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Original Article

Comparison of the wild and cultivated *Merrillanthus hainanensis* by DNA and HPLC fingerprint

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ABSTRACT

Merrillanthus hainanensis Chun et Tsiang is the only species in *Merrillanthus* genus of Asclepiadaceae family (currently considered a subfamily of Apocynaceae). It is also an endangered species distributed in China. The aim of this study is to compare the similarities of the wild and cultivated *M. hainanensis* by DNA and HPLC technologies.DNA sequencing is a useful and reliable method for the identification of species, and HPLC fingerprint shows the details of chemical composition. In this study, we sequenced the internal transcribed spacer (ITS) region of nuclear ribosomal RNA genes in the wild and cultivated types of *M. hainanensis*, and compared the sequences with other related species in Asclepiadaceae family. At the same time, we compared the HPLC fingerprints of the wild and the cultivated types of this plant. The sequencing results showed that the cultivated and wild-types of *M. hainanensis* have the same ITS sequences. Then the common ITS sequence was blasted in Genbank database. The highest similarity to this sequence is the ITS sequence of *Tylophora tenuis* (96.7%). Furthermore, comparison of HPLC fingerprint indicated that the wild type and the cultivated type were close like each other with twelve common peaks, which is consistent with the DNA sequencing. This work has shown that cultivation can be used to enlarge this endangered species.

Keywords: *Merrillanthus hainanensis*, DNA, ITS, HPLC fingerprint

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INTRODUCTION

In 1941, *Merrillanthus hainanensis* was discovered in Baisha, Hainan island of China [1]. It is an extremely rare vine [2]. In 2005, *M. hainanensis* was first discovered in Zhongshan City. At that time, the staff of Zhongshan State-owned Forest Resources Conservation Center conducted a comprehensive survey of wild plants in Zhongshan City. They found traces of hump vines near Cuishan Road in Wugui Mountain, and these plants were identified by South China Agricultural University.

However, in 2011, due to the lack of awareness of the protection of *M. hainanensis* and the need for urban construction, Zhongshan City built roads in the area where *M. hainanensis* grew, and it is difficult to find the trace of this plant. In the same year, the Zhongshan media reported that "the only *M. hainanensis* in Zhongshan was gone", so this plant was once considered extinct in Zhongshan city. In June 2015, the staff of Zhongshan forestry protection Center visited the Tianxin Forest Park and discovered a wild population with dozens of *M. hainanensis*. It was once again found that there are wild vines of *M. hainanensis*in Zhongshan.

In terms of biodiversity, the existence of each species has an unsightly effect on the ecological environment. *M. hainanensis* is a woody vine, a national secondary protected plant endemic to China. It is important to protect and expand its population. Many plants in *Asclepiadaceae* family have medicinal values, e.g. *Asclepias curassavica* [3,4]. However, there is very little research on *M. hainanensis* all over the world except for the distribution [5], phenological cycle [6] and tissue culture [7], and the medicinal value of *M. hainanensis* is still unknown. In this paper, DNA and HPLC analysis of wild *M. hainanensis* and its artificial cultivated species were carried out, and the genetic relationship was analyzed with other plants in the same family. This study is important for understanding the source of *M. hainanensis*, comparing the identity of wild and artificial cultivated strains, and expanding the population of *M. hainanensis*.

MATERIAL AND METHODS

Herbal materials: The cultivated *M. hainanensis* and wild *M. hainanensis* were collected in Zhongshan city, Guangdong province of China. These herbal materials were identified by Ms. Pan-Pan Liu of Zhongshan Management Centre of the Natural Protected Area. Herbal specimen for the wild and cultivated types of *M. hainanensis* were deposited in Zhongshan Management Centre of the Natural Protected Area.

Instruments and reagents:

Instruments: Electrophoresis System (BIO-RAD, USA); DNA Electrophoresis Cell (Tanon, Shanghai, China); JX-MINI-4K Mini Centrifuge (Jingxue Science, China); -20 °C Low Temperature Refrigerator (Haier, China); Constant Temperature Water Bath (Xinzhi, China); High Speed Refrigerated Centrifuge (Beckman, USA), Gel Imaging System (BIO-RAD, USA); PCR Instrument (Applied Biosystems, USA); Vortex-Genie2 (Scientific Industries, USA); Ultramicro Spectrophotometer (Thermo, USA); Gene sequencing (Beijing Qingke Biotechnology Co., Ltd. China)

Reagent: Plant Genomic DNA Extraction Kit (TransGen Biotech, China), rTaq enzyme and DNA marker DL2000 (Takara Bio, China); agarose (BIOWEST, USA); Primer synthesis (Beijing Qingke Biotechnology Co., Ltd. China).

Extraction of the genomic DNA of the cultivated and wild types of *M. hainanensis*

(1) Take 100 mg of fresh plant tissue and grind it thoroughly with liquid nitrogen；

(2) Add 250 μL of solution RB1 and mix thoroughly with 15 μL of RNase A；

(3) Incubate for 15 min in a 55 °C water bath；

(4) Centrifuge at 12,000 × g for 5 min, gently pipette the supernatant into a clean centrifuge tube ；

(5) Add 100 μL of PB1 solution, mix well, water bath for 5 min, centrifuge at 12,000 × g for 5 min. ；

(6) Pipette the supernatant into a clean centrifuge tube, add 375 μL of solution BB1, and mix thoroughly.；

(7) Pipette all the mixture into a spin column, centrifuge at 12,000 × g for 30 s, discard the effluent；

(8) Add 500 μL of solution CB1, centrifuge at 12,000 × g for 30 s, discard the effluent；

(9) Add 500 μL of solution WB1, centrifuge at 12,000 × g for 30 s, discard the effluent；

(10) Repeat step 9；

(11) Centrifuge (12,000×g,2 min) to remove WB1 completely

(12) Place the spin column in a clean centrifuge tube, add 20 μL of preheated EB (60 °C) to the center of the column, and let it stand for 3 min at room temperature., centrifuge 12,000×g (1 min) to elute DNA。

(13) Detected the genomic extraction using 1% agarose gel electrophoresis.

PCR amplification and sequencing: Amplification of the ITS sequence of *M. hainanensis* was performed with universal primers ITS5F and ITS4R using the extracted genomic as the template[8].PCR amplification system: genomic DNA 4 μL; rTaq 0.3 μL; ITS5F and ITS4R 0.5 μL, 10×buffer 2.5 μL, dNTP 1 μL,ddH_2O 16.2 μL.

Forward primer ITS5F: 5'-GGAAGTAAAAG CGTAACAAGG-3'

Reverse primer ITS4R: 5'-TCCTCCGCTATA TGATATGC-3'

The PCR amplification procedure: pre-denaturation at 94 °C for 5 min, template denaturation at 94 °C for 45 s, annealing at 56 °C for 30 s, and extension at 72 °C for 45 s. A total of 30 cycles were performed, and finally the gene was extended at 72 °C for 5 min. PCR amplification was detected using 1% agarose gel electrophoresis. Finally, the PCR product was sent to Beijing TsingKe Biological Technology Ltd for sequencing.

ITS sequences from related species of Asclepiadaceae family: The homology search was performed using the NCBI database to obtain the ITS sequence of the plant in Asclepiadaceae family. We obtained a total of 16 ITS sequences from other plants of this family. The Genbank accession numbers are shown in Table 1. The obtained sequence information and the sequence information of *M. hainanensis* obtained by gene sequencing were compared by ClustalX 2.1 software [9], and the corresponding common sequence was applied to subsequent genetic distance and cluster analysis.

Sequence No.	Latin name	GenBankcode
1	*Cryptostegi grandiflora*	DQ916844.1
2	*Cryptolepis sinensis*	KP764837.1
3	*Orbea pulchella*	KT795416.1
4	*Myriopteron extensum*	DQ916853.1
5	*Gomphocarpus fruticosus*	AM396906.1
6	*Cynanchum chinense*	GU951533.1
7	*Periploca sepium Bunge*	KP975389.1
8	*Gymnanthera oblonga*	DQ916850.1
9	*Asclepias curassavica*	AM396884.1
10	*Dregea volubilis*	MG818138.1
11	*Biondia chinensis*	HE793716.1
12	*Hoya carnosa*	DQ334464.1
13	*Brachystelma eduel*	AJ310798.1
14	*Graphistemma pictum*	LN896991.1
15	*Telosma cordata*	AM980873.1
16	*Tylophora tenuis*	AJ320468.1

Phylogenetics: The genetic distance analysis of the sequence after comparison was performed using MEGA 7.0 software. The cultivate and wild *M. hainanensis* and other 16 plants in Asclepiadaceae family were analyzed for genetic relationship and genetic kinship using the nearest distance method [10-11]. The phylogenetic tree [12] was shown in Figure 5.

HPLC Fingerprinting Analysis: The dry herbal materials of the wild type and the cultivated type were powdered. 0.5g of each sample was extracted with 10ml methanol under ultrasonic conditions for 1 hour. Then the solution was filtered with 0.22μ membrane and an aliquot of 10 μL was injected into HPLC for comparison. The chromatographic conditions were as follow. Agilent 1200 series, Phenomenex Luna C18 analytical column (250 mm×4.6 mm, 5 μm); mobile phase: water containing 0.1 % Formic acid (A)-acetonitrile (B); 10 to 90% B in 60min, flow rate 1.0 mL/min, temperature: 25 ℃

RESULTS

Extraction of genomic DNA: Electrophoresis (Figure 1) showed obvious bright bands above 10 kb in both the lanes of the cultivated and wild groups, and there was no tailing phenomenon, indicating that the genome extraction was successful. The DNAOD_{260}/OD_{280} values detected by the Ultramicro Spectrophotometer were 1.68 and 1.75, respectively, indicating that the extracted DNA can be used for the amplification in the next step.

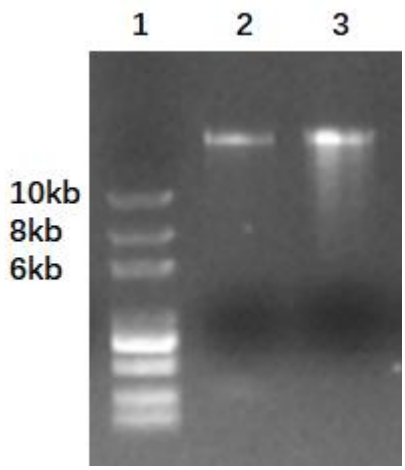

Figure 1 The gel electrophoresis of the genomic DNA from the cultivated and wild types of *M. hainanensis*. Lane 1: Markers; Lane 2: the genomic DNA of the cultivated type; Lane 3: the genomic DNA of the wild type.

ITS amplification and sequencing: Using the genome DNA extracted from the cultivated and wild *M. hainanensis* as the templates, and ITS 5F and ITS 4R as the primers, the PCR amplification was performed. The PCR results of were detected by 1% gel electrophoresis (Figure 2), which showed thatthere was an electrophoresis strip700 bpin both the cultivated and wild-type lanes,indicating that ITS sequence of *M. hainanensis*was successfully amplified.The sequencing results showed that the cultivated and wild-types of *M. hainanensis* have the same ITS sequences(Figure 3).Then the common ITS sequence was blasted in Genbank database. The highest similarity to this sequence is the ITS sequence of *Tylophora*, up to 96.7%.

The ITS sequence of the cultivated *M. hainanensis*:
CTTTTTGGTGAACTGCTGGAGGATCATTGT
CAAATCCTCGTGCCGAATGACCTGCGAACA
CGTTCCAAAAATCACAAGCGTTGTTGCGCC
CTTGCTTGGTGTGGGTCGGGGACTTGTTGC
CGGACGCGTCGAGCACGGAAAACGAAATC
CGGCGCGGGAAGCGCCAAGGACTAGCGAA
ATGGAGGATGGCCTTCCCGCGGCATCCTGG
CCGCGGGGGATTAAAGGGTCGTCGAATGA
AAATGTTATTCATACAGTACGACTCTCGGC
AACGGATATCTAGGCTCTCGCATCGATGAA
GAACGTAGCAAACTGCGATAGTTGGTGTGA
ATTGCAGAATCCCGTGAACCATGGAGTCTT
TGAAGGCAAGTTGCGCCCGAAGCCATTAGG
CCGAGGGCACGTCTGCCTGGGCGTCACGCA
TTGCGTCGTCCCCCCCTCACCCGTGTCCCGA
AAGGGTCGCGGGCGTTAGCGTTGGGGGGG
CGGAAGTTGGCTTCCCGTGCAGCGTTTGCG
GCTAGCCTGAAACAACGGTTCTCTCGGCGC
GGACGTAGCGACAAGTGGTGGTCGTCGGA
CGGGATTGTACGCGAGTTGCCGGGAAGCTG
CGTCGAGGAGAGCATTTGGACCCTGTGCGA
GACGAGTCCCTTCGGCGAGGGGCAATCGCA
ACGATTGCGACCCCAGGTCAGGCGAGGCA
GCCCCGCTG

The ITS sequence of the wild *M. hainanensis*:
CATCTCGTGCCGAAGACCTGCGACACGTTC
CAAAAATCACAAGCGTTGTTGCGCCCTTGC
TTGGTGTGGGTCGGGGACTTGTTGCCGGAC
GCGTCGAGCACGGAAAACGAAATCCGGCG
CGGGAAGCGCCAAGGACTAGCGAAATGGA
GGATGGCCTTCCCGCGGCATCCTGGCCGCG
GGGGATTAAAGGGTCGTCGAATGAAAATG
TTATTCATACAGTACGACTCTCGGCAACGG
ATATCTAGGCTCTCGCATCGATGAAGAACG
TAGCAAACTGCGATAGTTGGTGTGAATTGC
AGAATCCCGTGAACCATGGAGTCTTTGAAG
GCAAGTTGCGCCCGAAGCCATTAGGCCGAG
GGCACGTCTGCCTGGGCGTCACGCATTGCG
TCGTCCCCCCCTCACCCGTGTCCCGAAAGG
GTCGCGGGCGTTAGCGTTGGGGGGGCGGA
AGTTGGCTTCCCGTGCAGCGTTTGCGGCTA
GCCTGAAACAACGGTTCTCTCGGCGCGGAC
GTAGCGACAAGTGGTGGTCGTCGGACGGG
ATTGTACGCGAGTTGCCGGGAAGCTGCGTC
GAGGAGAGCATTTGGACCCTGTGCGAGAC
GAGTCCCTTCGGCGAGGGGCAATCGCAACG
ATTGCGACCCCAGGTCAGGGGGGGGCCAC
CTGTC

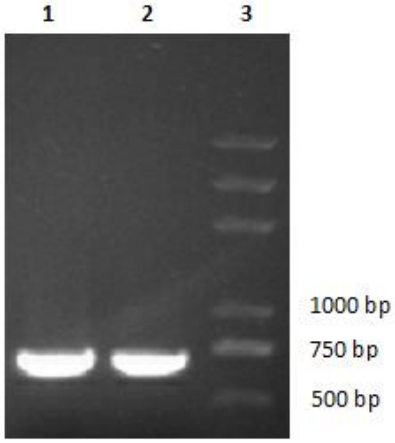

Figure 2 The gel electrophoresis of ITS DNA from the cultivated and wild types of *M. hainanensis.* Lane 1: cultivated type; Lane 2: wild type; Lane 3: markers.

Phylogenetic relationship between *M. hainanensis* and other species in Asclepiadaceae family: Based on the ITS sequences, a phylogenetic tree (Figure 4) was constructed using the Neighbor-Joining method (The bootstrap value was set 1000).The results showed that *M. hainanensis* and *Tylophora tenuis* was on the same branch with a similarity of 99%, indicating that these two plants have close phylogenetic relationship. In contrast, *Myriopteron extensum and Gymnanthera oblonga* were located at the other end of the phylogenetic tree, indicating that *M. hainanensis* is far from them.

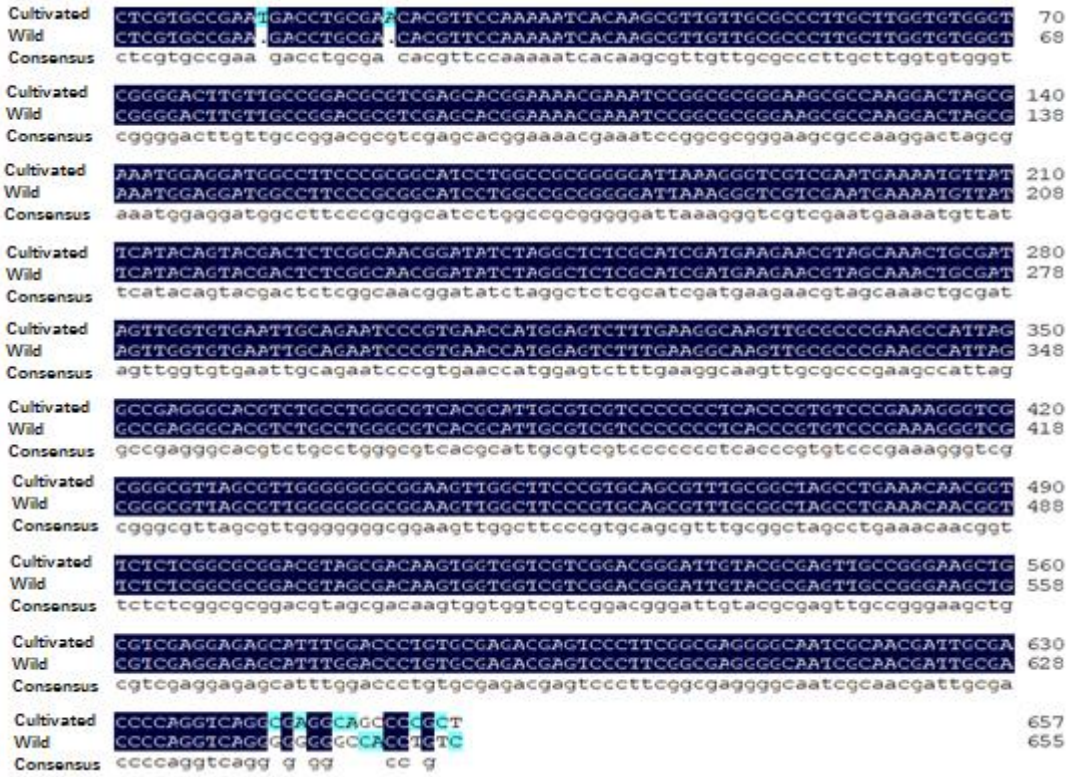

Figure 3 Comparison of the ITS sequences between the cultivated and wild types of *M. hainanensis*

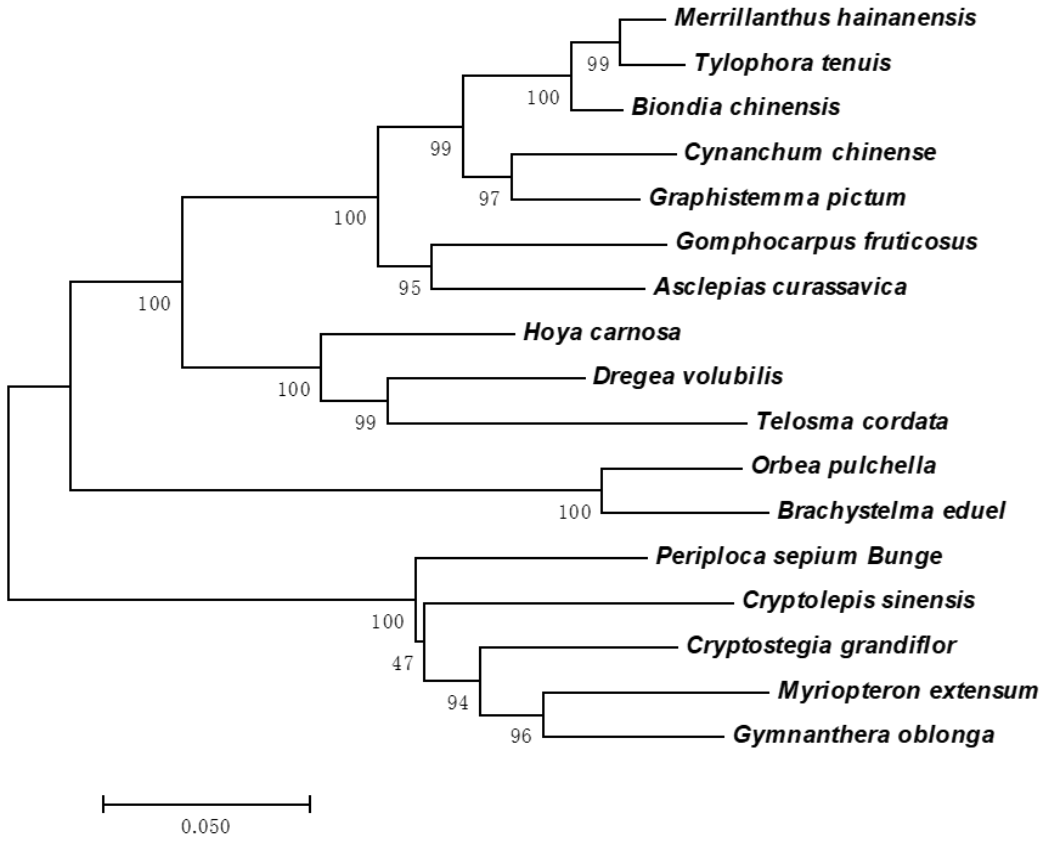

Figure 4 the phylogenetic tree based on the ITS sequences of 17 species in Asclepiadaceae family

HPLC fingerprints: 10 µL of the methanol solutions from the wild type and the cultivated type of *M. hainanensis* were injected into HPLC. The chromatograms were shown in Figure 5. The wild type and the cultivated type showed similar HPLC fingerprint pattern, and both contain 12 common peaks (1-12). Minor differences can be observed in the retention times 11-12 min and 20-22 min. Comparison of HPLC fingerprint indicated that the wild type and the cultivated type were close like each other, which is consistent to the ITS DNA study. Accordingly, cultivation can be used to enlarge this endangered species.

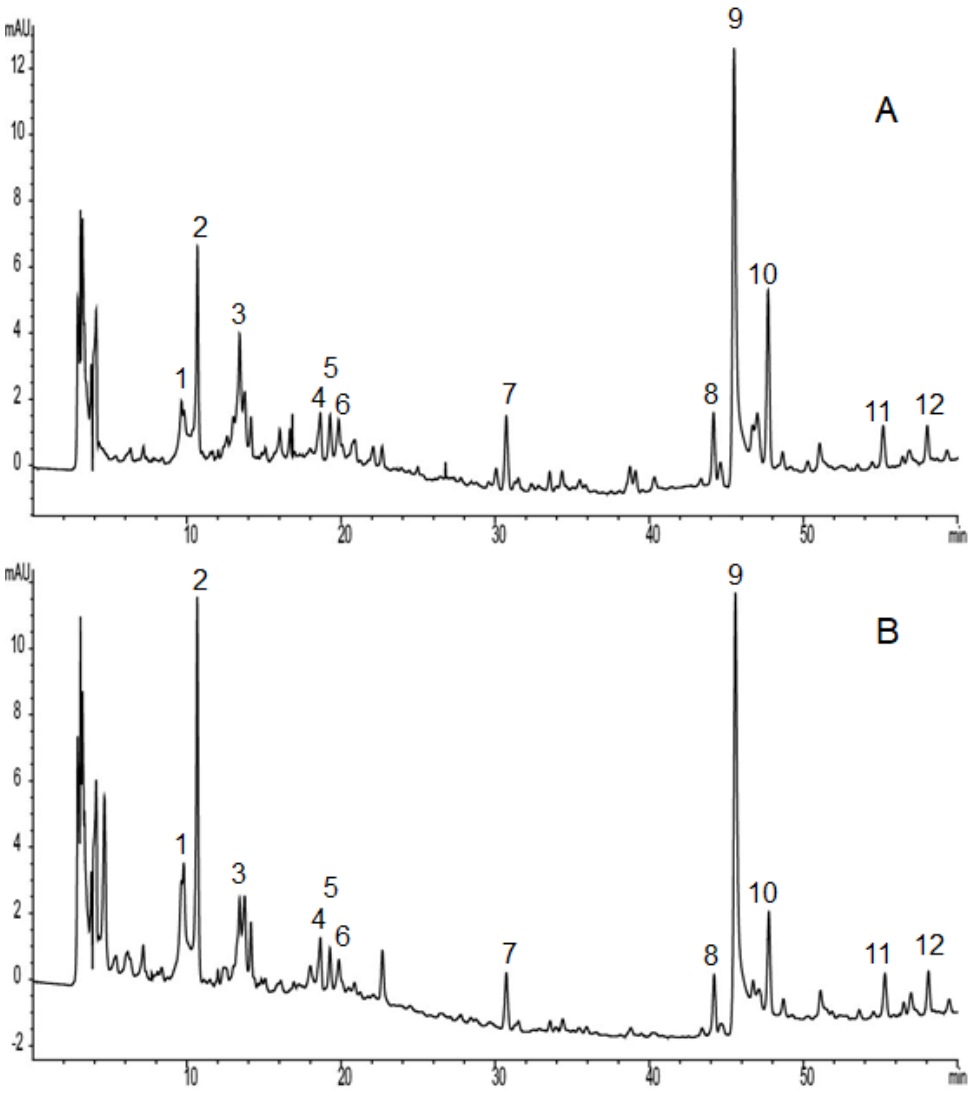

Figure 5 Comparison of the HPLC fingerprints of the wild-type (A) and cultivated-type (B) of *M. hainanensis*

DISCUSSION

Currently, *M. hainanensis* is an endangered species. Though wild species has ever been found in Burma and Cambodia, individuals cannot be found in some of the recording habitats due to modern constructions. Nowadays, this plant can only be found in Guangdong province and Hainan province of China. The Wugui mountain in Zhongshan city of Guangdong province might be the largest wildlife reserves found at present. Thus it is important to expand the population of this endangered plant. A battery of techniques has been developed for the authentication and standardization of herbal medicine in recent years. On one hand, chemical technologies were widely used because of the high efficacy in providing qualitative and quantitative assessment of the chemical composition of the material [13]. On the other hand, DNA technology is powerful in elucidating the identity of the material and the relationship of closed related species [14]. Thus it is necessary to combine both the chemical and DNA technologies. In this study, we sequenced the

internal transcribed spacer (ITS) region of nuclear ribosomal RNA genes in the wild and cultivated types of *M. hainanensis*, and compared the HPLC fingerprints of the wild and the cultivated types of this plant. The sequencing results showed that the cultivated and wild-types of *M. hainanensis* have the same ITS sequences. Furthermore, comparison of HPLC fingerprint indicated that the wild type and the cultivated type were close like each other with twelve common peaks, which is consistent with the DNA sequencing.

CONCLUSIONS

From both the DNA and HPLC study, the cultivated and wild-types of *M. hainanensis* are close like each other.Thus, cultivation can be used to expanding the population of the endangered plant *M. hainanensis*.

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CONFLICT OF INTEREST

No conflict of interest is associated with this study.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by them. Qian-Cai Jiang and Ren-Wang Jiang designed the study and the experiments, and prepared the manuscript. Qi-Qi Wang and Shu-Qin Qin assisted in DNA experimental work and Chun-Xia Xiao and Wei Xu assisted in HPLC experimental work. Zong-Jian Tan, Pan-Pan Liu, Hong-Mei Sun, Hao-Bing Liao and Yi Wu collected the wild type and cultivated types of *M. hainanensis*.

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