Application of Species-specific PCR in the Identification of *Hedyotis diffusa* Willd

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Abstract: *Hedyotis diffusa* Willd is an important traditional Chinese medicine but its adulteration is frequent. The aim of this paper is to provide a pair of the species-specific PCR primers and an effective method which may be used to identify *H. diffusa* by the molecular biological measure. *Hedyotis diffusa* Willd and its adulterants are collected from the commercial markets. DNA sequences of the internal transcribed spacer (ITS) from 9 species are significantly different from one another. Based on rDNA ITS sequences of all samples, a pair of species-specific primers, B174 and B53, are designed to authenticate *H. diffusa* from the other species. The results indicate that a 207 bp DNA fragment is amplified from *H. diffusa*, whereas no any fragment is amplified from the other 8 species under the same reaction condition. The primers designed in the present study are highly specific for *H. diffusa*. They could be used as key components in the *H. diffusa* identification kit.

Key words: *Hedyotis diffusa* Willd, Species-specific primer, Authentication

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物种特异性 PCR 在中药材白花蛇舌草鉴定中的应用

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[摘要] 提供中药材白花蛇舌草物种特异性鉴定引物以及建立简便、高效的特异性鉴定方法和研制鉴定试剂盒. 为此收集目前市场上出现的所有白花蛇舌草正伪品样品共 9 种,并测定所有样品的核糖体基因 ITS 区核苷酸序列,在此基础上设计一对物种特异性引物 B174 和 B53,特异性地鉴定白花蛇舌草. 表明用这对引物扩增白花蛇舌草,获得 207 bp 的特异性产物,而其它伪混品样品没有阳性产物. 可以认为用白花蛇舌草特异性鉴定引物 B174 和 B53 的所配制的鉴定试剂盒具有很好的应用前景.

[关键词] 白花蛇舌草,物种特异性引物,鉴定

0 Introduction

Hedyotis diffusa Willd is recognized as the original plant of Baihuasheshecao, a well-known traditional Chinese medicine. It grows widely in the south of the Yangtze River in China. The plant contains anthraquinones, lerpenoids, steroids, organic acid, polysaccharides, and so on [1]. Thus it exerts effects of inducing diuresis on

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reducing edema and removing toxic heat after it has been taken in clinic. Its extract has immuno-modulating activity and anti-tumor activity in vitro through stimulating the immune system to kill or engulf tumor cells [2].

Baihuasheshecao, at times, has been reported to be substituted for or adulterated by *Hedyotis tenelliflora*, *Hedyotis corymbosa* and *Hedyotis pinifolia* of Rubiaceae, *Sagina japonica*, *Stellaria alsine* and *Arenaria serpyllifolia* of Caryophyllaceae, and *Mollugo pentaphylla* and *Mollugo costata* of Mollugiaceae in markets [3,4]. Specially, the members of the *Hedyotis* genus show close resemblance to *H. diffusa*. The existence of these adulterants or substitutes jeopardizes the therapeutic value of *H. diffusa* in medicinal usages. Traditional ways of visual inspection and microstructure and chemical analyses are insufficient in detecting many of these adulterants [3,4], necessitating the development of molecular tools. Here, we describe sequencing of internal transcribed spacer regions (ITS) of rDNA in 9 species. Then, we utilize the sequence differences to design a pair of species-specific primers for identifying traditional Chinese medicine, Baihuasheshecao.

1 Materials and Methods

1.1 Samples

All materials were collected in China (Table 1). The dried plants were from Jiangsu Institute for Drug Control, Nanjing, China. The voucher specimens were identified by Prof. X Y Ding and deposited in Institute of Animal Molecular Biology, Yancheng Teachers College.

1.2 Isolation of DNA

The dried plants were frozen with liquid nitrogen and ground into powder, and genomic DNA was extracted as described previously [5].

1.3 Designing of species - specific primer

Sequences of rDNA ITS region from 9 species were determined. These sequences were aligned by the CLUSTAL X (1.8) program and the polymorphic sites were hunted for among the sequences.

The results showed that No. 174 base in ITS - 1 and No. 53 base in ITS - 2 region of *H. diffusa* are different from those in other 8 species´sequences. If the No. 174 and 53 bases of *H. diffusa* were used respectively as an end-base of up-and down-stream primer sequence (3´end) for polymerase chain reaction (PCR) in other species, this unmatched end-base should affect the progression of Taq polymerase and primer extension, and lead to amplification failure. Based on this assumption, a species-specific primer pair (B174 and B53) for a 207 bp fragment was designed by PrimerSelet software (Fig. 1)

Tab 1 Samples used in the present study

Species	Code	Source	Condition	Date of collection
Hedyotis diffusa	BH	Xiamen, Fujian	Fresh	2002/07/20
		Guangdong	Dried	1999/07
Hedyotis tenelliflora	QH	Guangdong	Dried	1999/07
Hedyotis corymbosa	SF	Guangdong	Dried	1999/07
Hedyotis pinifolia	SY	Guangdong	Dried	1999/07
Sagina japonica	QG	Huangshan, Anhui	Fresh	2002/06/27
Stellaria alsine	QS	Huangshan, Anhui	Fresh	2002/06/27
Arenaria serpyllifolia	ZZ	Huangshan, Anhui	Fresh	2002/06/27
Mollugo pentaphylla	SM	Hainan	Dried	No record
Mollugo costata	DL	Hainan	Dried	No record

1.4 PCR amplification

In order to exclude possible false negative, another pair of primers for an about 600 bp (different in different species) fragment from ITS region were designed. The upstream primer is P1 (5´- CGTAACAAGGTTTCCGTAGGTGAAC - 3´), and the downstream primer is P2 (5´- TTATTGATATGCTTAAACTCAGCGGG - 3´). The P1 and P2 primer pair was used to amplify and sequence in *H. diffusa*, *S. japonica*, *S. alsine* and *A. serpyllifolia*, while two pairs of primers (P1 and P3, P4 and P2) were used in *H. tenelliflora*, *H. corymbosa*, *H. pinifolia*,

M. pentaphylla and M. costata (Fig. 1). The P3 primer is 5′-GCTACGTTCTTCATCGAT-3′, and the P4 primer is 5′-CCATCAAGTCTTTGAACGCAA-3′. The PCR reaction followed that of the previous report [6].

1.5 Sequencing

PCR products were purified with a DNA purification kit (Shanghai Watson Bioengineering Inc.) according to the manufacturer's instruction, and then sequenced with BigDye™ on an ABI 310 Genetic Analyzer.

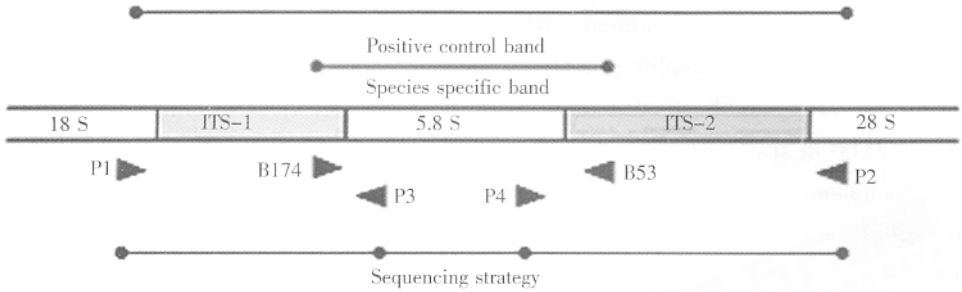

Fig.1 Diagrammatic illustration of rDNA ITS region

2 Results

2.1 Comparison of the ITS sequences

By PCR, a fragment of about 187-247 bp in the ITS-1 region and 219-244 bp in the ITS-2 region was amplified successfully. Direct sequencing of each sample was carried out and the sequences of the 9 species were deposited in the GenBank of NCBI under the following accession numbers: AY(438312~438325).

The aligned results showed 254 bps as consensus length, of which 80 (31.5%) bps were identical and 75 (29.5%) were similar and thus a total 39% were dissimilar in rDNA ITS-1 region. Similarly, they are 255 bps, 52 (20.4%), 83 (32.5%), and 47.1% respectively in ITS-2 region. There was a stretch of about 44 bps completely absent in the ITS-1 region of 4 species from *Hedyotis* genus and *M. costata*. Within the *Hedyotis* genus of Rubiaceae, the percentage differences between *H. diffusa* and other species range from 16% to 17% in the ITS-1 region, and 16% to 45.9% in the ITS-2 region. Thus the inter-specific variation in ITS regions in the 9 species is very high.

2.2 Authentication of Species-specific PCR

The about 600 bp fragment was successfully amplified from every specimen but no band in blank control by using P1 and P2 (Fig. 2), which demonstrates that the DNA extracted from each species can be utilized for further identification. After the B174 and B52 primers were used to amplify the 207 bp fragment from *H. diffusa* (Fig. 2), the specificity of the primer pair was verified. The PCR products appeared in the fresh plant and dried crude drug of *H. diffusa* (Fig. 2, 9 and 10). In contrast, there was no band in other species. This indicates that the primer pair is species-specific.

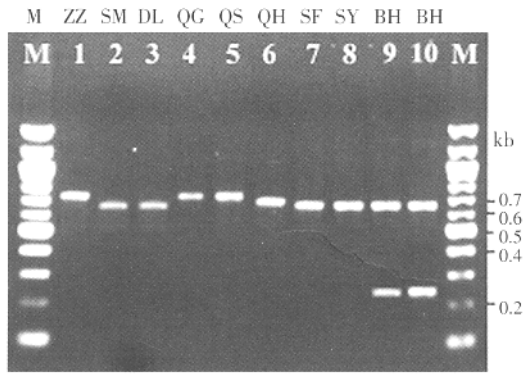

Fig. 2 Comparison of amplification patterns for 207 and around 600 bp fragments from all the samples

M: size marker, 9, 10: the dried crude drug and fresh plant of *H. diffusa*. All of codes(1-10) are given in table 1.

3 Discussion

The ITS region is widely applied in authentication and phylogenetic analysis of Chinese medicine [6]. Many copies of the rDNA are present in each plant genome. Within individuals and species, rRNA genes are highly homologous as the consequence of the homologous recombination and gene conversion [7].

In this research, the high-specific primers (B174 and B53) were designed to authenticate *H. diffusa*. The specific primer set was also used to amplify genomic DNA from 8 other different plant species but none of the species could show the expected amplification product. Positive controls for all DNA templates produced a DNA fragment about 600 – 660 bp in size, indicating that all templates are qualified for PCR. In the authentication of *H. diffusa*, all DNA templates from the medicinal materials are amplified by using the species-specific primer pair. The genuine *H. diffusa* would be identified if evident amplified band were observed.

Although the genuine *H. diffusa* can be clearly distinguished from its adulterants by comparing the DNA sequences, DNA sequencing is a time and cost consuming method. Therefore, based on the DNA sequence data of rDNA ITS region from *H. diffusa* and its adulterants, we designed a pair of specific primers to identify *H. diffusa* by PCR. Moreover, the method had also been successfully employed to authenticate crude drugs of tortoise plastrons, gecko and *Dendrobium officinale* [8-10]. In conclusion, the primers designed could be used as key components in *H. diffusa* identification kit.

DNA of crude drugs is usually degraded during preparation, transportation and storage of the medicinal materials. Taking this trait into account, the specific primers designed was employed to amplify about 207 bp fragment. Therefore, it would not impact the amplification of diagnostic fragment due to genomic DNA degradation, and be practically advantageous to apply. Furthermore, DNA fragment amplified with the designed primers also can't be less than 200 bp, otherwise which would be difficult to distinguish from primer dimers.

[References]

[1] Lu P, Dai Q H. Summarization on the chemical constituents of *Oldenlandia diffusa* Willd [J]. Journal of Beijing Polytechnic University, 2000, 26(3): 68 – 72.

[2] Shan B E, Zhang J Y, Du X N, et al. Immuno-modulatory activity and anti-tumor activity of *Oldenlandia diffusa* in vitro [J]. Chin J Integra Trad and West Med, 2001, 21(5): 370 – 374.

[3] Li X, Ju W J, Cui H X, et al. Identification of *Oldenlandia diffusa* (Wild.) Roxb. and its adulterants [J]. China J Chin Meter Med, 1996, 21(8): 460 – 470.

[4] Pan J Q. Pharmacognostical identification of *Oldenlandia diffusa* (Wild.) Roxb. and its adulterants[J]. J Chin Med Mater, 1995, 18(7): 344.

[5] Cai J N, Zhou K Y, Xu L S, et al. Ribosomal DNA ITS sequence analyses of Cnidium monnieri from different geographical origin in China [J]. Acta Pharm Sin, 2000, 35(1): 56 – 59.

[6] David T – W L, Shaw P C, Wang J, et al. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA [J]. Planta Med, 2001, 67(5): 456 – 460.

[7] Hillis D M, Dixon M T. Ribosomal DNA: molecular evolution and phylogenetic inference [J]. Q Rev Biol, 1991, 66(4): 411 – 453.

[8] Liu Z Q, Wang Y Q, Zhou K Y, et al. Study on highly specific diagnostic PCR traditional Chinese medicine tortoise plastron and its original animals [J]. Acta Pharm Sin, 1999, 34(12): 941 – 945.

[9] Liu Z Q, Wang Y Q, Zhou K Y, et al. Authentication of Chinese crude drug, Gecko, by allele-specific diagnostic PCR [J]. Planta Med, 2001, 67(4): 385 – 387.

[10] Ding X, Wang Z, Zhou K, et al. Allele-specific primers for diagnostic PCR authentication of *Dendrobium officinale* [J]. Planta Med, 2003, 69(6): 587 – 588.

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