

# Molecular identification of medicinal herbs, *Oldenlandia diffusa* and *Oldenlandia corymbosa* based on nrDNA ITS region sequence

Yan-Lin Sun · Dong Wang · Myung-Hun Yeom · Duck-Hee Kim · Han-Gon Kim · Soon-Kwan Hong

Received: 19 August 2011 / Accepted: 5 September 2011  
© Korean Society for Plant Biotechnology

**Abstract** The medicinal herb *Oldenlandia diffusa* is known as a folk medicine for the treatment of hepatitis, sore throat, appendicitis, malignant tumors and urethral infection in Southern China and Korea. Another species *O. corymbosa*, is also used for the therapy of the similar conditions, however, only *O. diffusa* is referred to the medicinal herb by Chinese Pharmacopoeia. Due to their similar morphology, *O. diffusa* and *O. corymbosa* are often misidentified. To easily identify *O. diffusa* from *O. corymbosa*, the phylogenetic utility of nuclear ribosomal DNA (nrDNA) internal transcribed spacers (ITS) were investigated among different *O. diffusa* and *O. corymbosa* populations in Korea. The nrDNA ITS sequence of *O. diffusa* contained 791 bp, with GenBank accession number of JF837601-JF837602. The nrDNA ITS sequence of *O. corymbosa* was 785–786 bp, with GenBank accession number of JF837603-JF837611. The results showed that there are some certain divergences in the ITS region sequence between both species, even among different populations of the same species. Particularly, *O. corymbosa* ST-4 population showed the highest dissimilarity of the ITS region sequence with other nine populations of *O. corymbosa* and two populations of *O. diffusa*. This consequence makes us further understand the molecular diversification between *O. corymbosa* and *O. diffusa*, and help to

promote the correct use and safety.

**Keywords** *Oldenlandia diffusa*, *Oldenlandia corymbosa*, internal transcribed spacer, ribosomal RNA gene, molecular analysis

## Introduction

The herb of *Oldenlandia diffusa* (also known by alternate *Hedyotis diffusa*) is known as a traditional, folk medicine in Southern China (Liang et al. 2008). It is traditionally used as an herbal tea component, and for the treatment of sore throat, infectious diseases and malignant tumors (Shan et al. 2001; Kitani et al. 2011; Wu et al. 2009; Xu et al. 1997). Another species, *O. corymbosa* (also known by alternate *Hedyotis corymbosa*) belonging to the same genus with *O. diffusa*, is also used in modern Chinese practice for the treatment of viral infections, cancer, some syndromes involving toxic heat, acne, boils, skin ailments, appendicitis, hepatitis, eye diseases and bleeding (Corbineau et al. 1980; Lin et al. 1987). Due to their similar morphological characters (Zhao and Li 2007), both *O. diffusa* and *O. corymbosa* are frequently confused with each other in the wholesale and food markets (Liang et al. 2006; Li et al. 2010). However, according to the Chinese Pharmacopoeia (The State Pharmacopoeia Committee of China 2005), the medical herb refers only to the dried herb of *O. diffusa* (Liang et al. 2006). The misusing has attracted an increasing attention on their use security. To make sure of their secure uses, clearer classification between these both medicinal herbs is urgently needed.

Many investigations have been reported to attempt to distinguish *O. diffusa* from *O. corymbosa* using various classification methods such as fluorescence microscopy (Lau et al. 2004), morphological analysis (Ko 1999; Liang et al. 2006), chemical fingerprints (Liang et al. 2008) and DNA

Y.-L. Sun  
School of Life Sciences, Ludong University, Yantai, 264-025, China

Y.-L. Sun · D. Wang · S.-K. Hong (✉)  
Department of Bio-Health Technology, College of Biomedical Science, Kangwon National University, Chuncheon, Kangwon-Do, 200-701, Korea  
e-mail: soonkwan@kangwon.ac.kr

M.-H. Yeom · D.-H. Kim · H.-G. Kim  
R&D Center, Amore Pacific Co. Yongin, 446-929, Korea

S.-K. Hong  
Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon, Kangwon-Do, 200-701, Korea

sequence analysis (Hao et al. 2004; Liu et al. 2004; Guo et al. 2011). Particularly, Liang et al. (2006) reported that the two species could be distinguished with each other according to transverse sections of stems; Liu et al. (2004) newly designed *O. diffusa*-specific PCR primers for internal transcribed spacer 2 (ITS2) region amplification to successfully identify *O. diffusa* from adulterated *O. corymbosa*; Guo et al. (2011) investigated five DNA barcoding regions including ITS, *matK*, *petD*, *trnH-psbA* and *rbcL* among 25 *Oldenlandia* species and constructed a phylogenetic tree. Nowadays, simple conventional morphological methodology for taxon classification has not satisfied the increasing demands of more refined identification. Compared to the conventional identification methods, molecular identification based on DNA cloning and sequencing has been considered a more efficient, faster means. By more rapidly providing more phylogenetic information, molecular marker identification method has been more frequently utilized for phylogenetic analysis. Among various molecular identification methods, the sequence analysis of ITS, a region of ribosomal RNA is considered as an effective method to give a higher number of informative sites at species levels (Kitani et al. 2009; Long et al. 2004).

In this study, we investigated the 18S-26S nuclear ribosomal DNA (nrDNA) ITS1 and ITS2 sequence variations between *O. diffusa* and *O. corymbosa*, to distinguish these two similar species in the molecular levels. This work would provide more ITS sequence information on *O. diffusa* and *O. corymbosa* species, help further understand the nucleotide variations among different Korea populations of *O. diffusa* and

*O. corymbosa*, and then identify the identification efficiency based on ITS region sequence between the both species.

## Materials and Methods

### Plant materials

Nine populations of *O. corymbosa* and two populations of *O. diffusa* collected from Korea, were used in this study. The populations were named as ST-L, ST-S, ST-1, ST-2, ST-3, ST-4, ST-5, ST-6 and ST-7 of *O. corymbosa*, and LT-L and LT-S of *O. diffusa*, for the convenience of sample management. Mature seeds of *O. diffusa* and *O. corymbosa* were provided by Research and Development Center of Amore-Pacific Co., 446-729, Yongin, Korea. The mature seeds of both species were surface-sterilized with 70% ethanol for 30 seconds and then rinsed with sterile water for five times. The sterilized seeds were then grown in a pot containing a mixture of sterilized soil and vermiculite (v:v, 3:1) in a green house condition with 40~60% relative wet rate, a 16/8 h (light/dark) photoperiod, and 24±2°C. After germination, the fresh leaf tissue was sampled and used for DNA extraction. The total or partial ITS sequences of *O. corymbosa* and *O. diffusa* have been published by GenBank, NCBI, with the accession numbers shown in Table 1.

### DNA extraction and PCR amplification

DNA was extracted from fresh leaves of *O. diffusa* and *O.*

**Table 1** ITS region sequence information about GenBank accession number, the length (bp) and (G+C) % of ITS1, 5.8S and ITS2 of 9 populations of *O. corymbosa* and 2 populations of *O. diffusa*

Species	Population	Accession number	ITS1 integrity degree	ITS1 Length (bp)	ITS1 (G+C) %	ITS2 integrity degree	ITS2 Length (bp)	ITS2 (G+C) %	5.8S integrity degree	5.8S Length (bp)	5.8S (G+C) %
<i>O. diffusa</i>	LT-L	JF837601	+	328	54.27	+	300	60.00	+	163	53.37
	LT-S	JF837602	-	232	58.62	-	263	61.22	+	163	53.37
<i>O. corymbosa</i>	ST-L	JF837603	+	318	55.66	+	304	60.20	+	163	53.37
	ST-S	JF837611	-	233	58.80	-	268	61.19	+	163	53.37
	ST-1	JF837605	-	232	59.05	-	279	59.86	+	163	53.37
	ST-2	JF837606	-	233	57.94	-	263	61.60	+	163	53.37
	ST-3	JF837607	-	226	59.29	-	279	60.57	+	163	53.37
	ST-4	JF837608	-	152	62.50	-	259	58.69	+	163	53.37
	ST-5	JF837609	-	232	59.05	-	268	61.19	+	163	53.37
	ST-6	JF837610	-	234	58.97	-	279	59.86	+	163	53.37
	ST-7	JF837604	+	322	51.86	+	301	60.80	+	163	53.37

+ presents complete sequence, - presents partial sequence

*corymbosa* using the modified cetyltrimethylammonium bromide (CTAB) method described by Kang et al. (1998). Common ITS primer sets ITS5, 5'-GAA AGT AAA AGT CGT AAC AAG G-3' and ITS4, 5'- TCC TCC GCT TAT TGA TAT GC-3' were used to amplify the nrDNA ITS region including ITS1, 5.8S rRNA, ITS2 regions (White et al. 1990). PCR amplification was conducted using this set of primers with the following program: 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and a final extension step at 72°C for 1.5 min. All PCR products were purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at MACRO GEN Advancing through Genomics (Korea).

### Sequence analysis

Homology analysis of the 11 ITS region sequences were performed by DNAMAN 6.0 software according to the Observed Divergency distance method. The phylogenetic tree of *O. corymbosa* and *O. diffusa* were also constructed based on neighbor joining method using DNAMAN 6.0.

## Results and Discussion

### Length and G + C content

Total ITS region sequences were successfully amplified by PCR using common primer sets, ITS4/ITS5 from DNAs of nine *O. corymbosa* populations and two *O. diffusa* populations. Due to inefficient reading with nucleotide overlapping appeared in sequencing results, only three total ITS region

sequences were obtained from one *O. diffusa* population, LT-L and two *O. corymbosa* populations, ST-L and ST-7 (Table 1). According to the total ITS region sequences of *O. diffusa* LT-L population, the size of the total ITS region was 791 bp, of which 328 bp belonged to ITS1 region, 163 bp belonged to 5.8S rRNA gene, and 300 bp belonged to ITS2 region. For *O. corymbosa*, the total ITS region accounted for 785 or 786 bp varying in ST-L or ST-7 population, respectively, of which ITS1 region accounted for 318~322 bp, ITS2 region for 301~304 bp, and 5.8S rRNA region for 163 bp with the identical size of *O. diffusa* (Table 1). Not only sequence lengths but (G+C) contents (%) of ITS region sequences were different among different populations of *O. diffusa* and *O. corymbosa*. The (G+C) contents (%) ranged from 54.27% to 58.62% in ITS1 region of *O. diffusa* and ranged from 51.86% to 62.50% in *O. corymbosa* (Table 1). Most of ITS2 regions showed higher (G+C) contents (%) than ITS1 regions, ranged from 60.00% to 61.22% in *O. diffusa* and ranged from 58.69% to 61.60% in *O. corymbosa*, except for ST-4 and ST-6 populations in *O. corymbosa* (Table 1). In this study, expectedly, the (G+C) contents (%) of the 5.8S rRNA region were absolutely identical to 53.37% among 11 sequences of both species.

### Homology analysis of *O. diffusa* and *O. corymbosa*

Among nine *O. corymbosa* populations and two *O. diffusa* populations, ST-4 population of *O. corymbosa* had the lowest homology of 93.5% with ST-S population, according to the total ITS region sequence analysis (Table 2). There was a number of nucleotides and length variations in ITS1 region (Fig. 1A, Table 3), but this dissimilarity was mainly owing to the sequence variation of the two populations in ITS2 region, with a low homology of 89.1% (Table 4).

**Table 2** Homology matrix (%) of 11 total ITS region sequences

Population	LT-L	LT-S	ST-1	ST-2	ST-3	ST-4	ST-5	ST-6	ST-7	ST-L	ST-S
LT-L	100										
LT-S	97.0	100									
ST-1	96.3	99.8	100								
ST-2	99.2	98	98.2	100							
ST-3	94.5	97.3	97.4	96	100						
ST-4	94.0	94.0	94.0	94.2	93.9	100					
ST-5	97.0	100	99.8	98.0	97.4	94.0	100				
ST-6	96.1	100	99.9	98.0	97.3	94.0	100	100			
ST-7	94.9	95.7	95.9	96.0	97.6	93.9	95.9	95.8	100		
ST-L	98.4	96.4	96.3	98.5	94.1	94.0	96.4	96.1	95.3	100	
ST-S	98.8	96.7	96.8	98.5	94.6	93.5	96.6	96.7	94.9	97.7	100

**Table 3** Homology matrix (%) of 11 ITS1 region sequences

Population	LT-L	LT-S	ST-1	ST-2	ST-3	ST-4	ST-5	ST-6	ST-7	ST-L	ST-S
LT-L	100										
LT-S	99.7	100									
ST-1	91.5	91.5	100								
ST-2	99.1	99.1	91.5	100							
ST-3	92.0	92	96.4	92.9	100						
ST-4	95.4	95.4	93.4	95.4	95.4	100					
ST-5	95.7	95.7	93.7	96.5	95.1	95.4	100				
ST-6	95.7	95.7	93.3	96.6	94.7	95.4	100	100			
ST-7	74.8	73.3	100	89.6	96.4	93.4	91.7	91.3	100		
ST-L	80.1	79.6	91.0	97.0	90.6	94.1	93.9	94	72.5	100	
ST-S	95.7	95.7	93.3	96.6	94.7	95.4	100	100	91.3	94	100

**A**

LT-L	TG.TCGAATCCTGCAAAC.GACCCCGAACACGTTTTTATA	398
LT-S	-----	158
ST-1	g-----g-----a-----acg-----	79
ST-2	-----g-----t-----	80
ST-3	g-gc-----g-----t-aa-a-----acg-----	73
ST-4	-----	0
ST-5	g-g-----g-----t-----a-----acg-----	79
ST-6	g-g-----g-----t-----a-----acg-----	81
ST-7	g-----tg-----g-----aa-a-----acg-----	70
ST-L	-----g-----	82
ST-S	-----	79

**B**

LT-L	CCCTCGACCATGACCCCGAGGTCAGGCGGGACTACCCGCTG	955
LT-S	-----	715
ST-1	-----a-----cc-----ac-a-c-----	636
ST-2	-----ac-----	637
ST-3	-----a-----cc-----ac-a-c-c-----	630
ST-4	-a-a-a-c-a-a-c-a-a-----	559
ST-5	-----a-----cc-----ac-a-c-----	636
ST-6	-----a-----cc-----ac-a-c-----	638
ST-7	-----	627
ST-L	-----	639
ST-S	-----a-a-a-ca-----c-a-----	636

**Fig. 1** DNA alignment of *O. diffusa*, *O. corymbosa* using total ITS region. Partial alignment of the total ITS regions showing notable base polymorphisms among different populations of *O. diffusa* and *O. corymbosa* in ITS1 (A) and ITS2 (B) regions. The letters on the top line represent the base letters in sequence alignment. Sequences different from the uppermost sequence are marked as their own sequences in lowercase. ‘-’ represents the base being identical to the uppermost sequence. ‘.’ represents gap

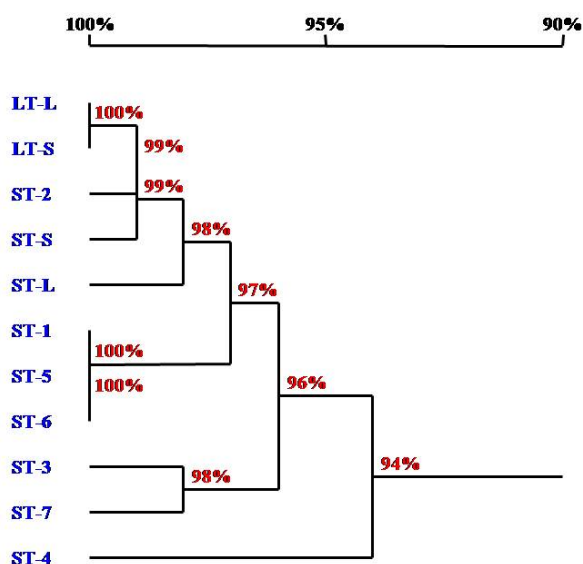
ST-4 population showed relatively low homology with all the other populations of either *O. corymbosa* or *O. diffusa*, except for ST-S population, according to the total ITS region sequence analysis (Table 2). Similar results were obtained from homology compared with the ITS2 region sequence. ST-4 population not only showed relatively low homology with other populations, but also appeared more information sites when aligned with other ITS2 region sequences (Fig. 1B). However, according to the ITS1 region sequence analysis, ST-4 population showed 93.4~95.4%

identity with other populations, while ST-7 having 100% homology with ST-1, showed relatively low homology with other populations, especially two populations of *O. diffusa* and ST-L population (Table 3). These results were not congruent with the evidence of Li et al. (2010) that *O. diffusa* and *O. corymbosa* could be clearly authenticated by either ITS1 or ITS2 region sequence. In the report of Li et al. (2010), *O. diffusa* showed its own informative nucleotide sites, different from those of *O. corymbosa*, through which even confused *O. diffusa* or *O. corymbosa* starting materials could be authenticated. In our study, both LT-L and LT-S populations belonged to the same species, *O. diffusa*, however, did not show identical nucleotide sequences in the ITS1 and ITS2 regions (Fig. 1A, B). In addition, ST-1 and ST-7 both belonging to the same species, *O. corymbosa*, showed 100% identity in ITS1 region, as well as ST-5, ST-6, and ST-S (Table 3). In the ITS2 region, ST-1, ST-5, ST-6, and ST-S populations of *O. corymbosa* showed 100% identity with each other (Table 4).

Summarizing the above results, it concluded that the ITS1 and ITS2 region sequences of both *O. diffusa* populations used in this experiment were intra-specifically conserved, while those of *O. corymbosa* populations were not intra-specifically conserved. This suggestion was not supported by the evidence of Guo et al. (2011) that the total ITS region of *O. diffusa* and *O. corymbosa* were intra-specifically conserved but inter-specifically variable, thus, enough to differentiate these both species. In our study, the nucleotide variations appeared in the total ITS regions of different *O. corymbosa* populations, and some populations showed more similar ITS regions with *O. diffusa* populations, e.g. ST-2 had higher homology with LT-T and LT-S compared to other *O. corymbosa* populations.

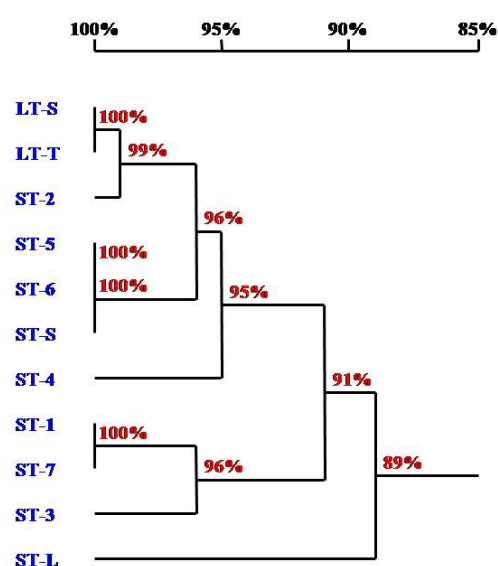
**Table 4** Homology matrix (%) of 11 ITS2 region sequences

Population	LT-L	LT-S	ST-1	ST-2	ST-3	ST-4	ST-5	ST-6	ST-7	ST-L	ST-S
LT-L	100										
LT-S	100	100									
ST-1	95.6	94.3	100								
ST-2	98.9	98.9	98.1	100							
ST-3	93.4	92.1	97.8	96.2	100						
ST-4	89.8	89.8	89.1	89.5	88.7	100					
ST-5	96.3	96.3	100	98.1	97.8	89.1	100				
ST-6	95.6	94.3	100	98.1	97.8	89.1	100	100			
ST-7	96.7	91.7	96.1	98.1	96.8	89.8	95.9	96.1	100		
ST-L	98.5	91.7	93.9	98.9	91.8	89.8	96.3	93.9	89.4	100	
ST-S	96.3	96.3	100	98.1	97.8	89.1	100	100	95.9	96.3	100

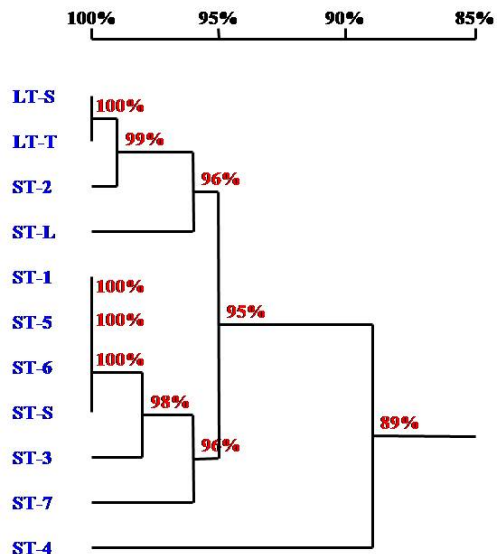
**Fig. 2** Phylogenetic tree constructed by total ITS region sequences of nine populations of *O. corymbosa* and two populations of *O. diffusa*

#### Phylogeny of *O. diffusa* and *O. corymbosa*

Based on the phylogenetic tree constructed by 11 total ITS region sequences, *O. diffusa* and *O. corymbosa* had not been anticipated divided into two clades, on the contrary, the LT-L population of *O. diffusa* population formed one monophyletic group with LT-S, near to ST-2 population of *O. corymbosa*, and ST-S population sharing 99% similarity (Fig. 2). The ST-4 population of *O. corymbosa* showed the highest dissimilarity with all populations, sharing 94% similarity according to clustering analysis of the total ITS region sequence. From the phylogenetic tree constructed by ITS1 region sequence, the ST-L population of *O. corymbosa* as the Outgroup shared 89% similarity with two

**Fig. 3** Phylogenetic tree constructed by ITS1 region sequences of nine populations of *O. corymbosa* and two populations of *O. diffusa*

monophyletic groups respectively formed by three *O. corymbosa* populations and the other *O. corymbosa* populations inter-fused with *O. diffusa*. The group simply formed by *O. corymbosa* populations shared the lowest similarity of 96%, while the other group shared 95% similarity between ST-4 and other populations (Fig. 3). In the ITS2 region phylogenetic tree, ST-4 population of *O. corymbosa* became the Outgroup, showing 89% similarity with other populations (Fig. 4). All populations of *O. diffusa* together with populations ST-2 and ST-L of *O. corymbosa* formed one monophyletic group, showing 96% identity in the lowest level, while other *O. corymbosa* populations formed another monophyletic group, showing 96% identity in the lowest level as well (Fig. 4).



**Fig. 4** Phylogenetic tree constructed by ITS2 region sequences of nine populations of *O. corymbosa* and two populations of *O. diffusa*

The clustering analysis on *O. diffusa* and *O. corymbosa* species showed that all species were not form distantly separate clusters, contrarily *O. diffusa* clade interlaced with some *O. corymbosa* populations, formed one multi-level group. This suggesting was not congruent with the evidence of Li et al. (2010) that *O. diffusa* and *O. corymbosa* could be recognized as distinct species in the genus *Oldenlandia* phylogenetic tree. The results could be comprehended by the currently available limited sampling of Asian species. To further understand the phylogeny of *O. diffusa* and *O. corymbosa*, a broader concept of samples could be investigated in the recent molecular phylogenetic analysis.

In conclusion, the total ITS sequences of *O. diffusa* and *O. corymbosa* populations collected from Korea were successfully amplified using common primer set, ITS4/ITS5. However, due to currently limited sampling of both species, *O. diffusa* and *O. corymbosa* could not yet be authenticated only based on ITS region sequences in the present study. To ensure proper use of herbal materials for efficient treatment and correct identification of the two medicinal herbs, *O. diffusa* and *O. corymbosa*, additional markers and identification methods should be applied to achieve correct authentication.

### Acknowledgements

This research was supported by Nutraceutical Bio Brain Korea 21 Project Group.

### References

- Corbineau F, Côme D (1980) Principaux paramètres de la germination des graines d'*Oldenlandia corymbosa* L. (Rubiaceae tropicale). *Physiol Vég* 18:259-273
- Guo X, Simmons MP, But PPH, Shaw PC, Wang RJ (2011) Application of DNA barcodes in *Hedyotis* L. (Spermacoaceae, Rubiaceae). *J Syst Evol* 49:203-212. doi:10.1111/j.1759-6831.2011.00130.x
- Hao MG, Liu ZQ, Wang JL (2004) Application of the sequences of rDNA ITS to identify Chinese crude drug *Hedyotis diffusa*. *J Anhui Norm Univ (Nat Sci)* 27:188-191
- Kang HW, Cho YG, Yoon UH, Eun MY (1998) A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed. *Plant Mol Biol Rep* 16:1-9
- Kårehed J, Groeninckx I, Dessein S, Motley TJ, Bremer B (2008) The phylogenetic utility of chloroplast and nuclear DNA markers and the phylogeny of the Rubiaceae tribe Spermacoaceae. *Mol Phylogenet Evol* 49:843-866. doi:10.1016/j.ympev.2008.09.025
- Kitani Y, Shu Z, Batkhui J, Sanchir C, Komatsu K (2011) Genetic diversity of *Ephedra* plants in Mongolia inferred from internal transcribed spacer sequence of nuclear ribosomal DNA. *Biol Pharm Bull* 34: 717-726
- Kitani Y, Shu Z, Batkhui J, Sanchir C, Komatsu K (2011) Genetic diversity of *Ephedra* plants in Mongolia inferred from internal transcribed spacer sequence of nuclear ribosomal DNA. *Biol Pharm Bull* 34:717-726
- Kitani Y, Zhu S, Omote T, Tanaka K, Batkhui J, Sanchir C, Fushimi H, Mikage M, Komatsu K (2009) Molecular analysis and chemical evaluation of *Ephedra* plants in Mongolia. *Biol Pharm Bull* 32:1235-1243
- Ko WC (1999) *Hedyotis*. In: Luo XR (ed) *Flora Republicae Popularis Sinicae*. Science Press, Beijing, vol 71, pp 32-77
- Lau P, Peng Y, Zhao Z (2004) Microscopic identification of Chinese patent medicine (1) - Wu Zi Yan Zong Wan. *Nat Med* 58:258-265
- Li M, Jiang RW, Hon PM, Cheng L, Li LL, Zhou JR, Shaw PC, But PP (2010) Authentication of the anti-tumor herb Baihuas-heshcao with bioactive marker compounds and molecular sequences. *Food Chem* 119:1239-1245. doi:10.1016/j.foodchem.2009.09.113
- Liang ZT, He MF, Fong WF, Jiang ZH, Zhao ZZ (2008) A comparable, chemical and pharmacological analysis of the traditional Chinese medicinal herbs *Oldenlandia diffusa* and *O. corymbosa* and a new valuation of their biological potential. *Phytomedicine* 15:259-267. doi:10.1016/j.phymed.2008.01.003
- Liang ZT, Jiang ZH, Leung KSY, Peng Y, Zhao ZZ (2006) Distinguishing the medicinal herb *Oldenlandia diffusa* from similar species of the same genus using fluorescence microscopy. *Microsc Res Tech* 69:277-282. doi:10.1002/jemt.20312
- Lin CC, Chen FY, Namba T (1987) Development of crude drug resources from Taiwan: pharmacognostical studies on a Chinese crude drug. *Shoyakugaku Zasshi* 41:180-188

- Liu ZQ, Hao MG (2005) Determination of rDNA ITS sequences in *Hedyotis diffusa*. Shaanxi J Tradl Chin Med 26:167-169
- Liu ZQ, Hao MG, Wang JL (2004) Application of allele-specific primer in the identification of *Hedyotis diffusa*. Zhong Yao Cai 27:484-487
- Long CF, Kakiuchi N, Takahashi A, Komatsu K, Cai SQ, Makage M (2004) phylogenetic analysis of the DNA sequence of the non-coding region of nuclear ribosomal DNA and chloroplast of *Ephedra* plants in China. Planta Med 70:1080-1084. Doi:10.1055/s-2004-832651
- Shan BE, Zhang JY, Du XN (2001) Immunomodulatory activity and anti-tumor activity of *Oldenlandia diffusa* in vitro. Zhongguo Zhong Xi Yi Jie He Za Zhi 21:370-374
- The State Pharmacopoeia Committee of China (2005) The appendix of Chinese pharmacopoeia. The Chemical Industry Press, Beijing, vol I, pp 22
- White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols—a guide to methods and applications. Academic Press, San Diego, Calif, pp 315-322
- Wu PK, Tai WCS, Liang ZT, Zhao ZZ, Hsiao WLW (2009) Oleanolic acid isolated from *Oldenlandia diffusa* exhibits a unique growth inhibitory effect against *ras*-transformed fibroblasts. Life Sci 85:113-121. Doi:10.1016/j.lfs.2009.04.025
- Xu GJ, Xu LS, Wang ZT (1997) Species systematization and quality evaluation of commonly used Chinese traditional drugs. South-China Edition, Fujian: Fujian Science and Technology Publisher, Fuzhou, China, vol 3-4
- Zhao ZZ, Li YS (2007) Easily confused Chinese medicines in Hong Kong. Hong Kong: Chinese Medicine Merchants Association Ltd., Hong Kong