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A Sensitive and Robust DNA Method for Authenticity Determination of Glehnia littoralis and Its Food Products

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Research Article

Keywords: Glehnia littoralis, Single nucleotide polymorphism, TrnL-F, Species-specific PCR, Authenticity determination

Posted Date: December 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2361738/v1

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Abstract

Glehnia littoralis has been widely recognized for its comprehensive nutritional properties and pharmaceutical effects. However, the adulteration practices with cheaper or very similar species in its food products lead to unfair market competition and severe health risks to the consumers. In this study, two species-specific PCR systems were constructed for molecular discrimination of *G. littoralis* from its adulterants based on the single nucleotide (SNP) markers exploited from ribosomal ITS and chloroplast trnL-F regions, respectively. The species-specific PCR assay utilizing trnL-F region was able to detect 0.1% of intentional adulteration of genomic DNA with a detection limit of 0.01 ng. The developed method, mitigating the adverse effects of DNA degradation and false negative results, was proved to be effective for botanical origin authentication of *G. littoralis* food products in a single reaction. Therefore the present study provides a sensitive and robust DNA method for authenticity determination of *G. littoralis* and its food products, and the established method can be employed to determine the authenticity and adulteration of other agro-products with high accuracy and reliability.

Introduction

G. littoralis, a perennial herb growing at coastal dunes in Eastern Asia, has been widely used as a diaphoretic, antipyretic, and analgesic for treating 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). Nowadays, *G. littoralis* has been widely used to develop a variety of functional foods, popular drugs, and skin care products owing to its comprehensive nutritional properties and pharmaceutical effects (Choe et al. 2019). With the increasing market demand and the beach destruction, the 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 have profoundly affected the efficacy and safety of *G. littoralis* products, 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* food 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 and straightforward, but it is challenging to identify processed materials when they are in powdered form or shredded pieces. In the past few years, great attention has been paid to develop physicochemical analysis methods, for instance, 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) 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 causing a high signal-to-noise ratio.

Compared with traditional and chemical analysis methods, DNA-based technologies are less affected by physiological conditions, processing state, and environmental factors (Samuel 2021). The primary practical advantages of rich polymorphism, genetic stability, and high accuracy associated with DNA markers have enhanced their widespread use in authenticity 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 enabled 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. In routine work, however, these methods are challenging in the authenticity or adulteration determination of commercial food products with high accuracy due to the DNA degradation during processing. In this study, we respectively exploited *G. littoralis*-specific SNP markers from ITS and chloroplast trnL-F regions, and developed a highly sensitive and robust protocol that can be used in the authenticity detection of *G. littoralis* food products.

Materials And Methods

Materials and DNA Isolation

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. The commercial products of *G. littoralis*, *A. stricta*, and *C. tangshen* were purchased from different biotechnology and pharmaceutical companies. All the voucher specimens were identified and deposited in School of Life Sciences, Yantai University. The detailed information of the samples and their origins is shown in Table 1. 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 food products was performed using a Magnetic Universal Genomic DNA Kit (Tiangen, China), following the manufacturer’s protocols.

Table 1
Plant samples used in this study.

Species/Products	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 slice	GS-02	Shandong	8
Glehnia littoralis powder	GS-03	Shandong	8
Glehnia littoralis extract	BT-01	Shandong	8
Adenophora stricta powder	NP-01	Shanxi	8
Adenophora stricta extract	NP-02	Shanxi	8
Codonopsis tangshen powder	CP-06	Sichuan	8
Codonopsis tangshen extract	CP-07	Sichuan	8

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).

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 the Basic Local Alignment Search Tool against the NCBI sequence database, and the query sequences were identified to the species level with the highest similarity. Multiple sequence alignment was conducted by using the 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.

Multiplex Species-specific Pcr

Molecular discrimination of *G. littoralis* from its five adulterants was 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 one 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, the 20 μL reaction mixture consists of 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 those of ITS region, except that the annealing temperature was reduced to 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 determine their detailed sequences.

Lod Test And Application In Food Products

To eliminate the possible false negative results, chloroplast trnL-F region was selected for botanical origin authentication of commercial products, by which *G. littoralis* can produce longer specific fragments than those of the adulterants. A total amount of 10 ng binary DNA mixtures containing 0.1%, 1%, 2%, 5%, 10%, and 50% of non-target DNA were respectively used for the limit of detection (LOD) test. To confirm the practicality of the method, genomic DNAs extracted from commercial food products of *G. littoralis*, *A. stricta*, *C. tangshen*, and their extract mixtures 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 raised to 40, to mitigate the adverse effect of DNA degradation of commercial products.

Results

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 expected, the multiple sequence alignment result 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 are 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. Similarly, 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 were located at nucleotide positions 88 (C/T), 281 (G/A), and 506 (C/A), respectively (Fig. 2). Consequently, species-specific primers for molecular discrimination of *G. littoralis* and its five adulterants can be designed based on these SNP sites exploited in ITS and trnL-F regions.

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 its specific sequences compared with those 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 their SNP sites located at nucleotide position 88. According to the SNP sites at nucleotide positions 281 and 506, primers BTF and BTR were respectively designed for specific authentication of *G. littoralis* (Fig. 2). The sequences of these specific primer pairs were shown in Table 2. The substitutions of A for C in primer NTF and A for T in primer BTR, were additional mismatches introduced deliberately to ensure reliable allelic specificity for target species.

Table 2
Nucleotide sequences of specific primers

Location	Primer	Nucleotide sequence*	Mismatch
ITS	ASF	5'-TCATTGTCGAAACCTGCA**CA**-3'	CA(TA)/AT
	ASR	5'-AGAGCCGAGATATCCGTTG**C**-3'	C/T
	BSF	5'-TCTTGTCCACAAACCACTCA-3'	Natural
	BSR	5'-GTTCCTGGAGCTCGCTAGG-3'	Natural
trnL-F	NTF	5'-TTCAAATTCAGAGAAACAC**T**-3'	ACT/CCC
	NTR	5'-TCCATTGAGTCTCTGCACCT-3'	No
	BTF	5'- ACTTCCGAGAAGGATATG**CG**-3'	CG/GA(AA)
	BTR	5'- TCGTCCGATTAATTAGTTCA**G**-3'	AG/TT

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

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 a size of 187 bp. 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 a size of 211 bp, while *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). No fragment was amplified when the same primer pairs were cross-challenged against DNAs from non-target species, and the minor 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.

Lod Test And Botanical Origin Authentication Of Commercial Products

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 than the shorter fragment. Therefore, chloroplast trnL-F

region is selected for botanical origin authentication of commercial products because the five adulterants yielded shorter amplicons than those from *G. littoralis*. A total amount of 10 ng binary DNA mixtures containing 0.1%, 1%, 2%, 5%, 10%, and 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* down to 0.01 ng level of template DNA. To confirm the practicality of the developed method in botanical origin authentication, genomic DNAs of commercial products in the forms of slice, powder, and extract were subjected to the developed PCR assay. As expected, the commercial products yielded their species-specific amplicons representing their botanical origins, which were consistent with that described in Fig. 4. For the mixed samples of *G. littoralis* and adulterant extracts, two fragments with dim brightness were detected for each sample (Fig. 6), indicating the existence of adulterant and the DNA degradation in their extracts. The findings highlight that this approach is effective for botanical origin authentication of commercial *G. littoralis* food products and can be used for authenticity and adulteration determination of *G. littoralis* food products.

Discussion

In recent years, scientific research has demonstrated the pharmacological values of *G. littoralis*. However, the broader application of *G. littoralis* in food and pharmaceutical industries has been restricted due to the adulteration practices. In the market, it is common to find that several different species share the same name “Sha shen” with *G. littoralis*. In order to establish a healthy system for the practical application of *G. littoralis*, it is crucial to precisely authenticate its raw materials and botanical origin authenticity of commercial food products. Although various morphological and physicochemical analysis methods have been developed for authentication of *G. littoralis*, these methods have limitations in authenticity detection because geographical environment, storage conditions, and processing procedures may affect the phenotypic markers. In comparison, DNA-based approaches are more reliable due to their high sensitivity and resistance against degradation. DNA-based techniques can thus provide consistent authentication results irrespective of physiological conditions, environmental factors, storage and processing methods of the samples (Yip et al. 2007). Among these, DNA barcoding is the most commonly used technique for the genetic identification of *G. littoralis* from its adulterants. Nevertheless, DNA barcoding technology requires repeat operations of barcode amplification, sequencing, and clustering analysis of the samples to be tested for each identification. From botanical materials to commercial products, *G. littoralis* undergoes a series of 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 and can detect trace differences in base pair sequence. Therefore, the selection of

suitable gene sequences for analysis is critical for the exploitation of DNA polymorphisms. Although *G. littoralis* and its five adulterants belong to different taxonomic levels, the exploitation of polymorphic sites that are unanimously specific to the five adulterants can be challenging. In the present study, 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* could be easily discriminated from the five adulterants 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*, the extract is the most important, which can be used as intermediate for other final commercial food products. However, the manufacturing processes including hot temperature extraction and drying procedures result in the degradation of DNA into small fragments, making botanical identification with DNA molecular markers particularly challenging. In this study, the specific primers designed in chloroplast trnL-F region were selected for botanical authentication of *G. littoralis* food products. Firstly, chloroplast trnL-F region is multi-copy in the cell, PCR amplification of partial trnL-F region is more degradation-tolerant. Moreover, the absence of the longer PCR product on an agarose gel could be explained by the template DNA degradation, the design of a longer amplicon size for *G. littoralis* than the other adulterants in trnL-F region can thus avoid false negative authentication result. As a result, 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 products 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. In addition, the shorter size of the adulterants than *G. littoralis* eliminates the possible false negative outcomes resulting from DNA degradation. Therefore the present study provides a sensitive and robust DNA method for authenticity determination of *G. littoralis* and its food products, and the established method can be employed to determine the authenticity and adulteration of other agro-products with high accuracy and reliability.

Conclusions

The present study provides a sensitive and robust DNA species-specific PCR method for authenticity and adulteration determination of *G. littoralis* and its food products, based on the specific SNP markers exploited from chloroplast trnL-F region. The species-specific PCR assay, mitigating the adverse effects of DNA degradation and false negative results, can detect 0.1% of intentional adulteration with a detection limit of 0.01 ng template DNA in a single reaction. The established assay was proved to be effective for botanical origin authentication of *G. littoralis* food products, and the method can be employed to determine the authenticity and adulteration of other agro-products with high accuracy and reliability.

Declarations

Ethics Approval and Consent to Participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable.

Competing Interests The authors declare no competing interests.

Author Contributions

Mengchen Lin and Dandan Wang wrote the main manuscript text. Qiaoning Yu performed the experiments and was a major contributor in writing the manuscript. Hongtao Wang investigated the resources and prepared Table 1. Mengchen Lin validated the results and prepared figures 1-6. Dandan Wang was the supervisor of the proposed work. All authors reviewed the manuscript.

Funding

This study was funded by Shandong Provincial Natural Science Foundation (ZR2022MH270).

Data Availability

All analyzed data generated in this study are available within the article.

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Figures

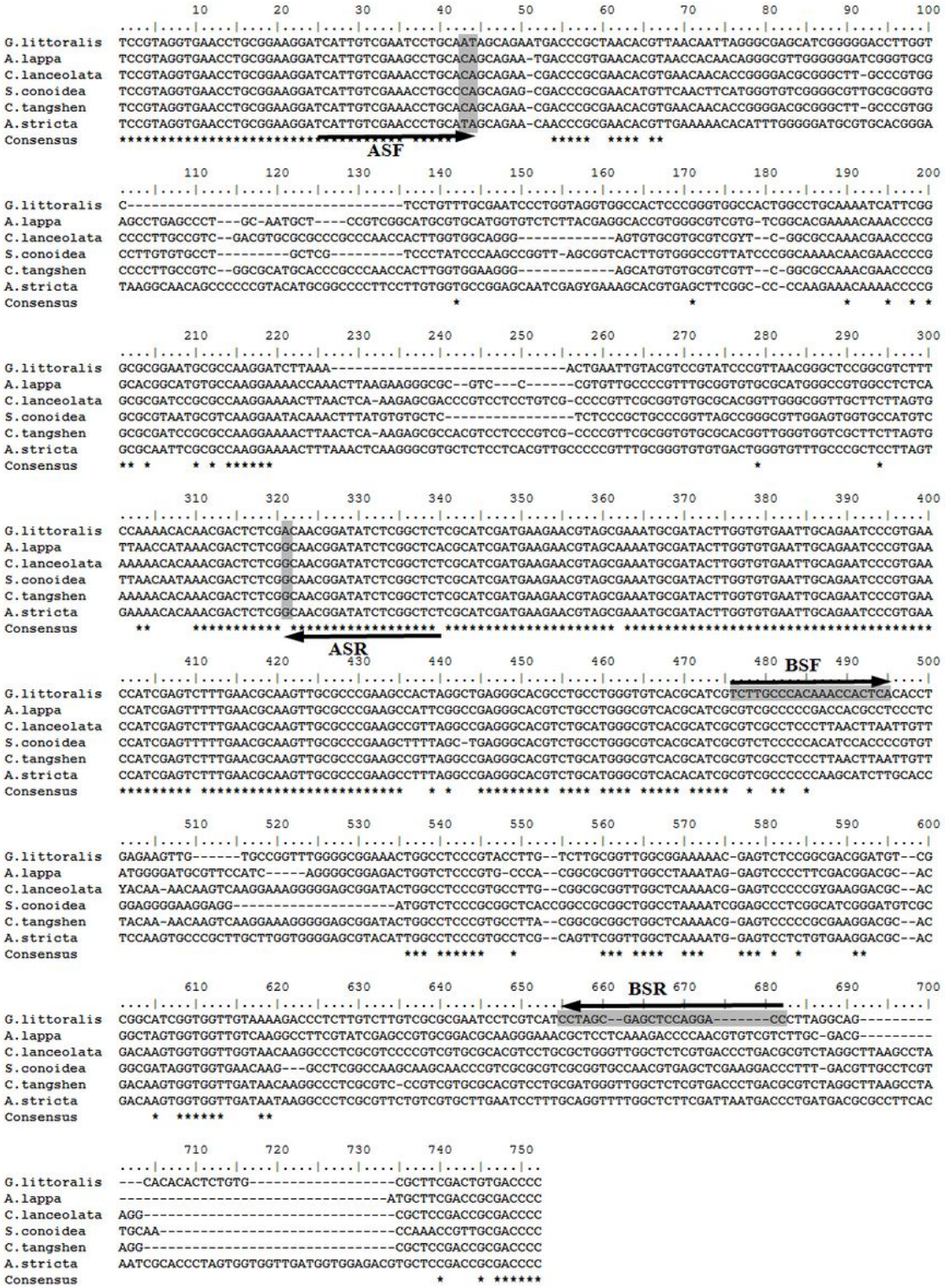


Figure 1

Multiple sequence alignment of ITS sequences.

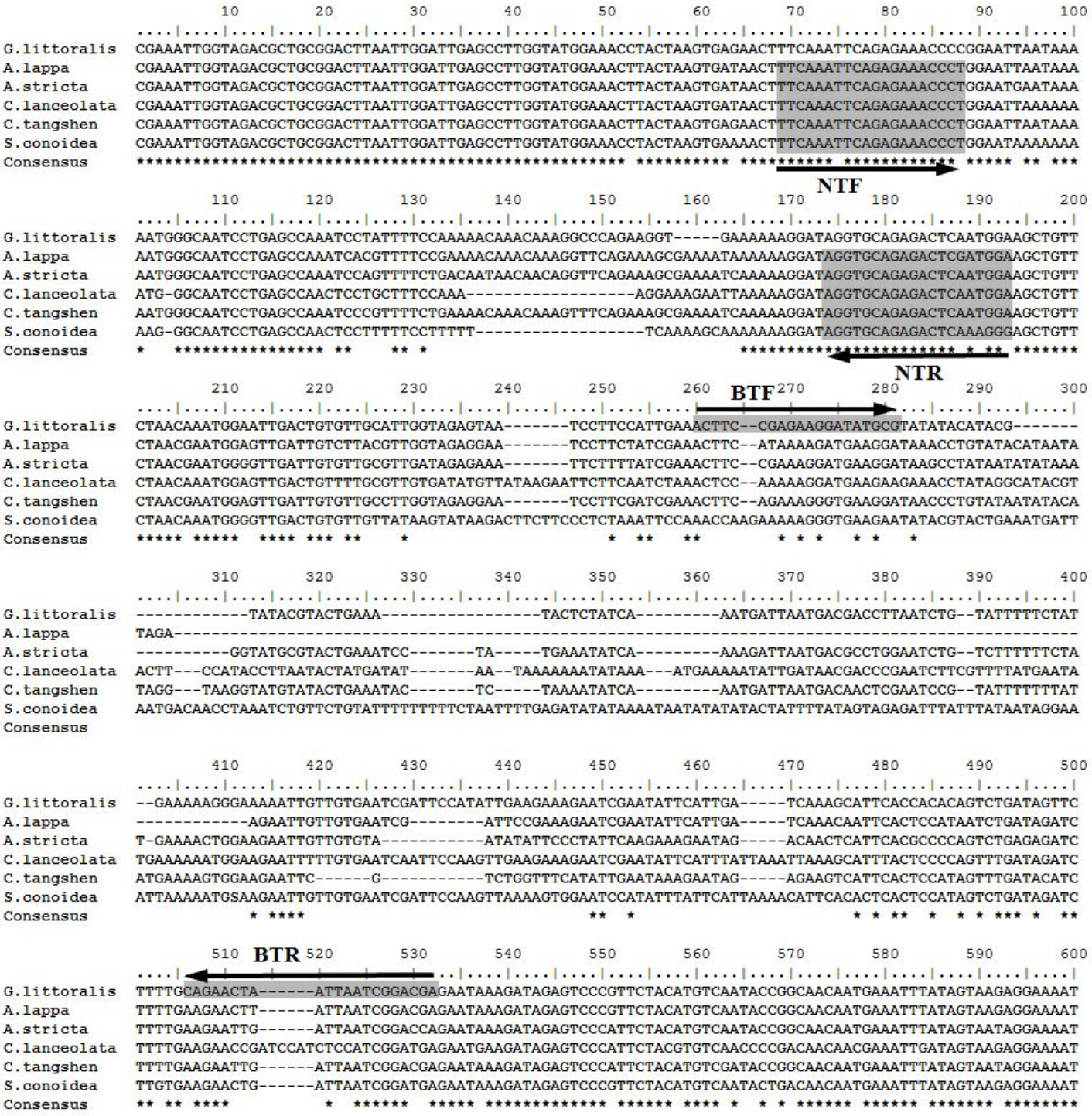


Figure 2

Multiple sequence alignment of trnL-F sequences.

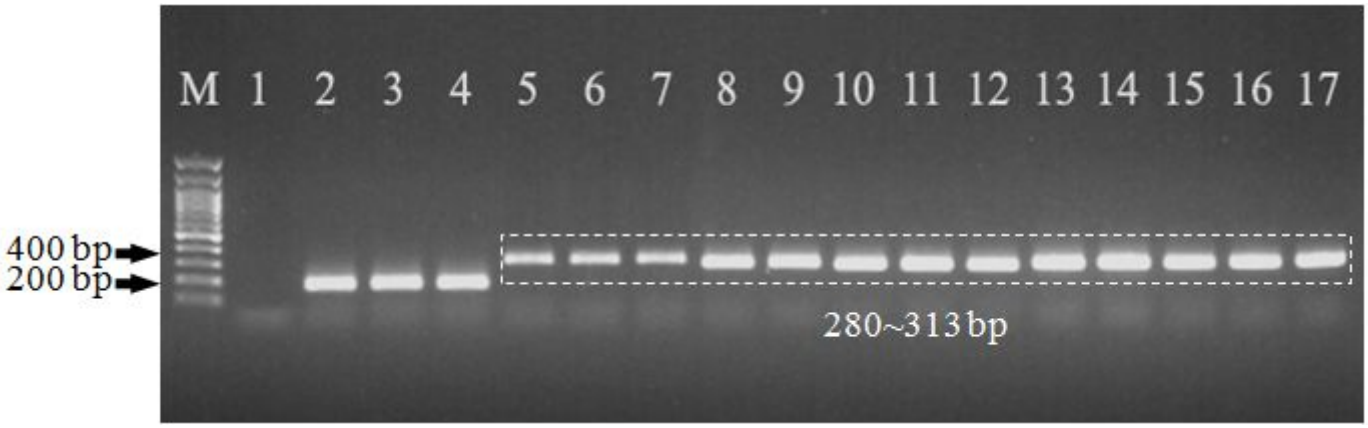


Figure 3

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*.

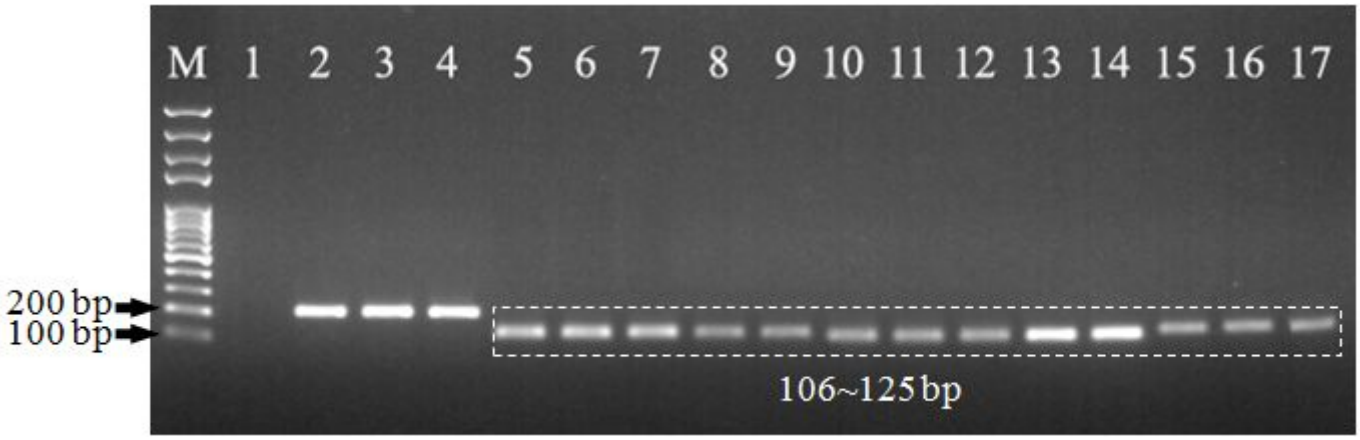


Figure 4

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*.

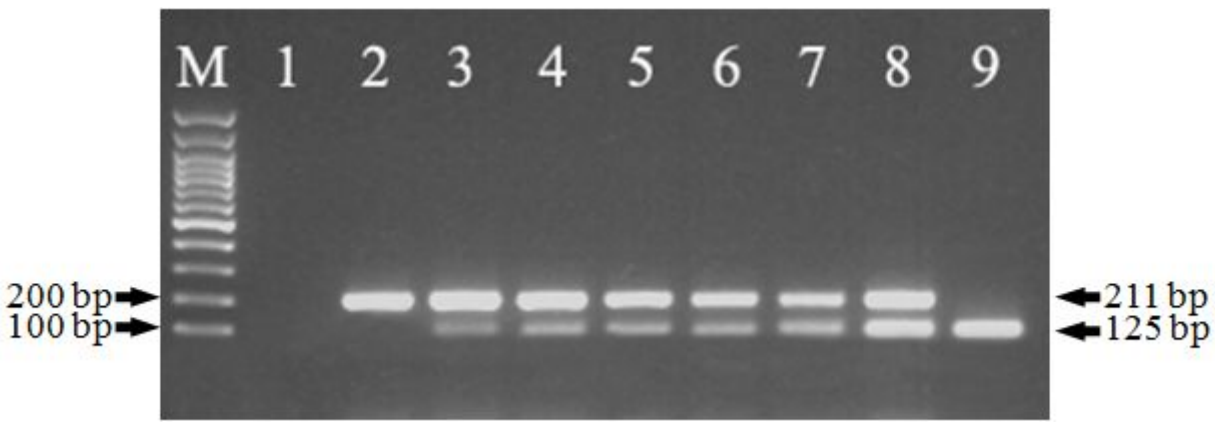


Figure 5

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*.

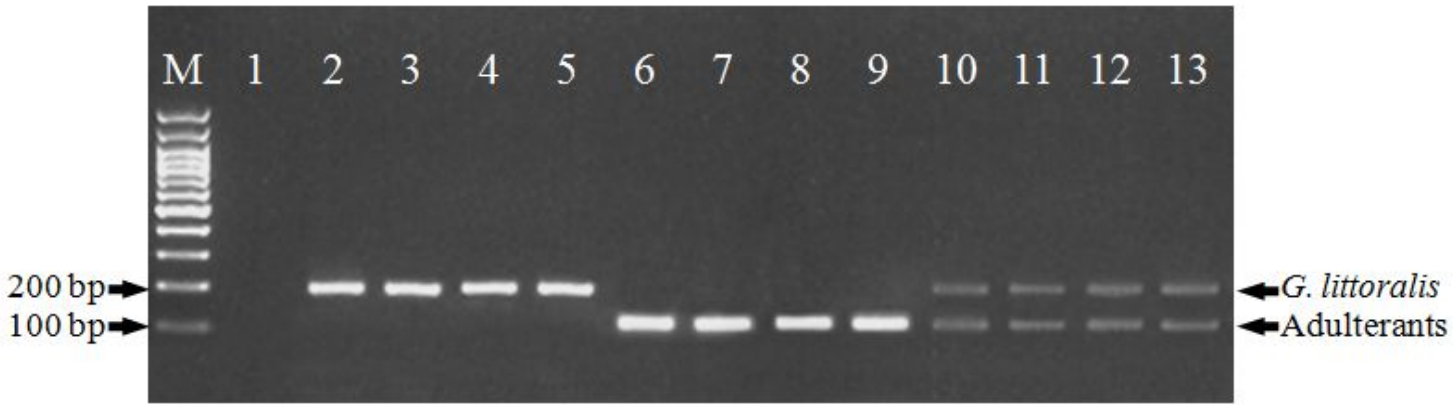


Figure 6

Multiplex species-specific PCR results of commercial products. M: 100 bp Plus II DNA Ladder; 1: Negative control; 2-3: *G. littoralis* slice; 4-5: *G. littoralis* powder; 6-7: *A. stricta* powder; 8-9: *C. tangshen*powder; 10-11: Mixture of *G. littoralis*and *A. stricta* extracts (20:1); 12-13: Mixture of *G. littoralis* and *C. tangshen* extracts (20:1).