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Development of a reliable DNA method for quality control of Glehnia littoralis and its industrial extracts

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Abstract

As a medicinal and edible plant, *Glehnia littoralis* has been widely recognized for its comprehensive nutritional properties and pharmaceutical effects, but it has also been the target of fraudulent practices. To overcome the difficulties in unanimous polymorphism exploitation of the adulterants and DNA degradation of industrial extracts, ribosomal ITS and chloroplast trnL-F gene sequences were targeted for molecular analysis. Based on the developed single nucleotide polymorphism (SNP) markers, two species-specific PCR systems were respectively established and proved to be effective for molecular discrimination of *G. littoralis* from its adulterants. The SNP marker exploited in trnL-F region was able to detect 0.1% of intentional adulteration of genomic DNA with a detection limit of 0.01 ng, and it is effective for botanical origin authentication of *G. littoralis* industrial extracts either in single or mixed plant samples. Compared with the other molecular protocols so far used, the established DNA method enabled the accurate authentication and standardization of routine work for quality control of *G. littoralis* and its industrial products.

Introduction

Glehniae Radix is the dried root and rhizome of *G. littoralis* Fr. Schmidt ex Miq, a perennial herb growing at coastal dunes in Eatsern Asia. Glehniae Radix has been widely used as a diaphoretic, antipyretic, and analgesic for the treatment of respiratory and immune-related diseases in Asian countries (Tamura et al. 2022). More recently, the pharmaceutical effects of *G. littoralis* have been demonstrated by a host of studies, including bidirectional regulation of immunity, anti-inflammatory, anti-radiation, anti-oxidation, anti-mutation, and other functions (Jing et al. 2022; Ng et al. 2004; Wu et al. 2018; Yoon et al. 2010; Yuan et al. 2002). It was widely used to develop a variety of popular drugs, food, functional foods, and skin care products (Choe et al. 2019), which prove the vast potential of *G. littoralis*. However, with the increasing market demand and the beach destruction, overharvesting of *G. littoralis* has already threatened the existence of this wild species and resulted in frequent fraudulent adulteration. For example, Glehniae Radix is often adulterated with the roots of *Adenophora stricta, Aucklandia lappa Decne, Codonopsis lanceolata, Codonopsis tangshen*, and *Silene conoidea* due to their similarity in appearance. The substitutions and unscrupulous adulterations profoundly affects the efficacy and safety of *G. littoralis*, in some cases lead to herbal poisoning. Therefore, a reliable and accurate authentication method for botanical origin authenticity control is clearly desirable to ensure the safety and quality of *G. littoralis* commercial products.

Traditional methods to identify *G. littoralis* in the market mainly rely on morphological and histological characteristics, including traits identification and microscopic inspection (Chen et al. 2021). These methods are simple, but it is difficult to identify processed materials when they are in powdered form or in shredded pieces. With the development of modern analytical techniques, a variety of physicochemical analysis methods, including high performance liquid chromatography (HPLC) coupled with photodiode array (Seo et al. 2016), fourier-transform infrared spectroscopy (FT-IR) (Jing et al. 2022), and thin-layer chromatography (Su et al. 2013) have been used to evaluate the quality of *G. littoralis* products.

Nevertheless, these products undergo a series of processing procedures resulting in the alteration of the marker compounds contents. Additionally, such approaches are difficult to distinguish closely related species, many of which contain similar chemical components.

Compared with traditional and chemical analysis methods, DNA-based technologies are less affected by the physiological conditions, processing state, and environmental factors (Samuel 2021). The major practical advantages of rich polymorphism, genetic stability, and high accuracy associated with DNA markers have enhanced their widespread use in medicinal herb identification (Kiran et al. 2010; Vannozzi et al. 2018). Wang et al. (2016) investigated the genetic diversity within and among natural populations of *G. littoralis* using SRAP markers and found that high levels of genetic variation are present within and among populations. Besides, the DNA barcoding technique has also allowed for the genetic identification of *G. littoralis* by analyzing unknown sample sequences against known database sequences. Zhu et al. (2015) discriminated *G. littoralis* from its adulterants using DNA barcoding method based on ITS2 sequences. However, in routine work, DNA barcoding technology needs repeat operations of barcode amplification, sequencing, and clustering analysis of the sample to be tested for each identification. In this study, we respectively exploited *G. littoralis*-specific SNP markers from ITS and chloroplast trnL-F regions, and developed a highly sensitive protocol that can be used in the authentication of *G. littoralis* as well as its commercial products.

1 Materials And Methods

1.1 Materials

The plant materials of *G. littoralis* and its five adulterants (*A. stricta*, *A. lappa Decne*, *C. lanceolata*, *C. tangshen*, and *S. conoidea*) were sampled from different locations in China. All the voucher specimens were identified and deposited in School of Life Sciences, Yantai University. The commercial extracts of *G. littoralis* and its five adulterants were purchased from different biotechnology and pharmaceutical companies. The samples and their origins are shown in Table 1.

Table 1
Plant samples used in this study.

Species/Extracts	Voucher	Origin	Number of samples
Glehnia littoralis	BS-01	Shandong	10
Adenophora stricta	NS-02	Jiangsu	10
Aucklandia lappa Decne	YM-03	Yunnan	10
Codonopsis lanceolata	LY-04	Jilin	10
Codonopsis tangshen	CT-05	Chongqing	10
Silene conoidea	MP-06	Shandong	10
Glehnia littoralis extract	BT-01	Shanxi	8
Adenophora stricta extract	NT-01	Shanxi	8

1.2 Method

1.2.1 DNA Isolation

The root samples of *G. littoralis* and its five adulterants were cut into pieces and ground to a fine powder in liquid nitrogen. Genomic DNA was isolated from approximately 30 mg of root powder using a Hi-DNAsecure Plant Kit (Tiangen, China) according to the manufacturer's instructions. DNA extraction of commercial extracts of above 6 species was performed by using a Magnetic Universal Genomic DNA Kit (Tiangen, China), following the manufacturer’s protocols.

1.2.2 PCR Amplification and Sequencing

According to the taxonomic levels of *G. littoralis* and its five adulterants, ribosomal ITS and chloroplast trnL-F spacer regions were selected for DNA polymorphism exploitation. The primer pairs for PCR amplification of ITS and trnL-F spacer regions were ITSP1 (5’-TACCGATTGAATGRTCCG-3’) and ITS4R (5’-TCCTCCGCTTATTGATATGC-3’), trnL (5’-CGAAATCGGTAGACGCTACG – 3’) and trnF (5’-ATTTGAACTGGTGACACGAG-3’), respectively (Moon et al. 2017; Tanaka et al. 2020). PCR amplification was carried out in a total volume of 20 μL containing approximately 20 ng of genomic DNA, 5 μM each of the forward and reverse primers, 10 μL of 2×Tap Plus PCR Mix (Tiangen, China). The reaction was performed in a T100 Thermal Cycler (Bio-Rad, USA) with the following cycling parameters: one cycle of pre-denaturation at 94°C for 4 min; 33 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final extension of 7 min at 72°C. PCR products were purified by a PCR Purification Kit (TransGen, China) and sequenced in both directions with PCR-derived primers on an automatic DNA sequencer (ABI PRISM 3700, USA) by using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA).

1.2.3 Sequencing Analysis and Design of Species-Specific Primers

The DNA sequences of ITS and trnL-F regions from the bidirectional sequencing results were compiled using SeqMan software. The sequences were submitted to Basic Local Alignment Search Tool against the NCBI sequence database, and the query sequences were identified to species level with the highest similarity. Multiple sequence alignment was conducted by using Clustal Omega program (Sievers et al. 2011). Species-specific primer pairs were designed according to the specific SNP sites of *G. littoralis* and its five adulterants. The primers were designed using Primer Premier 5 program, and unfavorable factors including false priming, hairpin, and cross dimer were minimized to ensure amplification specificity and efficiency.

1.2.4 Multiplex Species-Specific PCR

Molecular discrimination of *G. littoralis* from its five adulterants were conducted by multiplex species-specific PCR with two primer pairs designed in ITS and trnL-F regions, respectively. Multiplex species-specific PCR reaction mixture of ITS region contained 10 ng of template DNA, 0.25 μM of BSF, 0.25 μM of BSR, 0.5 μM of ASF, 0.5 μM of ASR, and 10 μL of 2×Tap Plus PCR Mix (Tiangen, China) to a total volume of 20 μL. PCR was conducted using 1 cycle of 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 65°C, 30 s at 72°C, and a final extension at 72°C for 5 min. For multiplex species-specific PCR reaction of trnL-F region, a 20 μL reaction mixture comprising 10 ng of template DNA, 0.25 μM of BTF, 0.25 μM of BTR, 0.75 μM of NTF, 0.75 μM of NTR, and 10 μL of 2×Tap Plus PCR Mix (Tiangen, China). The reaction profiles were identical with that of ITS region, except that the annealing temperature was 58°C. PCR products were electrophoresed and visualized on 1% agarose gels stained with ethidium bromide. The species-specific fragments were purified by a Gel Extraction kit (TransGen, China) followed by sequencing to confirm their sequences.

1.2.5 Limit of Detection Test and Application on Commercial Extracts

To avoid false negative results, chloroplast trnL-F region was selected for botanical origin authentication of commercial extracts because *G. littoralis* can produce longer specific fragment than that of the adulterants in trnL-F region. A total amount of 10 ng binary DNA mixtures containing 0.1%, 1%, 2%, 5%, 10%, 50% of non-target DNA were respectively used for limit of detection test. To confirm the practicality of the method, genomic DNAs extracted from commercial extracts of the six species were also used as templates for multiplex species-specific PCR. The reactions were carried out with the same reaction mixtures and thermal cycling parameters as described in 1.2.4, except that the cycling number was added to 40, to mitigate the adverse effect of DNA degradation of commercial extracts.

2 Results

2.1 Sequence Analysis

The ribosomal ITS sequences of *G. littoralis* and its five adulterants, *A. lappa Decne*, *C. lanceolata*, *S. conoidea*, *C. tangshen*, and *A. stricta*, were determined to be 609 bp, 647 bp, 664 bp, 632 bp, 663 bp, and

714 bp, respectively. As had been expected, multiple sequence alignment results showed that a host of DNA polymorphisms were detected in ITS region. In service of our objective to develop a DNA marker for discrimination of *G. littoralis* from its five adulterants, we tried to exploit polymorphic sites that unanimously specific to the five adulterants. As shown in Fig. 1, G. *littoralis* contains AT at nucleotide position 43–44, but the five adulterants were replaced with CA or TA in the same position. Likewise, the five adulterants contain nucleotide G in the 321st position, while *G. littoralis* was replaced with C at the same location. Similar to ITS region, the chloroplast trnL-F sequences also showed a variety of different sizes among *G. littoralis* and its five adulterants resulting from nucleotide insertions/deletions. The PCR products of *G. littoralis*, *A. lappa Decne*, *C. lanceolata*, *S. conoidea*, *C. tangshen*, and *A. stricta*, were determined to be 993 bp, 955 bp, 1021 bp, 1060 bp, 999 bp, and 990 bp, respectively. Three SNP sites that unanimously specific to the five adulterants were exploited, which located at nucleotide positions 88 (C/T), 281 (G/A), and 506 (C/A) (Fig. 2). Therefore, species-specific primers for molecular discrimination of *G. littoralis* and its five adulterants can be designed based on these SNP sites discovered both in ITS and trnL-F regions.

2.2 Species-Specific Primer Design

Based on the species-specific SNP sites delineated in 2.1, two specific primer pairs were designed for molecular discrimination of *G. littoralis* and its five adulterants in ITS and trnL-F regions, respectively. In ITS region, primers ASF and ASR were designed for specific identification of the five adulterants based on the SNP sites located at nucleotide positions 43, 44, and 321. Primers BSF and BSR were designed for specific authentication of *G. littoralis* according to *G. littoralis*-specific sequences compared with that of the five adulterants (Fig. 1). In chloroplast trnL-F region, primers NTF and NTR were designed for specific identification of the five adulterants based on the SNP sites located at nucleotide position 88. According to the *G. littoralis*-specific SNP sites located at nucleotide positions 281 and 506, primers BTF and BTR were respectively designed (Fig. 2). The sequences of these specific primer pairs were shown in Table 2. The substitutions of A for C in primer NTF and of A for T in primer BTR, were additional mismatches introduced deliberately to ensure reliable allelic specificity for target species.

Table 2
Sequences of specific primers

Location	Primer	Nucleotide sequence*	Introduced mismatch
ITS	ASF	5'-TCATTGTCGAAACCTGCA**CA**-3'	
	ASR	5'-AGAGCCGAGATATCCGTTG**C**-3'	
	BSF	5'-TCTTGTCCACAAACCACTCA-3'	
	BSR	5'-GTTCCTGGAGCTCGCTAGG-3'	
trnL-F	NTF	5'-TTCAAATTCAGAGAAACAC**T**-3'	C→A
	NTR	5'-TCCATTGAGTCTCTGCACCT-3'	
	BTF	5'- ACTTCCGAGAAGGATATG**CG**-3'	
	BTR	5'- TCGTCCGATTAATTAGTTCA**G**-3'	T→A

*Bolded nucleotides indicate the species-specific SNP sites at the 3’ terminal. Underlined bases indicate the deliberate mismatches.

2.3 Molecular Authentication of G. littoralis

Two species-specific primer pairs designed in ITS and trnL-F regions were respectively used in multiplex PCR for molecular discrimination of *G. littoralis* from its five adulterants. In ITS region, the combination of two primer pairs amplified different fragment patterns for *G. littoralis* and its five adulterants. As shown in Fig. 3, the *G. littoralis* samples produced unique bands with size of 187 bp, and the five adulterants, *A. lappa Decne*, *C. lanceolata*, *S. conoidea*, *C. tangshen*, and *A. stricta*, generated their species-specific amplicons with sizes of 295 bp, 295 bp, 280 bp, 295 bp, and 313 bp, respectively. Similarly, with the two species-specific primer pairs designed in trnL-F region, *G. littoralis* samples amplified unique fragments with size of 211 bp, *A. lappa Decne*, *C. tangshen*, and *A. stricta* produced species-specific bands of 125 bp, and *C. lanceolata* and *S. conoidea* generated their amplicons of 106 bp (Fig. 4). As shown in Fig. 3 and Fig. 4, no fragment was amplified when the same primer pairs were cross-challenged against DNAs from non-target species, and the small differences in the amplicon sizes among five adulterants will not interfere with the authentication of *G. littoralis* on an agarose gel. Molecular discrimination of *G. littoralis* were repeated many times with the samples listed in Table 1, and the results were further validated with expected reproducibility. Therefore, the developed multiplex species-specific PCR was effective for molecular discrimination of *G. littoralis* from its five adulterants.

2.4 Limit of Detection Test and Botanical Origin Authentication of Commercial Extracts

The absence of the longer PCR product on an agarose gel could be explained by the template DNA degradation, something that will be less visible for the shorter fragment. To avoid false negative results,

chloroplast trnL-F region is selected for botanical origin authentication of commercial extract because the five adulterants yielded shorter amplicons than *G. littoralis*. A total amount of 10 ng binary DNA mixtures containing 0.1%, 1%, 2%, 5%, 10%, 50% of non-target DNA were respectively checked by the developed multiplex species-specific PCR. As shown in Fig. 5, as the content of non-target DNA increased, species-specific bands of the adulterant gradually became brighter. The results showed that the developed method could detect 0.1% of adulteration in *G. littoralis* with a detection limit of 0.01 ng. To confirm the practicality of the developed method in botanical origin authentication, genomic DNAs of commercial extracts were subjected to the developed PCR assay. As shown in Fig. 6, the commercial extracts yielded their species-specific amplicons as described in Fig. 4 which representing their specific botanical origins. For the extract mixtures of and its adulterants, two species-specific fragments were amplified. Therefore, this approach is effective for botanical origin authentication of commercial extracts of and can be used for quality control of *G. littoralis* industrial products.

3 Discussion

In recent years, scientific research has confirmed the pharmacological values of *G. littoralis*. However, the lack of proper quality control of *G. littoralis* restricts its wider application in food and pharmaceutical industries. In the market, it is common to find that several different medicinal plants share the same name with *G. littoralis*. In order to establish a healthy system for the application of *G. littoralis*, it is crucial to precisely authenticate its raw materials and commercial extract products. Although a variety of morphological and physicochemical analysis methods have been developed for authentication of *G. littoralis*, these methods have limitations in accurate authentication because geographical environment, storage condition, and processing procedures may affect the phenotypic markers. In comparison, DNA markers are more reliable and provide consistent authentication results irrespective of physiological conditions, environmental factors, storage and processing methods of the samples (Yip et al. 2007). DNA barcoding is the most commonly used technique for genetic identification of *G. littoralis* from its adulterants. However, DNA barcoding technology requires repeat operations of barcode amplification, sequencing, and clustering analysis of the sample. From botanical materials to commercial extracts, *G. littoralis* undergoes a series treatment procedures including hot extraction, vacuum concentration, and spray-drying, which result in the degradation of DNA into shorter fragments. Therefore, full-length DNA barcoding sequences are very difficult to be amplified from deeply processed products.

Species-specific PCR refers to PCR amplification using specific primers for distinguishing related samples (Wang et al. 2018). This method is based on the fact that mismatches of specific primers can lead to failure in amplification. Therefore, the selection of suitable gene sequences for analysis is critical for exploitation of DNA polymorphisms. Although *G. littoralis* and its five adulterants belong to different taxonomic levels, the exploitation of polymorphic sites that unanimously specific to the five adulterants can be challenging. The highly variable ribosomal ITS and chloroplast trnL-F regions were targeted for molecular analysis and, as expected, showed sufficient polymorphic nucleotides among *G. littoralis* and its five adulterants. The introduction of additional mismatches in primer NTF and BTR ensured absolute allelic specificity, and *G. littoralis* and its adulterants could be easily identified by the presence of their

species-specific PCR products. The authentication results from the two markers respectively developed in ITS and trnL-F regions were consistent with each other, indicating that the developed species-specific PCR methods were effective for discrimination of *G. littoralis* from its adulterants.

Among the commercial products of *G. littoralis*, *G. littoralis* extract is the most important industrial product which can be used as intermediates of other final commercial products. However, the manufacturing processes including hot temperature extraction and drying procedures result in degradation of DNA into small fragments, making botanical identification with DNA molecular markers is relatively difficult. In this study, specific primers designed in chloroplast trnL-F region was selected for botanical authentication of *G. littoralis* extract. First, ribosomal ITS and chloroplast trnL-F genes are multi-copy in the cell, PCR amplification of partial ITS and trnL-F regions are more degradation-tolerant. Second, the absence of the longer PCR product on an agarose gel could be explained by the template DNA degradation, the design of longer amplicon size for *G. littoralis* than the other adulterants can thus avoid false negative authentication result. As expected, the developed method can detect 0.1% of adulteration with a detection limit of 0.01 ng and is effective for botanical origin authentication of commercial extracts of *G. littoralis*. It turned out that the disadvantage of DNA degradation in deeply processed products can be mitigated by choosing multi-copy DNA sequences and raising PCR cycles. Therefore, the present study provide a sensitive and accurate DNA molecular method for quality control of *G. littoralis* and its industrial products, in comparison with the other molecular protocols so far used.

4 Conclusions

In this study, two species-specific PCR systems were developed for molecular discrimination of *G. littoralis* from its adulterants based on ribosomal ITS and chloroplast trnL-F regions. The DNA marker in trnL-F region was able to detect 0.1% of intentional adulteration of genomic DNA with a detection limit of 0.01 ng, and it is effective for botanical origin authentication of *G. littoralis* extracts either in single or mixed plant samples. It is suggested that the established method as a sensitive and accurate DNA approach for quality control of *G. littoralis* and its industrial products.

Declarations

Author Contribution Mengchen Lin: methodology, validation, data curation, writing—original draft preparation. Hongtao Wang: conceptualization. Qiaoning Yu: software, validation, investigation. Dandan Wang: conceptualization, resources, writing—review and editing, supervision.

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Data Availability The datasets generated for this study are available on request to the corresponding author.

Conflicts of Interest The authors declare no conflict of interest.

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Figures

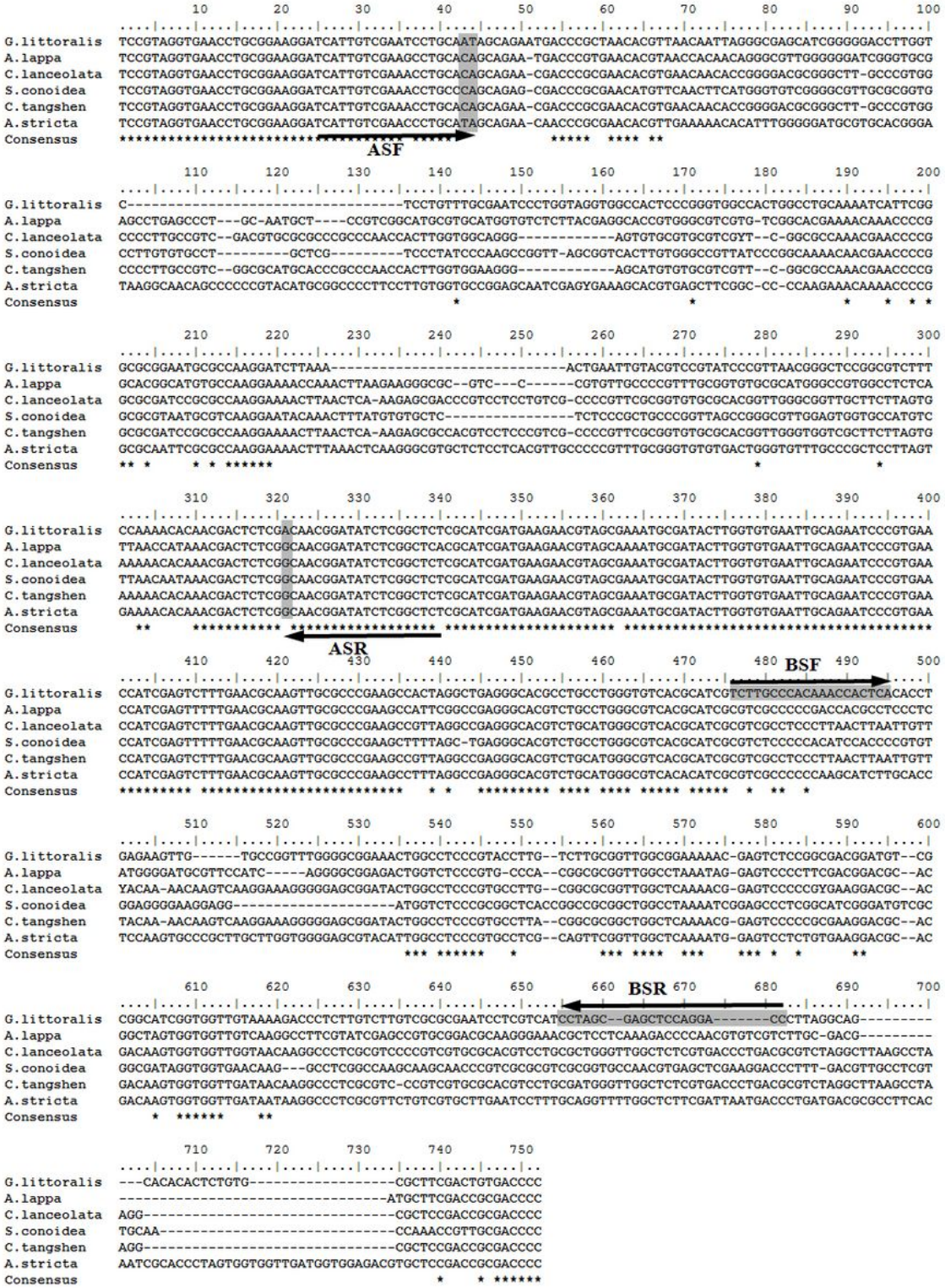


Figure 1

Multiple sequence alignment of ITS sequences.

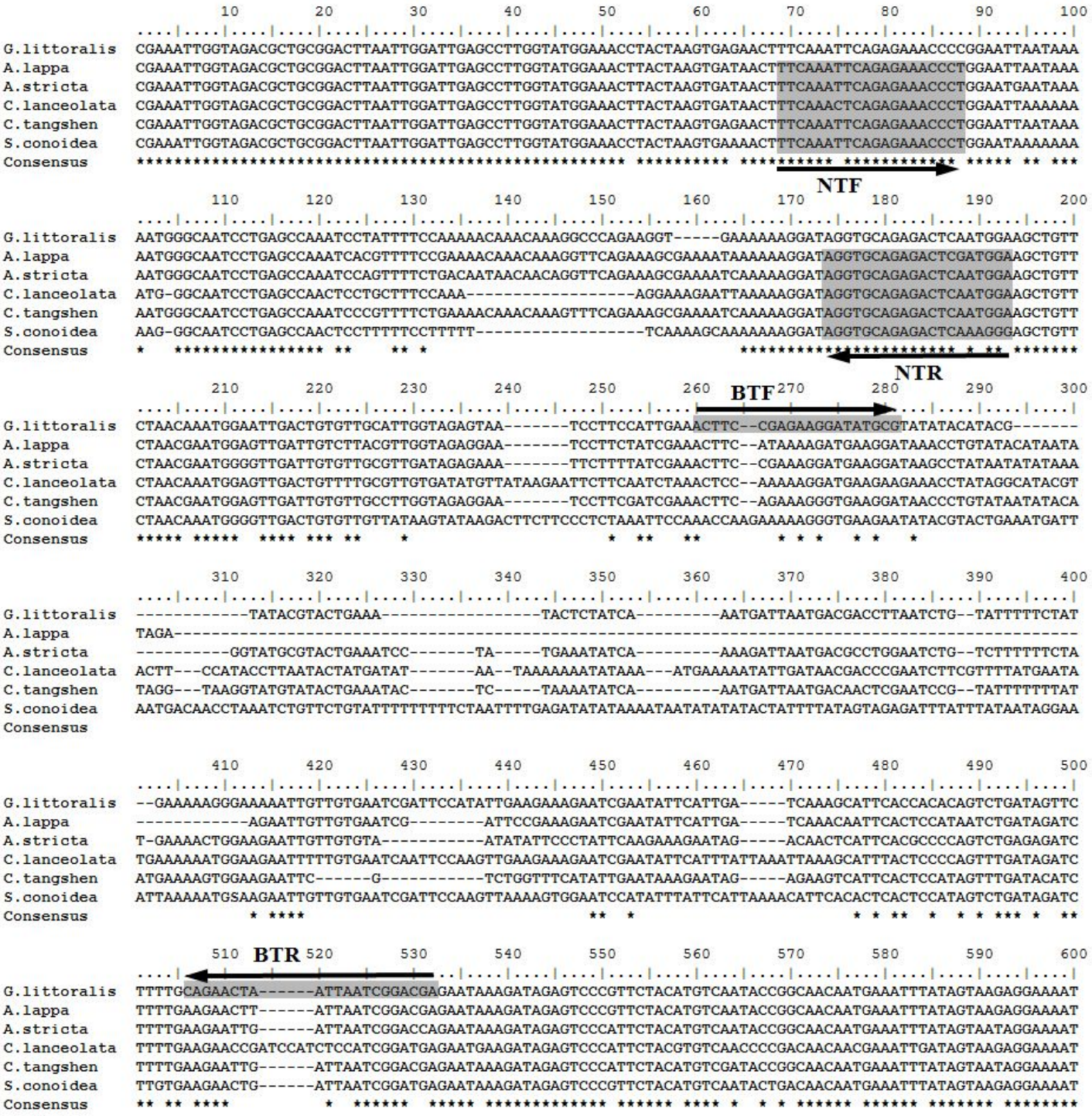


Figure 2

Multiple sequence alignment of trnL-F sequences.

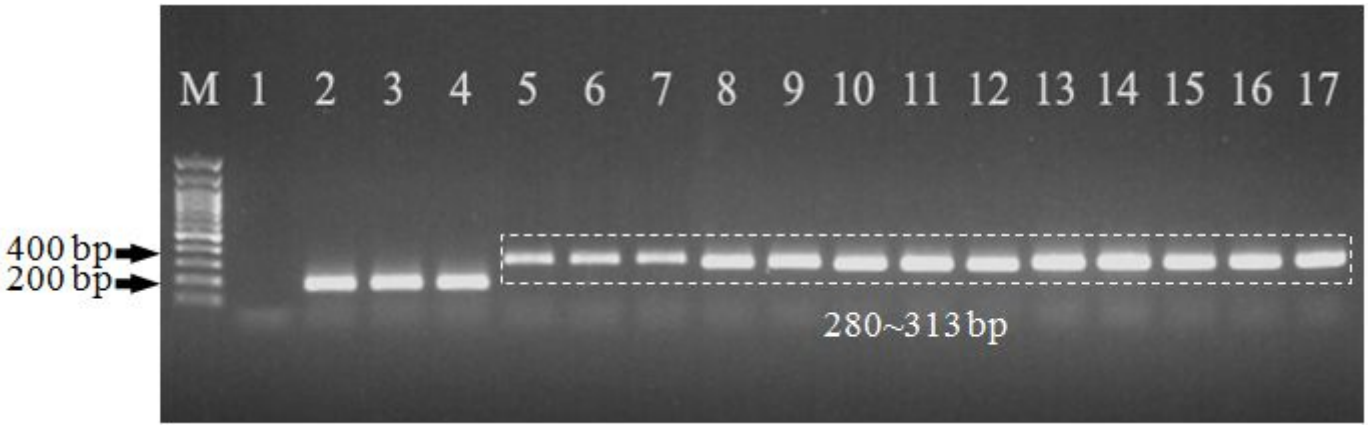


Figure 3

Agarose gel image of multiplex species-specific PCR with primers ASF, ASR, BSF and BSR in ITS region. M: 100 bp Plus II DNA Ladder; 1: Negative control; 2-4: *G. littoralis*; 5-7: *A. stricta*; 8-9: *A. lappa Decne*; 10-12: *S. conoidea*; 13-14: *C. lanceolata*; 15-17: *C. tangshen*.

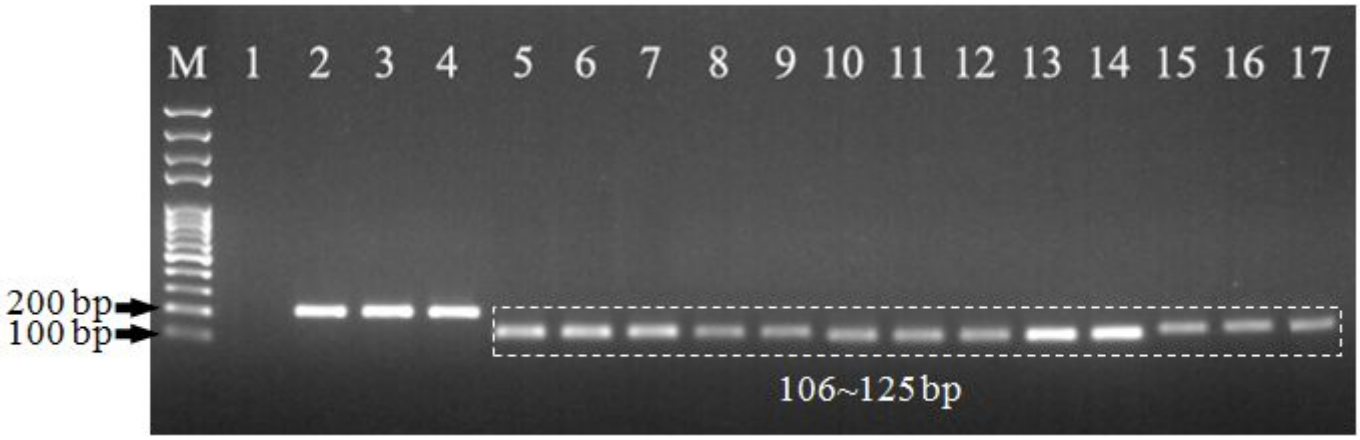


Figure 4

Agarose gel image of multiplex species-specific PCR with primers NTF, NTR, BTF and BTR in trnL-F region. M: 100 bp Plus II DNA Ladder; 1: Negative control; 2-4: *G. littoralis*; 5-7: *A. stricta*; 8-9: *A. lappa Decne*; 10-12: *S. conoidea*; 13-14: *C. lanceolata*; 15-17: *C. tangshen*.

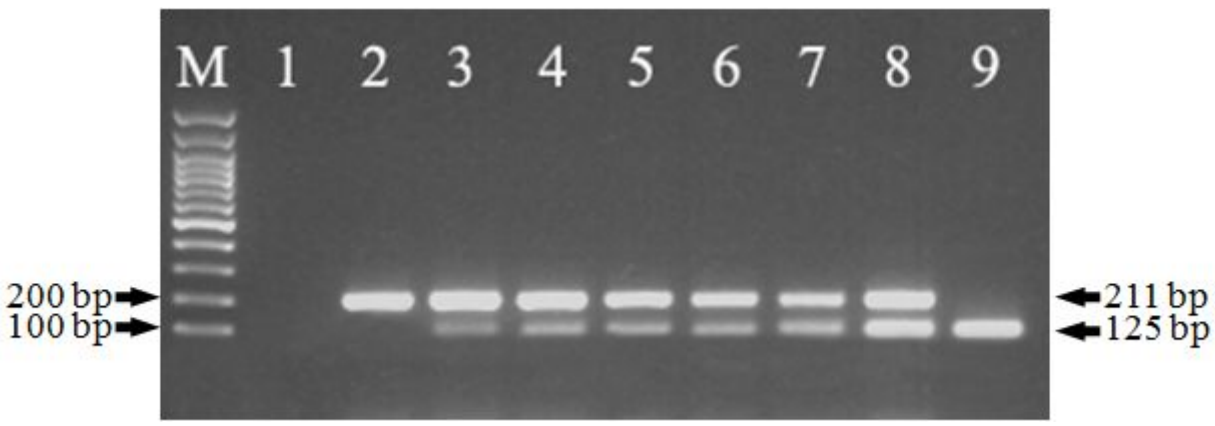


Figure 5

LOD test of multiplex species-specific PCR with primers NTF, NTR, BTF and BTR in trnL-F region. M: 100 bp Plus II DNA Ladder; 1: Negative control; 2: *G. littoralis*; 3-8: binary DNA mixtures of *G. littoralis* and *A. stricta* containing 0.1%, 1%, 2%, 5%, 10%, 50% amount of *A. stricta*, respectively; 8: *A. stricta*.

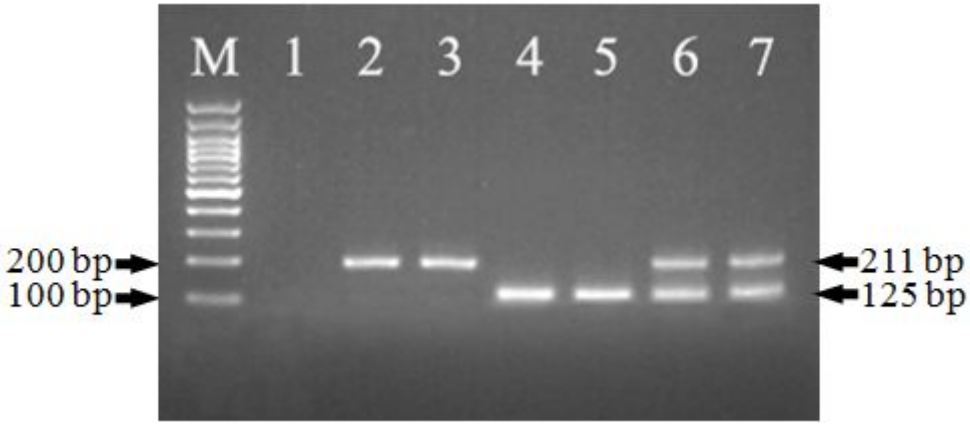


Figure 6

Multiplex species-specific PCR results of commercial extracts. M: 100 bp Plus II DNA Ladder; 1: Negative control; 2-3: Extracts of *G. littoralis*; 4-5: Extracts of *A. stricta*; 6-7: Mixtures of *G. littoralis* and *A. stricta*extracts.