragments of ribosome (*AtI03* and *sqd1*). Liu *et al.* (2015) found that the combined sequencing of *rbcL* and ITS2 was the best in evaluating the diversity of tropical plants in classifying subtropical forests with the characteristic gene fragments of *rbcL*, *matK*, ITS1, ITS2 and trnH-psbA.

In 1991, *C. changshan-huyou* was recognized as a new species of the *Citrus* genus (Zhang, 1991), but to date its origin remains to be clarified. The oldest citrus seedling plant of *C. changshan-huyou* (*i.e.*, the "ancient plant") is 113 years old (as of 2019) and currently grows in Chentang Village of the Chngshan County. Local farmers believe that all younger *C. changshan-huyou* plants were originally reproduced and developed from this tree (Zhong 2002; Yu *et al.* 2006; Li *et al.*, 2017), and the analysis of this ancient plant is essential in investigating the evolution of *C. changshan-huyou.* Our results showed that the ancient plant had close relationship with pomelo (*C. maxima*) according to the sequencing result of the ribosome ITS2 and the chlorophyll *rbcL*, *rpoC1*, and *rpoB*. Since the chlorophyll genes come from the female parent, it could be inferred that pomelo was a parent (female parent) in the evolution of *C. changshan-huyou*. Besides, the ancient plant also had close relationship with sweet orange (*C. sinensis* Osbeck) according to the sequencing results of ribosome ITS1, chlorophyll *rpoC1* and trnH-psbA, and mitochondria nad1 exonB-C, indicating that sweet orange was also a parent (male or female). The sequencing results of ribosome ITS1 and mitochondria nad1 exonB-C showed that the ancient plant was genetically closely associated with ponkan (*C. ponki*), which was likely a male parent of the ancient plant, since the mitochondrial gene fragment comes from the male parent. Especially, the Cuihong cultivar of *C. changshan-huyou* seemed to differ slightly from the ancient plant in terms of origin, probably because the breeding process of the Cuihong cultivar involved constant hybridization with other species such as mandarin orange, sweet orange, ponkan, *etc.* Therefore, it could be argued that the origin of *C. changshan-huyou* involved the hybridization of multiple sources, including pomelo, sweet orange, ponkan *etc.*

Conclusion

(1) Pomelo was a female parent of *C. changshan-huyou* based on sequences of ITS2, *rbcL*, *rpoC1*, and *rpoB.* (2) Sweet orange was either a male or female parent of *C. changshan-huyou* based on sequences of ITS1, *rpoC1*, trnH-psbA, and nad1 exonB-C. (3) Ponkan was a male parent of *C. changshan-huyou* based on sequences of ITS1 and nad1 exonB-C. It could be argued that the origin of *C. changshan-huyou* involved the hybridization of multiple sources, including pomelo, sweet orange and ponkan.

Acknowledgements

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