Species-Specific Polymerase Chain Reaction for the Detection of *Anethum graveolens* Adulteration in *Cuminum cyminum* Seeds

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Abstract

Cuminum cyminum (cumin) is a widely used culinary spice valued for its distinct flavor, aromatic profile, and nutritional benefits, making it a staple in global cuisine and traditional medicine. However, due to its higher market value, cumin is often a target for economic adulteration. One of the most reported adulterants is *Anethum graveolens* (dill), a lower-cost herb known as sathakuppa in Ayurveda. While dill seeds closely resemble cumin morphologically, they pose a significant health risk due to their abortifacient properties, which have been historically documented in Sri Lanka and India. Dill has been traditionally used for treating menstrual disorders due to its bioactive compounds that influence endometrial contractions, making its unintentional consumption particularly concerning for pregnant women.

This study aimed to develop a reliable molecular technique to differentiate *A. graveolens* from *C. cyminum* to detect dill adulteration in cumin samples, ensuring food authenticity and preventing economic fraud. Species-specific primers were designed targeting the Internal Transcribed Spacer (ITS) region of *A. graveolens* to ensure specificity without cross-reactivity with *C. cyminum*. Polymerase Chain Reaction (PCR) assays were performed, followed by agarose gel electrophoresis to visualize amplified DNA fragments and confirm the presence of *A. graveolens* in cumin samples. For further confirmation, amplified DNA products have been followed by sanger sequencing, providing additional molecular evidence of species differentiation.

The molecular approach successfully detected *A. graveolens* adulteration in cumin samples at levels as low as 1%, demonstrating high sensitivity and specificity. The PCR-based method effectively distinguished between the two species, overcoming the limitations of conventional morphological identification techniques. PCR-based detection, complemented by sequencing, provides a robust tool for identifying *A. graveolens* adulteration in cumin, ensuring food authenticity and product integrity. The findings highlight the importance of molecular authentication techniques in preventing spice adulteration and mitigating associated health risks.

1. Introduction

Cumin (*Cuminum cyminum*) is a highly valued spice, cherished for its distinctive aroma and flavor that enhance a wide range of cuisines, including Indian, Middle Eastern, and Mexican dishes. Beyond its culinary significance, cumin has played a notable role in traditional and modern medicine, owing to its bioactive compounds with antioxidant, antimicrobial, and anthelmintic properties (Madhavan & Tharakan, 2017). The seeds of the cumin plant are harvested, dried, and used in whole or powdered form, contributing to its status as both a kitchen staple and a functional food ingredient. However, the growing demand and economic importance of cumin have made it increasingly vulnerable to adulteration an issue that affects not only market integrity but also consumer safety.

A prominent and concerning adulterant in cumin is *Anethum graveolens*, commonly known as dill (Pastor, 2023). Both cumin and dill belong to the Apiaceae family and bear seeds that are strikingly

similar in size, shape, and color, making them difficult to differentiate not only in powdered products but even in whole seed form as illustrated in Fig. 1.1. This morphological resemblance facilitates both accidental contamination and intentional adulteration, which often goes unnoticed in visual inspections.

Dill, is not only valued for its culinary and aromatic properties but also holds therapeutic importance in traditional systems such as Ayurveda (Chahal et al., 2017). In Ayurvedic medicine, dill seeds have been traditionally used to manage menstrual cycle irregularities, including amenorrhea and excessive bleeding (Atta-ur-Rahman, M. Iqbal Choudhary and Yousuf, 2020). A commonly recommended remedy involves consuming powdered dill seeds with jaggery to help regulate cycles and ease menstrual discomfort. Modern research supports these uses, showing that dill seed extracts can influence the estrous cycle and hormone levels, suggesting potential roles in cycle regulation and even antifertility effects (Malihezaman Monsefi et al., 2015). These traditional and scientific perspectives together highlight dill's multifaceted potential as a natural therapeutic agent.

Dill (*Anethum graveolens*), while widely used for its culinary and medicinal properties, may pose certain health risks, particularly related to its abortifacient potential (Rahmdel et al., 2018). Some compounds in dill, such as alkenylbenzenes, have raised safety concerns due to their toxicological properties. Studies suggest that consumption of plant food supplements containing dill can contribute to cumulative exposure to these compounds, posing a potential reproductive health risk, especially in sensitive populations such as pregnant women (Alajlouni et al., 2017). As such, caution is advised regarding the use of dill-based supplements or high doses during pregnancy due to its possible uterotonic and abortifacient effects.

The widespread consumption of cumin across diverse populations amplifies the risk associated with such adulteration. Economically, it enables suppliers to substitute a cheaper ingredient (dill) for a more valuable one (cumin), undermining consumer trust and product authenticity. Consequently, accurate and reliable methods for detecting adulteration are essential for maintaining food safety and regulatory compliance.

Various detection techniques have been explored to address this issue. Traditional methods such as microscopy offer rapid visual analysis based on morphological traits. However, due to the high resemblance between cumin and dill seeds especially when ground microscopy often lacks the resolution needed for definitive identification. Chemical-based approaches offer greater specificity, with techniques such as high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC-MS), and liquid chromatography–mass spectrometry (LC-MS) being commonly employed. LC-MS, in particular, is highly sensitive and capable of generating detailed chemical fingerprints, allowing for the differentiation of plant species based on unique metabolite profiles (He et al., 2020). However, despite its accuracy, LC-MS is expensive and requires sophisticated instrumentation and technical expertise, limiting its accessibility for routine screening.

Other chemical methods, including thin-layer chromatography (TLC) and Fourier-transform infrared spectroscopy (FTIR), have also been used for spice authentication. TLC offers a relatively simple and

low-cost way to separate and identify chemical constituents, but it may not be sufficiently sensitive for detecting low levels of adulterants (He et al., 2020). FTIR spectroscopy can provide rapid, non-destructive chemical analysis based on molecular vibrations, but like TLC, it may struggle to distinguish between chemically similar species without advanced spectral libraries.

In contrast to these chemical methods, DNA-based approaches have emerged as highly reliable tools for species identification, particularly in cases of processed or morphologically similar materials (Mahadani and Ghosh, 2013). Polymerase chain reaction (PCR) techniques offer unparalleled specificity and sensitivity by amplifying trace amounts of DNA unique to each species (Ichim, 2019). Several PCR-based approaches are available for food authentication, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), real-time quantitative PCR (qPCR), and DNA barcoding.While these methods vary in complexity, cost, and resolution, their common advantage lies in their ability to identify adulterants at the genetic level, regardless of the physical state of the sample (Sun, 2018).

Species-specific PCR stands out among these techniques due to its ability to selectively amplify DNA sequences unique to the target species (Wallinger et al., 2012). This is achieved through the design of primers that bind only to regions found exclusively in the adulterant's genome, thereby avoiding amplification of the host species. In the context of cumin adulteration, this approach ensures that even low levels of *Anethum graveolens* DNA can be detected in a background of *Cuminum cyminum*, making it highly suitable for both qualitative and quantitative analysis of adulteration. Compared to other methods, species-specific PCR offers a cost-effective, rapid, and highly accurate solution with minimal need for post-PCR processing (Pereira, Carneiro and Amorim, 2008).

The reliability of species-specific PCR depends heavily on the selection of appropriate genetic markers with sufficient variability between the species of interest. While chloroplast regions such as *rbcL* and *matK* are widely used for general plant identification (Wallinger et al., 2012), their sequences are highly conserved between cumin and dill, with more than 98% similarity. This makes them unsuitable for developing species-specific primers in this case. Instead, the internal transcribed spacer (ITS) region of nuclear ribosomal DNA was selected due to its higher interspecific variability, allowing for clearer discrimination between closely related species.

To further improve specificity in species identification, DNA barcoding based on sequencing of the internal transcribed spacer (ITS) region can be employed. In this approach, PCR amplification of the ITS region is followed by sequencing and comparison with reference databases such as GenBank, enabling accurate species-level identification based on genetic similarity.

In this study, a molecular method based on species- specific PCR amplification followed by sequencing of the ITS region was developed for the detection of *Anethum graveolens* adulteration in cumin. Species-specific identification was achieved through sequence alignment with authenticated *Anethum graveolens* reference sequences. This assay proved to be sensitive, cost-effective, and applicable to both seed and powdered forms of the spice. The study adds to the expanding toolkit of molecular techniques

for food authentication and supports global efforts to ensure consumer protection and food integrity within the spice trade.

2. Methodology

The experimental workflow employed in this study is illustrated in Fig. 2.1. This method outlines the step-by-step process beginning with seed sample collection, followed by DNA extraction, PCR amplification using species-specific primers, and analysis through agarose gel electrophoresis. Depending on the presence or absence of amplification bands, further analysis was conducted using either sequencing or restriction fragment length polymorphism (RFLP).

2.1 Sample Collection and Preparation

Authentic samples of *Cuminum cyminum* (cumin) and *Anethum graveolens* (dill) were collected and confirmed using Liquid Chromatography–Mass Spectrometry (LC-MS) to ensure purity and eliminate the possibility of prior adulteration (Liang et al., 2006). Adulterated samples were prepared by mixing dill seeds into cumin at concentrations of 25%, 10%, and 1% (w/w). A 100g portion was taken from each sample and subjected to partial grinding using a household grinder to facilitate further processing. Each adulteration level was prepared in triplicate to ensure reproducibility.

2.2 DNA Extraction

Genomic DNA was extracted from pure and adulterated seed samples using the Phytospin D™ Plant DNA Extraction Kit. Prior to extraction, the 5g of Partially ground seeds were ground into a fine powder under liquid nitrogen to break down cell walls and improve DNA yield. All extractions were performed following the manufacturer's instructions.

2.3 Primer Designing

ITS region sequences of *Anethum graveolens* and *Cuminum cyminum* were retrieved from the NCBI GenBank database and aligned using BioEdit software (Ibis Therapeutics, Carlsbad, CA, USA) to identify polymorphic regions. *A. graveolens*-specific primers (Fig. 2.2) were designed manually and screened for primer species specificity (Fig. 2.3) and cross-reactivity in an *in silico* analysis using the local nucleotide alignment tool "BLAST" (http://www.ncbi.nlm.nih.gov/blast). Candidate primers (Table 2.1) were evaluated for the presence of secondary structures such as hairpin loops, self-dimers, using the Benchling online molecular biology suite (Benchling, San Francisco, CA, USA). To improve amplification fidelity and reduce the risk of truncated products, HPLC-graded primers were synthesized (Cha and Thilly, 1993).

2.4 PCR Amplification

PCR amplification of the internal transcribed spacer (ITS) region was conducted in a 25 µL reaction volume comprising 10.0 µL of GoTaq® Green Master Mix (2×; Promega), 2.5 µL of forward primer (10 µM), 2.5 µL of reverse primer (10 µM), 2.5 µL of DNA template, and 7.5 µL of PCR-grade water. Negative

controls were included in each PCR assay by substituting the DNA template with 2.5 µL of nuclease-free water, in order to monitor for potential contamination or non-specific amplification and DNA extracted from a pure dill sample served as the positive control. PCR reactions were carried out in Heal Force Thermal cycler T960, conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 7 minutes.

2.5 Agarose Gel Electrophoresis

PCR products were analyzed using 1% agarose gel electrophoresis. Gels were stained with ethidium bromide and visualized under UV light. A 100 bp DNA ladder was included in each run to determine the size of the amplified fragments.

2.6 DNA Barcoding ; Sequencing

The purified PCR products were subjected to Sanger sequencing using a SeqStudio™ Genetic Analyzer (Applied Biosystems, USA) at Genelab Medicals (Sri Lanka). Sequencing was performed in both forward and reverse directions to ensure the accuracy of the obtained sequences.

3. Results and Discussion

Primer specificity is critical in developing accurate molecular diagnostics, particularly for distinguishing closely related plant species with similar morphology (Mafra, Ferreira and Oliveira, 2007). As shown in Fig. 3.1, PCR amplification produced a distinct band only in the *Anethum graveolens* sample, with no amplification observed in *Cuminum cyminum* (Al-Hadeithi & Jasim, 2021). This indicates a high degree of specificity, with primers annealing and amplifying only the target *Anethum* DNA sequence.

The absence of amplification in *Cuminum* confirms that the primers do not bind non-specifically under the tested PCR conditions. Such specificity is essential to avoid false positives, particularly in mixed samples or when the target species is present at low abundance (Zagon et al., 2017). These findings support the primer set's suitability for reliable identification of *Anethum graveolens* in food authentication applications.

To evaluate the sensitivity of the assay, PCR was performed using DNA extracted from seed mixtures adulterated with *Anethum graveolens* at concentrations of 25%, 10%, and 1% (w/w). As results shown in Fig. 3.1, clear amplicon bands were observed in all dill included samples, including the 1% sample. This indicates that the developed primer set can reliably detect *Anethum* DNA even at very low concentrations, demonstrating high analytical sensitivity.

The ability to identify the target species at just 1% adulteration is particularly significant for food authentication, where trace detection is often required to verify label claims and detect fraudulent substitutions. These results affirm the method’s suitability for detecting low-level adulteration in complex mixtures, reinforcing its application in quality control and regulatory settings.

It is important to note that this assay is qualitative in nature, aiming to confirm the presence or absence of *Anethum graveolens* rather than quantify its exact proportion within a mixture. In the context of food adulteration, such qualitative detection is highly relevant (Callao and Ruisánchez, 2018). Adulteration is often economically motivated. In this case, the substitution of *Cuminum cyminum* with the comparatively cheaper *Anethum graveolens* can lead to significant profit margins. Given that such fraudulent practices typically involve substitution at levels exceeding 1%, the demonstrated sensitivity of this method is sufficient to reliably identify economically driven adulteration events in commercial spice products.

To validate the presence of *Anethum graveolens* in the adulterated samples, PCR products obtained from positive amplifications of the ITS region were subjected to Sanger sequencing (Mafra, Ferreira and Oliveira, 2007). The resulting DNA sequences were analyzed using the BLASTn tool against the NCBI GenBank database. The sequences showed a 100% identity match with authenticated *Anethum graveolens* reference sequences, thereby confirming the species identity and the target sequence of the amplified product. This molecular confirmation demonstrates the reliability of the assay in detecting *A. graveolens* even in samples with low levels of adulteration and highlights the applicability of DNA barcoding for authenticating spice ingredients in both seed and powdered forms.

Although this study employed DNA barcoding of the ITS region followed by sequence alignment for species identification, *in silico* analysis revealed the presence of a unique restriction site for the AatII enzyme within the *Anethum graveolens* ITS region which is able to cleaved amplified DNA fragment to two distinguished fragments. This site was absent in the corresponding ITS region of *Cuminum cyminum* (cumin), suggesting the potential utility of restriction fragment length polymorphism (RFLP) as an additional validation method.

In future applications, when it requires a second validation method, digestion of PCR amplicons with AatII could serve as a rapid, cost-effective approach to confirm *Anethum* adulteration, especially in routine screening or in settings where sequencing facilities are limited. Incorporating such enzyme-based assays could enhance the specificity and robustness of molecular authentication strategies in the spice industry (Aly, Levin and Xu, 2018).

Conclusion

The present study demonstrates the development of a species-specific PCR assay for the detection of *Anethum graveolens* in spice mixtures, with high specificity and sensitivity. The primers successfully amplified target DNA exclusively from *Anethum*, with no cross-reactivity observed in *Cuminum cyminum*, ensuring reliable discrimination between the two morphologically similar species. Sensitivity analysis using w/w% adulterated seed mixtures revealed that the assay could detect *Anethum* presence down to 1%, which is sufficient to identify economically motivated adulteration practices commonly exceeding this threshold. Furthermore, confirmation by sequencing of PCR products reinforced the accuracy of species identification. As a qualitative method, this assay offers a rapid and cost-effective approach for

detecting adulteration in commercial spice products, supporting food authenticity and consumer protection efforts.

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Table 1

Table 2.1. Primer sequences used for PCR amplification targeting the internal transcribed spacer (ITS) region.

Primer Name	Sequence (5′→3′)	Length (bp)	Tm (°C)	GC Content (%)	Target Region
ITS Fw	GTAAACACATTGGGCAAGCTTCA	23	56.3	43.48	ITS
ITS Rv	GACACAACGACCGCACAAC	19	56.9	57.89	ITS

Figures

Figure 1

Figure 1.1. Morphological comparison between seeds of *Anethum graveolens* (dill) (a) and *Cuminum cyminum* (cumin) (b), illustrating their visual similarity in size, shape, and color.

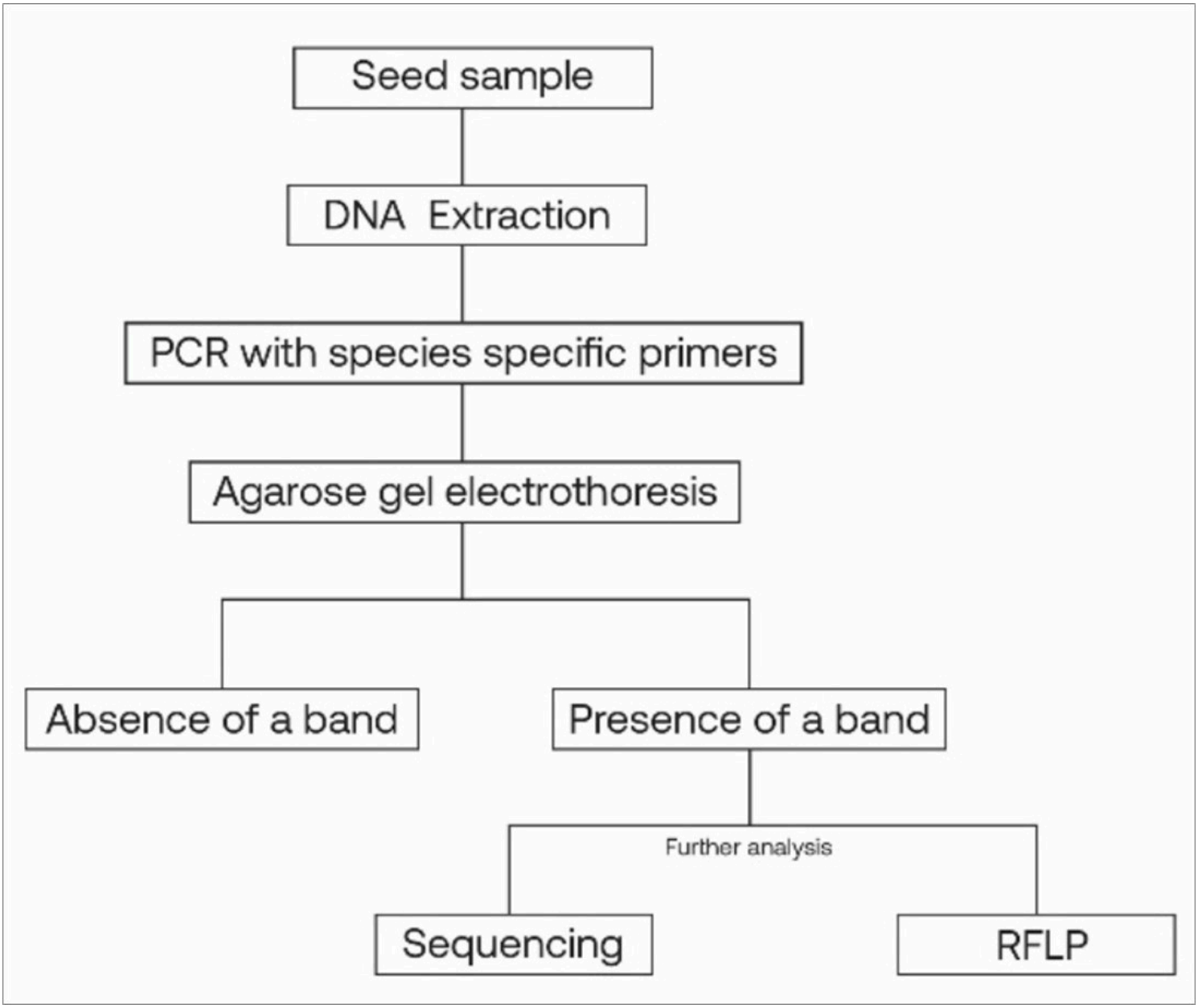

Figure 2

Figure 2.1. Flow diagram illustrating the general methodology used for detecting *Anethum graveolens* adulteration in *Cuminum cyminum* seeds. DNA was extracted from seed samples and subjected to PCR amplification using species-specific primers. Agarose gel electrophoresis was used to visualize the PCR products. The presence of a band indicates the presence of *A. graveolens* DNA, suggesting an adulterated sample, whereas the absence of a band confirms no adulteration. If further confirmation is required, sequencing or restriction fragment length polymorphism (RFLP) can be employed.

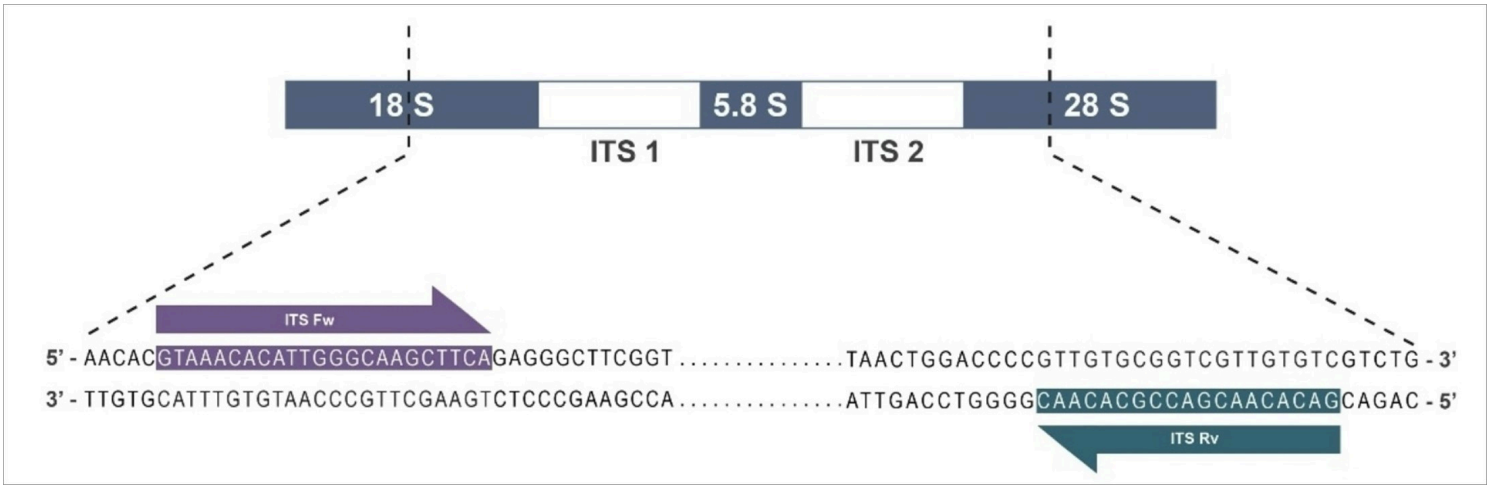

Figure 3

Figure 2.2. Schematic representation of primer annealing sites targeting the ITS region of *Anethum graveolens*, with ITS Fw and ITS Rv newly designed species – specific primers flanking ITS1 and ITS2.

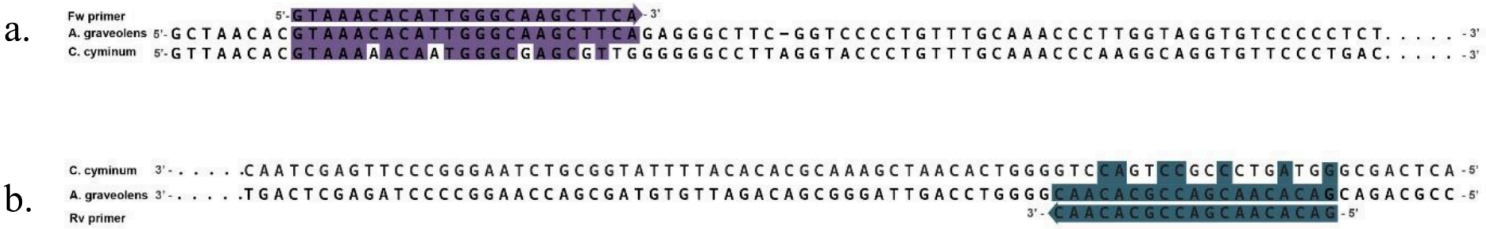

Figure 4

Figure 2.3. Sequence alignment showing the species-specific binding of (a.) forward and (b.) reverse primers designed for *Anethum graveolens* (dill) in contrast to *Cuminum cyminum* (cumin). The highlighted regions indicate primer binding sites, demonstrating mismatches in the cumin sequence that prevent primer annealing, thereby confirming primer specificity for *A. graveolens* target DNA.

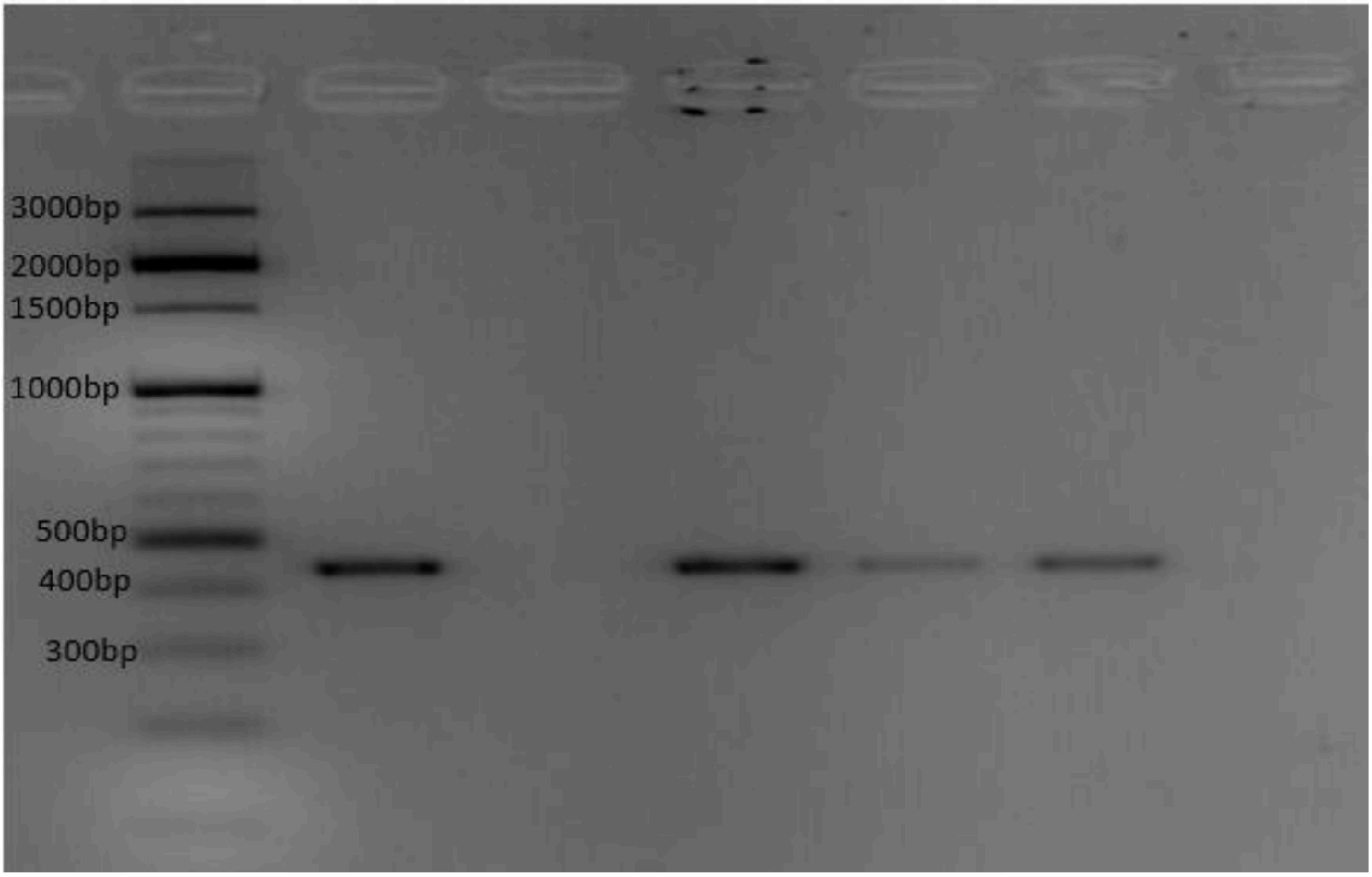

Figure 5

Figure 3.1. Agarose gel electrophoresis of PCR-amplified products using species-specific primers to detect adulteration in cumin with dill. Lane 1: 100 bp DNA ladder; Lane 2: pure dill sample; Lane 3: pure cumin sample; Lane 4: cumin sample adulterated with 25% dill; Lane 5: cumin sample adulterated with 10% dill; Lane 6: cumin sample adulterated with 1% dill.