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Value of FINS Depends on Choice of DNA Locus as Shown in Baihuasheshecao

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ABSTRACT

Forensically informative nucleotide sequencing (FINS) is a molecular approach to identify biological materials based on DNA sequences. It focuses on the clustering of samples with reference species through an analysis of the phylogenetic relationship among the species. The choice of loci is critical to the successful use of FINS for identification. In this study, we investigated the influence of six loci (ITS, *matK*, *rbcL*, *rps16*, *trnH-psbA* and *trnL-trnF*) on the application of FINS for the identification of traditional Chinese medicine as demonstrated in Baihuasheshecao, a traditional anti-tumor herb derived from *Hedyotis diffusa* willd. A total of 36 plant samples of *Hedyotis* species and eight samples of Baihuasheshecao commodities were collected from Hong Kong, Macao, Taiwan, mainland China and the USA. Internal transcribed spacers (ITS) sequences displayed the highest rate of formation of single species cluster (89%), followed by *rps16* (78%), *matK* (74%), *trnL-trnF* (72%), *trnH-psbA* (67%) and *rbcL* (61%). All the loci could discriminate *H. diffusa* from the common substitute species *H. corymbosa, H. pinifolia and H. tenelliflora*. FINS identification based on ITS, *trnH-psbA* and *trnL-trnF* revealed that five Baihuasheshecao commodities were genuine herb derived from *H. diffusa*, while the other three commodities were substitutes derived from *H. corymbosa*. *MatK*, *rbcL* and *rps16* could not be amplified from all commodity samples and were thus inapplicable for FINS identification of dried the samples. In conclusion, the success of FINS identification depends on the DNA loci as they show different abilities to differentiate genuine species from substitutes, to discriminate closely related species and to generate quality sequences. In this study, the eligibility of DNA locus for FINS identification of Baihuasheshecao commodities, in descending order, is ITS > *trnL-trnF* > *trnH-psbA* > *rps16* > *matK* > *rbcL*.

Key words: Baihuasheshecao, forensically informative nucleotide sequencing (FINS), *Hedyotis diffusa*, molecular authentication

INTRODUCTION

Forensically informative nucleotide sequencing (FINS) is a molecular approach to trace the source of biological material based on the informative sites in DNA sequence and clustering revealed in phylogenetic trees[(1)]. It focuses on the clustering of samples with reference species through an analysis of the phylogenetic relationship among the species[(2)]. In the past decade, FINS has been applied to identify the source of food products and herbal medicines using various DNA loci[(3-11)]. DNA of testing materials is extracted, which is followed by amplification and sequencing of a portion of DNA. This DNA sequence is compared with reference DNA sequences of the same locus and the identity of the testing material can be determined by phylogenetic analysis[(1)]. The resolution of FINS depends on the variability and the number of informative sites in the DNA sequences of testing samples and reference materials[(2)]. Since the evolutionary rates of different DNA loci vary among different taxa, it is essential to select DNA loci with sufficient variability to give appropriate resolution in FINS. In recent years, several DNA loci have been suggested by the Consortium for the Barcode of Life for barcoding every species, including mitochondrial cytochrome c oxidase 1 gene (*CO1*), chloroplast large subunit of ribulose-bisphosphate carboxylase gene (*rbcL*), chloroplast maturase K coding region (*matK*), chloroplast *trnH-psbA* intergenic spacer (*trnH-psbA*) and nuclear internal transcribed spacer (ITS)[(12-14)]. Other DNA loci, such as chloroplast *rps16* and chloroplast *trnL-trnF* region, are also commonly used to differentiate medicinal materials[(15,16)]. All these DNA loci have different success rates of PCR amplification, genetic divergence levels and identification

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values[13,17]. Consequently, it is necessary to explore the most suitable DNA loci for FINS and rate their success rates as primary DNA targets. In this study, the influence of six DNA loci (ITS, *matK*, *rbcL*, *rps16*, *trnH-psbA* and *trnL-trnF*) was compared on the application of FINS for identifying traditional Chinese medicines (TCM) using Baihuasheshecao as a demonstration.

According to the *Pharmacopoeia of the People's Republic of China*[18], Baihuasheshecao is derived from the whole plant of *Oldenlandia diffusa* (willd) Roxb., which is regarded as a synonym of *Hedyotis diffusa*[19,20]. Traditionally, it has been used for relieving 'internal heat', detoxification, facilitation of diuresis and for elimination of 'internal wetness'. Recent studies have shown that it has anti-tumor, immunomodulatory[21-24], anti-inflammatory, anti-bacterial, hepatoprotective[25,26], anti-oxidative[27-29] and neuroprotective properties[28]. In the market, there are several common substitutes derived from various species in the same genus, particularly *H. corymbosa, H. pinifolia* and *H. tenelliflora* (syn. *H. angustifolia* Cham. & Schlecht), and the species from the families Caryophyllaceae, Molluginaceae and Loganiaceae[30]. Traditional classification of *Hedyotis* showed several Sections in *Hedyotis*, including Hedyotis, Diplophragma, Dimetia, Euoldenlandia and Gonotheca[19]. More updated taxonomy review showed that the classification of *Hedyotis* is more complicated because of the reduced morphological characters and unsettled nomenclatural issues[20]. Among four *Hedyotis* species (*H. diffusa*, *H. corymbosa*, *H. pinifolia* and *H. tenelliflora*), they share similar appearances and thus are easily confused as Baihuasheshecao commodities. Among them, *H. diffusa* and *H. corymbosa* are common species distributed widely in China. The two species look very similar except for the number of axillary flowers, which may vary with geographical and environmental factors. Despite the similar morphology, the medicinal values of *H. diffusa* and *H. corymbosa* are somewhat different[25]. Consequently, correct identification of the genuine herb is important for therapeutic applications.

MATERIALS AND METHODS

I. *Samples Studied*

A total of 36 plant samples covering 19 *Hedyotis* species were collected from Hong Kong, Macao, Taiwan and mainland China. A total of 35 plant samples were collected in field and deposited in Shiu-Ying Hu Herbarium, School of Life Sciences, The Chinese University of Hong Kong. One specimen sample of *H. vestita* R. Br. (HK36764) was obtained from Hong Kong Herbarium, Agriculture, Fisheries and Conservation Department. Eight herb samples of Baihuasheshecao were purchased from herb stores in Hong Kong, mainland China and USA and were deposited in the Museum, Institute of Chinese Medicine, The Chinese University of Hong Kong (Table 1).

II. *DNA Extraction, Amplification, Cloning and Sequencing*

Approximately 10 mg of sample was subjected to DNA extraction by the modified CTAB (cetyltrimethylammonium bromide) extraction method as previously described[9]. Target DNA loci were amplified by polymerase chain reaction (PCR) in a 25 μL reaction mixture containing 17.3 μL double distilled water, 2.5 μL 10× PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 25 mM $MgCl_2$, 0.001% gelatin], 1 μL 2.5 mM dNTPs, 0.5 μL each 10 mM forward and reverse primer, 1 unit *Taq* polymerase and 1.5 μL DNA extract. The primers for ITS are RUT-ITSF1 (5′- TCG CGA GAA GTC CAC TGA -3′) and ITS4 (5′- CAC ACC GCC CGT CGC TCC TAC CGA -3′)[31]; for *matK* are matK_2F (5′- GTT CAC TAA TTG TGA AAC GT -3′) and matK_3R (5′- GAT CCG CTG TGA TAA TGA GA -3′)[32]; for *rbcL* are rbcL_Z674F (5′- TTT ATA AAT CAC AAG CCG AAA CTG GTG AAA TC -3′) and rbcL_Z1375R (5′- AAT TTG ATC TCC TTC CAT ATT TCG CA -3′)[33]; for *rps16* are rps16_F (5′- GTG GTA GAA AGC AAC GTG CGA CTT -3′) and rps16_R3 (5′- CGA TAG ACG GCT CAT TGG GAT A -3′)[33,34]; for *trnH-psbA* are trnHR (5′- CGC GCA TGG TGG ATT CAC AAA TC -3′) and psbAF (5′- GTT ATG CAT GAA CGT AAT GCT C -3′)[32]; for *trnL-trnF* are TabC (5′- CGA AAT CGG TAG ACG CTA CG -3′) and TabF (5′- ATT TGA ACT GGT GAC AC GAG -3′)[35]. PCR was performed in a thermal cycler through 32 cycles of 95°C for 45 sec, 50°C or 53°C for 1 min, and 72°C for 1 min. PCR products were analyzed by gel electrophoresis and purified by Gel-M™ Gel Extraction System (Viogene) following the manufacturer's instructions. PCR products were cloned using pGEM®-T Easy Vector System I (Promega) and transformed into *Escherichia coli* (strain DH5α) competent cells. Purified PCR products and bacterial clones were sent to either Macogen Inc. or Beijing Genomics Institute for DNA sequencing.

III. *Data Analysis*

For each DNA locus, sequences of all samples were aligned and edited using BioEdit 7.0[36] and ClustalX 2.0[37]. The evolutionary divergences among sequences were calculated under the maximum parsimony criterion for phylogenetic trees construction with bootstrap assessment by 1000 replications using MEGA5[38]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The maximum parsimony tree was obtained using the Close-Neighbor-Interchange algorithm[39] with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated.

RESULTS AND DISCUSSION

In this study, we have used six DNA loci from the nuclear and chloroplast genomes (ITS, *matK*, *rbcL*, *rps16*, *trnH-psbA* and *trnL-trnF*) for the authentication of Baihuasheshecao

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commodities using FINS approach. All the samples were successfully amplified with the six DNA loci. The only exception was *H. vestita* (YU26), which was derived from an old voucher specimen stored in Hong Kong Herbarium, Agriculture, Fisheries and Conservation Department; only ITS and *matK* were successfully amplified from this specimen (Table 1). The sizes of the amplicon were 659-669 bp for ITS, 265-475 bp for *trnH-psbA*, 853-906 for *trnL-trnF*, 636-671 bp for *rps16*, 670 bp for *rbcL* and 874 bp for *matK*. The numbers of informative sites/aligned sites were 210/689 for ITS, 215/547 for *trnH-psbA*, 176/999 for *trnL-trnF*, 94/697 bp for *rps16*, 39/670 bp for *rbcL* and 114/874 bp for *matK*.

Table 1. Plant samples and Baihuasheshecao commodities used in this study

Species / sample	Code	Collection place	GenBank accession number					
			ITS	*trnH-psbA*	*trnL-trnF*	*rps16*	*rbcL*	*matK*
Sect. Hedyotis								
Hedyotis auricularia L.	MA17*	Saikung, Hong Kong, China	HQ148794	HM640352	HM752867	HM752952	HM753037	HM753122
Hedyotis auricularia L.	MA18*	Saikung, Hong Kong, China	HQ148795	HM640353	HM752868	HM752953	HM753038	HM753123
Hedyotis pinifolia Wall. ex G.Don	MA11*	Trilho do Nordeste de Coloane, Macau, China	HQ148788	HM640346	HM752861	HM752946	HM753031	HM753116
Hedyotis pinifolia Wall. ex G.Don	YU19*	Hong Kong, China	HQ148821	HM640379	HM752894	HM752979	HM753064	HM753149
Hedyotis tenelliflora Blume	TM05*	Taimoshan, Hong Kong, China	HQ148762	HM640320	HM752835	HM752920	HM753005	HM753090
Hedyotis tenelliflora Blume	TM06*	Taimoshan, Hong Kong, China	HQ148763	HM640321	HM752836	HM752921	HM753006	HM753091
Hedyotis verticillata (L.) Lam.	MA14*	Colina da Guia Municipal Park, Macau, China	HQ148791	HM640349	HM752864	HM752949	HM753034	HM753119
Hedyotis verticillata (L.) Lam.	YU12*	Lion Rock Country Park, Hong Kong, China	HQ148815	HM640373	HM752888	HM752973	HM753058	HM753143
Hedyotis vestita R. Br.	YU26#	Hong Kong Herbarium, Hong Kong, China	HQ148827	-	-	-	-	HQ225735
Sect. Diplophragma								
Hedyotis acutangula Champ. ex Benth.	HA01*	Tailongsaiwan, Hong Kong, China	HQ148753	HM640311	HM752826	HM752911	HM752996	HM753081
Hedyotis acutangula Champ. ex Benth.	HA02*	Tailongsaiwan, Hong Kong, China	HQ148754	HM640312	HM752827	HM752912	HM752997	HM753082
Hedyotis assimilis Tutch.	BW07*	Guangzhou, China	HQ148743	HM640301	HM752816	HM752901	HM752986	HM753071
Hedyotis assimilis Tutch.	BW09*	Guangzhou, China	HQ148744	HM640302	HM752817	HM752902	HM752987	HM753072
Hedyotis bodinieri H.Lév.	TM22*	Taimoshan, Hong Kong, China	HQ148777	HM640335	HM752850	HM752935	HM753020	HM753105
Hedyotis bodinieri H.Lév.	YU11*	Taimoshan, Hong Kong, China	HQ148814	HM640372	HM752887	HM752972	HM753057	HM753142
Hedyotis bracteosa Hance	MA01*	Trilho do Nordeste de Coloane, Macau, China	HQ148778	HM640336	HM752851	HM752936	HM753021	HM753106
Hedyotis bracteosa Hance	MA02*	Trilho do Nordeste de Coloane, Macau, China	HQ148779	HM640337	HM752852	HM752937	HM753022	HM753107
Hedyotis consanguinea Hance	BX03*	Patsinleng, Hong Kong, China	HQ148752	HM640310	HM752825	HM752910	HM752995	HM753080
Hedyotis consanguinea Hance	BW20*	Patsinleng, Hong Kong, China	HQ148748	HM640306	HM752821	HM752906	HM752991	HM753076
Hedyotis loganioides Benth.	YU16*	Ma On Shan, Hong Kong, China	HQ148819	HM640377	HM752892	HM752977	HM753062	HM753147
Hedyotis loganioides Benth.	YU17*	Ma On Shan, Hong Kong, China	HQ148820	HM640378	HM752893	HM752978	HM753063	HM753148
Hedyotis longiexserta Merr. & F.P.Metcalf	BW06*	Guangzhou, China	HQ148742	HM640300	HM752815	HM752900	HM752985	HM753070
Hedyotis shiuyingiae T. Chen	TM09*	Tai Mo Shan, Hong Kong, China	HQ148764	HM640322	HM752837	HM752922	HM753007	HM753092
Hedyotis shiuyingiae T. Chen	TM19*	Tai Mo Shan, Hong Kong, China	HQ148774	HM640332	HM752847	HM752932	HM753017	HM753102
Hedyotis uncinella Hook. & Arn.	TM16*	Tai Mo Shan, Hong Kong, China	HQ148771	HM640329	HM752844	HM752929	HM753014	HM753099
Hedyotis uncinella Hook. & Arn.	TM17*	Tai Mo Shan, Hong Kong, China	HQ148772	HM640330	HM752845	HM752930	HM753015	HM753100
Hedyotis vachellii Hook. & Arn.	YU21*	Lan Tau Peak, Hong Kong, China	HQ148823	HM640381	HM752896	HM752981	HM753066	HM753151
Hedyotis vachellii Hook. & Arn.	YU22*	Lan Tau Peak, Hong Kong, China	HQ148824	HM640382	HM752897	HM752982	HM753067	HM753152

Table 1. Continued

Species / sample	Code	Collection place	GenBank accession number					
			ITS	*trnH-psbA*	*trnL-trnF*	*rps16*	*rbcL*	*matK*
Sect. Dimetia								
Hedyotis hedyotidea (DC.) Merr.	KL03*	Kei Leng, Hong Kong, China	HQ148756	HM640314	HM752829	HM752914	HM752999	HM753084
Hedyotis hedyotidea (DC.) Merr.	MA03*	Seac Pai Van Country Park, Macau, China	HQ148780	HM640338	HM752853	HM752938	HM753023	HM753108
Sect. Euoldenlandia								
Hedyotis corymbosa (L.) Lam.	MA25*	Encosta do Altinho de Coloane, Macau, China	HQ148802	HM640360	HM752875	HM752960	HM753045	HM753130
Hedyotis corymbosa (L.) Lam.	TA01*	Taiwan	HQ148757	HM640315	HM752830	HM752915	HM753000	HM753085
Hedyotis diffusa Willd.	MA19*	Lotus Bridge, Coloane, Macau, China	HQ148796	HM640354	HM752869	HM752954	HM753039	HM753124
Hedyotis diffusa Willd.	TY01*	Pai O, Lan Tau Island, Hong Kong, China	HQ148759	HM640317	HM752832	HM752917	HM753002	HM753087
Sect. Gonotheca								
Hedyotis biflora (L.) Lam.	MA08*	Jardim Lou Lim Iok, Macau, China	HQ148785	HM640343	HM752858	HM752943	HM753028	HM753113
Hedyotis biflora (L.) Lam.	YU20*	Lantau Island, Hong Kong, China	HQ148822	HM640380	HM752895	HM752980	HM753065	HM753150
Commodity sample								
-	DS01	Hangzhou, Zhejiang Province, China	HQ148828	HQ148844	HQ148861	-	-	-
-	DS03	Tianjin, China	HQ148830	HQ148846	HQ148863	-	-	-
-	DS08	Lengshuijiang, Hunan Province, China	HQ148835	HQ148851	HQ148868	-	-	-
-	DS10	Seattle, Washington State, USA	HQ148837	HQ148853	HQ148870	-	-	-
-	DS11	Guangzhou, Guangdong Province, China	HQ148838	HQ148854	HQ148871	-	-	-
-	DS12	Hong Kong, China	HQ148839	HQ148857	HQ148872	-	-	-
-	DS13	Hong Kong, China	HQ148840	HQ148858	HQ148873	-	-	-
-	DS15	Boston, USA	HQ148842	HQ148860	HQ148875	-	-	-

* Voucher specimens are stored in Shiu-Ying Hu Herbarium in the School of Life Sciences, The Chinese University of Hong Kong.
# Voucher specimen HK 36764 is stored in Hong Kong Herbarium, Agriculture, Fisheries and Conservation Department, Hong Kong SAR Government.

I. *Authentication Using FINS Approach*

The use of FINS for authentication focuses on the identity of the samples through an analysis of the molecular sequences and the phylogenetic relationship of the species concerned. The clustering of species and resolution for species identification in the phylogram is the major consideration, while the cladistics interpretation of the phylogenetic relationships among taxa is secondary[2]. The maximum parsimony tree constructed based on ITS showed that individual pairs of samples of *H. diffusa* (99%), *H. corymbosa* (99%), *H. pinifolia* (99%) and *H. tenelliflora* (99%), formed close groupings distinct from those of other species with the support of high bootstrap frequencies (Figure 1). Traditional taxonomy has classified *H. diffusa* and *H. corymbosa* in the same section of Euoldenlandia. Our results obviously showed that these two species are remotely related; similar interpretation was reported in a previous phylogenetic analysis[9]. A more thorough cladistics study is on-going with the aim to further clarify the phylogenetic relationship among the 400+ *Hedyotis* species.

Our FINS analysis indicated that five Baihuasheshecao commodities collected form Zhejiang (DS01), Tianjin (DS03), Hunan (DS08), Seattle (DS10) and Guangdong (DS11) were genuine and clustered with *H. diffusa*. In contrast, three commodities collected from Hong Kong (DS12 and DS13) and Boston (DS15) were substitutes derived from *H. corymbosa* (Figure 1). Similar conclusion was drawn from the maximum parsimony tree of *trnL-trnF*, in which the distinct clusters of *H. diffusa* (98%), *H. corymbosa* (99%), *H. pinifolia* (99%) and *H. tenelliflora* (99%) were well supported by high bootstrap frequencies, and the Baihuasheshecao commodities clustered with *H. diffusa* (DS01, DS03, DS08, DS10 and DS 11) and *H. corymbosa* (DS12, DS13 and DS15), respectively (Figure 2). For *trnH-psbA*, the distinct clusters of *H. diffusa* (100%), *H. corymbosa* (99%), *H. pinifolia* (100%) and *H. teneliflora* (91%) were clearly formed with high bootstrap support (Figure 3). FINS analysis based on *trnH-psbA* also

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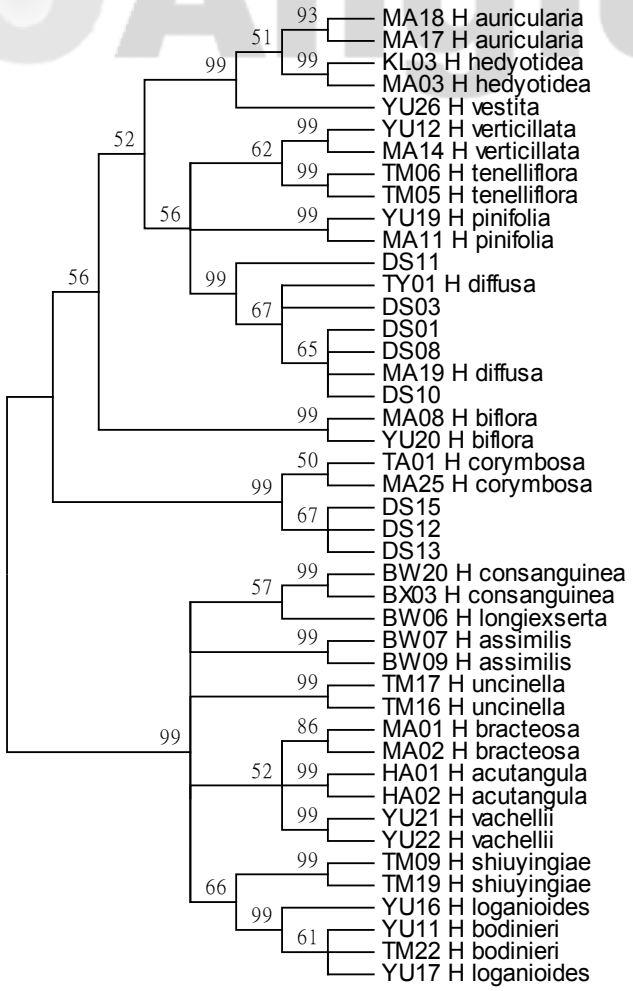

Figure 1. Maximum parsimony analysis of *Hedyotis* species based on ITS. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. A total of 44 taxa comprised with 36 plant samples of 19 *Hedyotis* species and eight samples of Baihuasheshecao commodity were included. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

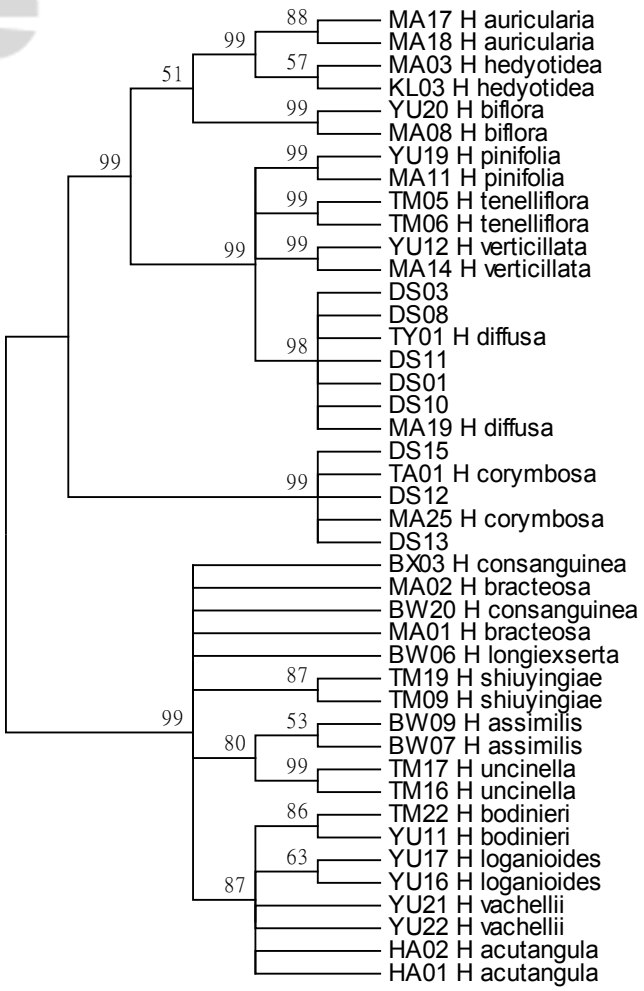

Figure 2. Maximum parsimony analysis of *Hedyotis* species based on *trnL-trnF*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. A total of 43 taxa comprised with 35 plant samples of 18 *Hedyotis* species and eight samples of Baihuasheshecao commodity were included. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

indicated that five Baihuasheshecao commodities (DS01, DS03, DS08, DS10 and DS11) were derived from *H. diffusa* and three Baihuasheshecao commodities (DS12, DS13 and DS15) are substitutes derived from *H. corymbosa* (Figure 3). The DNA loci *matK*, *rbcL* and *rps16* could be amplified in reference species only but not in Baihuasheshecao commodities, and therefore the identity of these commodities could not be revealed using these loci in this study (Figures 4-6). Generally, the DNA quality and quantity in less carefully preserved samples are low. Amplification of large DNA loci from dried commodity herbs remains very challenging.

II. *Eligibility of DNA Loci for FINS Identification*

The most crucial factor for successful FINS analysis is the choice of suitable DNA locus, which should have sufficient genetic divergences and should be easily amplified, sequenced and analyzed. In this study, all six DNA loci resolve *H. diffusa*, *H. corymbosa*, *H. pinifolia* and *H. tenelliflora* into distinct clusters (Figures 1-6). The phylogram of ITS shows the highest number of single species clusters except *H. bodinieri* and *H. longanioides* (17/19 species, 89%), followed by *rps16* (14/18 species, 78%), *matK* (14/19 species, 74%), *trnL-trnF* (13/18 species, 72%), *trnH-psbA* (12/18 species, 67%) and *rbcL* (11/18 species, 61%). In general, the higher number of single species clusters formed, the higher resolution value of FINS and the higher eligibility of the DNA loci for FINS. Consequently, the eligibility of DNA loci for FINS based on the genetic divergence is ITS > *rps16* > *matK* > *trnL-trnF* > *trnH-psbA* > *rbcL*. Amplification of the six loci was generally easy for most of the plant samples[40,41]. However, *matK*, *rbcL* and *rps16* were not amplified from the dried herbal commodities and therefore these three DNA loci were not eligible

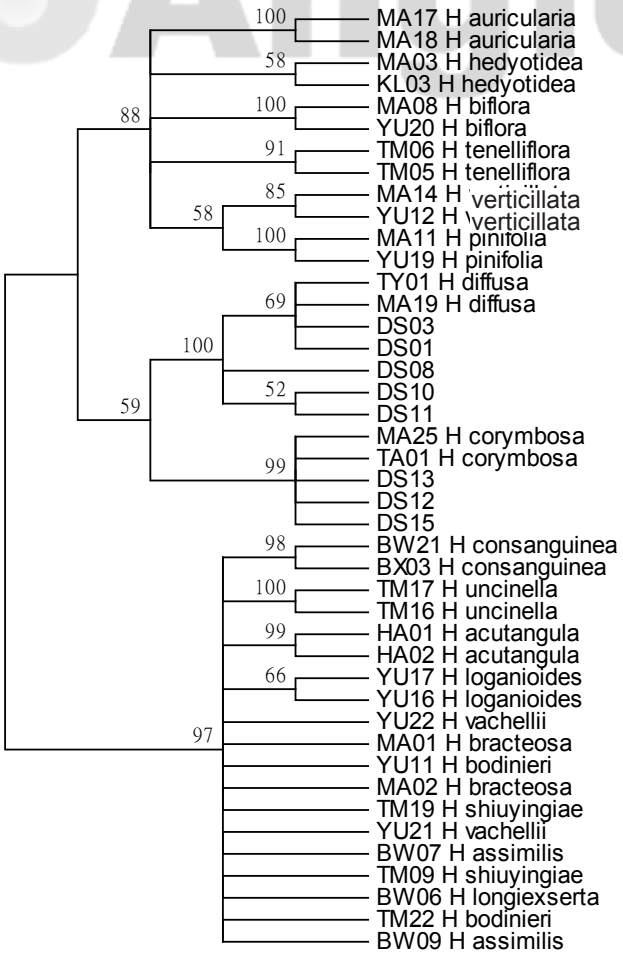

Figure 3. Maximum parsimony analysis of *Hedyotis* species based on *trnH-psbA*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. A total of 43 taxa comprised with 35 plant samples of 18 *Hedyotis* species and 8 samples of Baihuasheshecao commodity were included. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

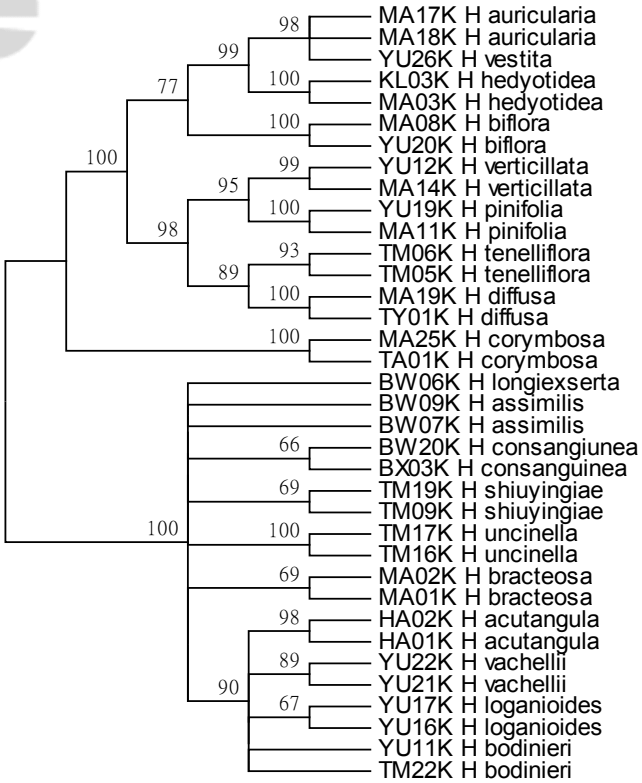

Figure 4. Maximum parsimony analysis of *Hedyotis* species based on *matK*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. A total of 36 plant samples of 19 *Hedyotis* species were included. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

for FINS identification of Baihusheshecao commodities in this study. DNA degradation may lead to poor DNA quality and quantity for subsequent molecular identification. Therefore, DNA locus with large size, such as *matK*, *rbcL* and *rps16*, may lead to unsuccessful amplification in commodity samples. Consequently, the eligibility of DNA loci for FINS identification of Baihusheshecao commodities should be ITS > *trnL-trnF* > *trnH-psbA* > *rps16* > *matK* > *rbcL*. It is worthwhile to note that fungal contamination is frequently found in herbal commodity which is improperly processed and stored, thus generating possible false positive amplification results, especially for nuclear loci such as ITS. Plant-specific primers should be designed in this case to eliminate the false positive amplification. For DNA sequencing, the presence of multiple copies, pseudogenes, and secondary structure of ITS sometimes lead to poor quality of sequence data[42-44]. *TrnH-psbA* sometimes does not generate bidirectional unambiguous sequences due to the presence of a poly-A/T structure which lowers the successful rate of DNA sequencing[45]. Although molecular cloning of *trnH-psbA* prior to DNA sequencing can improve the DNA sequencing results, the presence of large number of indels makes sequence alignment and data analysis difficult.

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REFERENCES

1. Bartlett, S. E. and Davidson, W. S. 1992. FINS (Forensically Informative Nucleotide Sequencing): A procedure for identifying the animal origin of biological specimens. Biotechniques 12: 408-411.
2. Li, M., Zhang, Y. B., But, P. P. H. and Shaw, P. C. 2011. Forensically informative nucleotide sequencing (FINS) for the authentication of Chinese medicinal materials. Chin. Med. 6: 42.
3. Chan, W. H., Ling, K. H., Chiu, S. W., Shaw, P. C. and

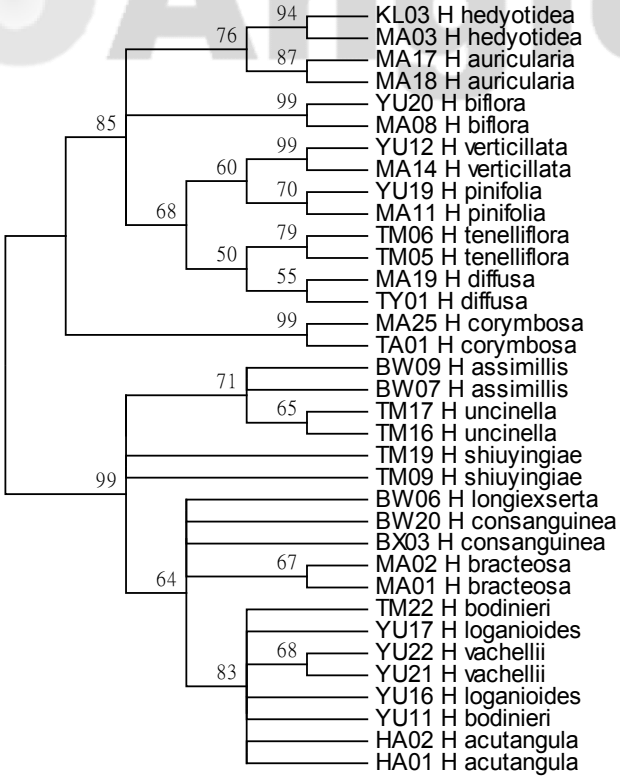

Figure 5. Maximum parsimony analysis of *Hedyotis* species based on *rbcL*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. A total of 35 plant samples of 18 *Hedyotis* species were included. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

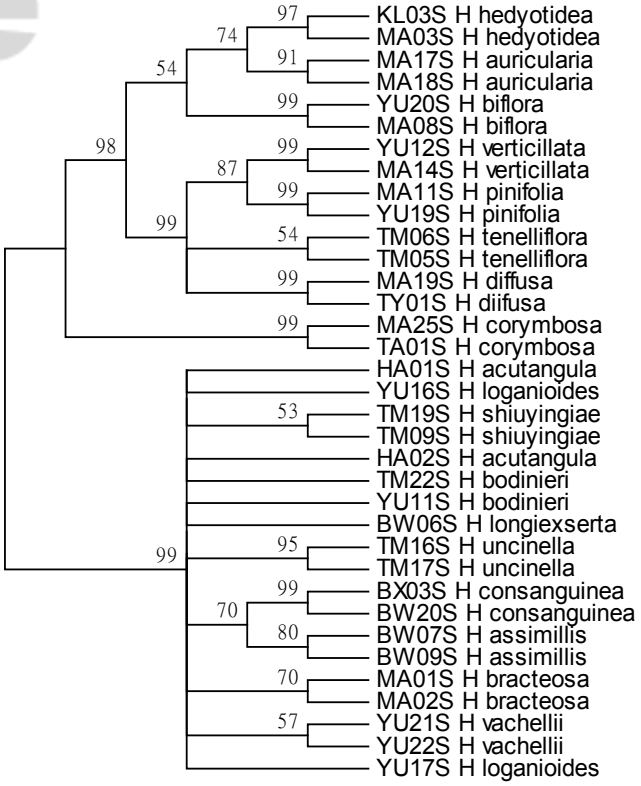

Figure 6. Maximum parsimony analysis of *Hedyotis* species based on *rps16*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. A total of 35 plant samples of 18 *Hedyotis* species were included. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

But, P. P. H. 2011. Molecular analyses of *Cordyceps gunnii* in China. J. Food Drug Anal. 19: 18-25.

4. Chan, W. H., Ling, K. H., Shaw, P. C., Chiu, S. W. and But, P. P. H. 2012. Application of FINS and multiplex PCR for detecting genuine abalone products. Food Control 23: 137-142.
5. Chapela, M. J., Sotelo, C. G., Calo-Mata, P., Pérez-Martín, R. I., Rehbein, H., Hold, G. L., Quinteiro, J., Rey-Méndez, M., Rosa, C. and Santos, A. T. 2002. Identification of *Cephalopod* species (Ommastrephidae and Loliginidae) in seafood products by forensically informative nucleotide sequencing (FINS). J. Food Sci. 67: 1672-1676.
6. Guha, S. and Kashyap, V. K. 2006. Molecular identification of lizard by RAPD & FINS of mitochondrial 16s rRNA Gene. Leg. Med. 8: 5-10.
7. Lau, D. T. W., Shaw, P. C., Wang, J. and But, P. P. H. 2001. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA. Planta Med. 67: 456-460.
8. Law, S. K. Y., Simmons, M. P., Techen, N., Khan, I. A., He, M. F., Shaw, P. C. and But, P. P. H. 2011. Molecular analyses of the Chinese herb Leigongteng (*Tripterygium wilfordii* Hook.f.). Phytochemistry 72: 21-26.
9. Li, M., Jiang, R. W., Hon, P. M., Cheng, L., Li, L. L., Zhou, J. R., Shaw, P. C. and But, P. P. H. 2010. Authentication of the anti-tumor herb Baihuasheshecao with bioactive marker compounds and molecular sequences. Food Chem. 119: 1239-1245.
10. Li, M., Ling, K. H., Lam, H., Shaw, P. C., Cheng, L., Techen, N., Khan, I. A., Chang, Y. S. and But, P. P. H. 2010. *Cardiocrinum* seeds as a replacement for *Aristolochia* fruits in treating cough. J. Ethnopharmacol. 130: 429-432.
11. Santaclara, F. J., Espiñeira, M. and Vieites, J. M. 2007. Genetic identification of squids (families Ommastrephidae and Loliginidae) by PCR-RFLP and FINS Methodologies. J. Agric. Food Chem. 55: 9913-9920.
12. Hebert, P. D. N., Cywinska, A., Ball, S. L. and deWaard, J. R. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B 270: 313-321.
13. CBOL Plant Working Group. 2009. A DNA barcode for land plants. Proc. Natl. Acad. Sci. U.S.A. 106: 12794-12797.
14. Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A. and Janzen, D. H. 2005. Use of DNA barcodes to identify flowering plants. Proc. Natl. Acad. Sci. U.S.A. 102: 8369-8374.
15. Li, R. and Dao, Z. 2011 Molecular authentication of the traditional Tibetan medicinal plant, *Meconopsis impedita*.

African J. Biotechnology 10: 7493-7496.
16. Li, M., Cao, H., But, P. P. H. and Shaw, P. C. 2011. Identification of herbal medicinal materials using DNA barcodes. J. Syst. Evol. 49: 271-283.
17. Li, M., Wong, K. L., Chan, W. H., Li, J. X., But, P. P. H., Cao, H. and Shaw, P. C. 2012. Establishment of DNA barcodes for the identification of the botanicals sources of Chinese 'cooling' beverage. Food Control 25: 758-766.
18. Pharmacopoeia Commission. 2010. Pharmacopoeia of the People's Republic of China 2010. Chemical Industry Press. Beijing, P. R. China.
19. Ko, W. C. 1999. *Hedyotis*. In "Flora Republicae Popularis Sinicae". Vol. 71. pp. 32-77. Luo, X. R. ed. Science Press. Beijing, P. R. China.
20. Chen, T. and Taylor, C. M. 2011. *Hedyotis*. In "Flora of China". Vol. 19. pp. 147-174. Wu, Z. Y., Raven, P. H. and Hong, D. H. eds. Science Press, Beijing, and Missouri Botanical Garden Press, St. Louis.
21. Chan, K. 1995. Progress in traditional Chinese medicine. Trends Pharmacol. Sci. 16: 182-187.
22. Chung, H. S., Jeong, H. J., Hong, S. H., Kim, M. S., Kim, S. J., Song, B. K., Jeong, I. S., Lee, E. J., Ahn, J. W., Baek, S. H. and Kim, H. M. 2002. Induction of nitric oxide synthase by *Oldenlandia diffusa* in mouse peritoneal macrophages. Biol. Pharm. Bull. 25: 1142-1146.
23. Shan, B. E., Zhang, J. Y. and Du, X. N. 2001. Immunomodulatory activity and anti-tumor activity of *Oldenlandia diffusa* in vitro. Zhongguo Zhong Xi Yi Jie He Za Zi 21: 370-374.
24. Yoshida, Y., Wang, M. Q., Liu, J. N., Shan, B. E. and Yamashita, U. 1997. Immunomodulating activity of Chinese medicinal herbs and *Oldenlandia diffusa* in particular. Int. J. Immunopharmacol. 19: 359-370.
25. Lin, C. C., Ng, L. T., Yang, J. J. and Hsu, Y. F. 2002. Anti-inflammatory and hepatoprotective activity of peh-hue-juwa-chi-cao in male rats. Am. J. Chin. Med. 30: 225-234.
26. Wang, Y. L., Zhang, Y., Fang, M., Li, Q. J., Jiang, Q. and Ming, L. 2005. Anti-inflammatory and antibacterial effects of total flavones of *Oldenlandia diffusa* Willd. Chin. Pharmacol. Bull. 21: 348-350.
27. Kim, D. H., Lee, H. J., Oh, Y. J., Kim, M. J., Kim, S. H., Jeong, T. S. and Baek, N. I. 2005. Iridoid glycosides isolated from *Oldenlandia diffusa* inhibit LDL-oxidation. Arch. Pharm. Res. 28: 1156-1160.
28 Kim, Y., Park, E. J., Kim, J., Kim, Y., Kim, S. R. and Kim, Y. Y. 2001. Neuroprotective constituents from *Hedyotis diffusa*. J. Nat. Prod. 64: 75-78.
29. Lu, C. M., Yang, J. J., Wang, P. Y. and Lin, C. C. 2000. A new acylated flavonol glycoside and antioxidant effects of *Hedyotis diffusa*. Planta Med. 66: 374-377.
30. Su, X. J. 1999. Authentication of Baihuasheshecao and their adulterants. Modern Chinese Medicine. 1: 44.
31. White, T. J., Burns, T., Lee, S. and Taylor, J. 1990. PCR Protocols: A Guide to Methods and Applications. pp. 315-322. Innis, M., Gelfand, D., Sninsky, J. and White, T. eds. Academic: San Diego, U.S.A.
32. Sang, T., Crawford, D. J. and Stuessy, T. F. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). Am. J. Bot. 84: 1120-1136.
33. Bremer, B., Bremer, K., Heidari, N., Erixon, P., Olmstead, R. G., Anderberg, A. A., Källersjö, M. and Barkhordarian, E. 2002. Phylogenetics of asterids based on 3 coding and 3 noncoding chloroplast DNA makers and the utility of non-coding DNA at higher taxonomic levels. Mol. Phylogenet. Evol. 24: 274-301.
34. Oxelman, B., Liden, M. and Berglund, D. 1997. Chloroplast *rps16* intron phylogeny of the tribe Silenese (Caryophyllaceae). Plant Syst. Evol. 206: 393-410.
35. Taberlet, P., Gielly, L., Patou, G. and Bouvet, J. 1991. Universal primers for amplification of three non–coding regions of chloroplast DNA. Plant Mol. Biol. 17: 1105-1109.
36. Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. (Oxf.) 41: 95-98.
37. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.
38. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731-2739.
39. Nei, M. and Kumar, S. 2000. Molecular Evolution and Phylogenetics. p.128. Oxford University Press. New York, U.S.A.
40. Kårehed, J., Groeninckx, I., Dessein, S., Motley, T. and Bremer, B. 2008. The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoceae. Mol. Phylogent. Evol. 49: 843-866.
41. Guo, X., Simmons, M. P., But, P. P. H., Shaw, P. C. and Wang, R. J. 2011. Application of DNA barcodes in *Hedyotis* L. (Spermacoceae, Rubiaceae). J. Syst. Evol. 49: 203-212.
42. Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S. and Donoghue, M. J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. 82: 247-277.
43. Alvarez, I. and Wendel, J. F. 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29: 417-434.
44. Stuessy, T. F. 2009. Plant taxonomy: the systematic evaluation of comparative data. pp. 273-294. Columbia University Press. New York, U.S.A.
45. Zhu, Y. J., Chen, S. L., Yao, H., Tan, R., Song, J. Y., Luo, K. and Lu, J. 2010. DNA barcoding the medicinal plants of the genus *Paris*. Yao Xue Xue Bao 45: 376-382.